SEROTONIN HAS EARLY, CILIA-INDEPENDENT ROLES IN XENOPUS LEFT-RIGHT PATTERNING

Laura N. Vandenberg, Joan M. Lemire and Michael Levin*

Center for Regenerative and Developmental Biology, and Department of Biology
Tufts University
200 Boston Ave. Suite 4600
Medford, MA 02155 U.S.A.

* Author for Correspondence
Tel. (617) 627-6161
Fax: (617) 627-5035
email: michael.levin@tufts.edu

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Abstract

Consistent left-right (LR) patterning of the heart and viscera is a crucial part of normal embryogenesis. Because errors of laterality form a common class of birth defects, it is important to understand the molecular mechanisms and stage at which LR asymmetry is initiated. Frog embryos are a system uniquely suited to the analysis of the mechanisms involved in orientation of the LR axis because of the many genetic and pharmacological tools available for use and the fate-map and accessibility of early blastomeres. Two major models exist for the origin of LR asymmetry and both implicate pre-nervous serotonergic signaling. In the first, the charged serotonin molecule is instructive for LR patterning – it is redistributed asymmetrically along the LR axis and signals intracellularly on the right side at cleavage stages. A second model suggests serotonin is a permissive factor required to specify the dorsal region of the embryo containing chiral cilia that generate asymmetric fluid flow during neurulation – a much later process. We performed theory-neutral experiments designed to distinguish between these models. The results uniformly support a role for serotonin in the cleavage stage embryo, long before the appearance of cilia, in ventral right blastomeres that do not contribute to the ciliated organ.
Introduction

Understanding how consistent left-right (LR) asymmetry of the body-plan is established is important for developmental biology and medicine. Individuals with LR patterning defects including heterotaxia (the loss of concordance among the visceral organs), and isomerisms (loss of asymmetry), have grave medical consequences (Hackett, 2002; Peeters and Devriendt, 2006; Zhu et al., 2006). The frog Xenopus laevis has proven to be an excellent model for the study of LR asymmetry because of the vast number of developmental, molecular-genetic, physiological, and pharmacological tools that are available for use in this organism. Specific benefits of this model system also include the large number of eggs available for study at the earliest of stages (which allowed the discovery of many very early asymmetry mechanisms that have so far been too difficult to study in mice), and a well-defined fatemap that allows targeting left- and right-side cells at will (which is not possible in zebrafish for example). Thus, the frog embryo is a uniquely powerful model in which to study the earliest events that link biophysical chirality to asymmetric gene expression and subsequent organ situs.

There are currently two competing paradigms of how the LR axis is established. One model proposes that symmetry is first broken during neurulation, when cilia localized to a node-like structure generate a chiral fluid flow that asymmetrically distributes a morphogen, or causes asymmetric bending of sensory cilia (reviewed in (Basu and Brueckner, 2008; Hashimoto and Hamada, 2010)). This model implies considerable evolutionary divergence among phyla; since many different model systems (including amniotes such as the chick and pig) orient their asymmetry without the benefit of cilia (Levin and Palmer, 2007; Speder et al., 2007; Gros et al., 2009), it becomes unclear which species is the best model for human disease-relevant symmetry breaking.

The other model proposes that a highly-conserved chiral intracellular cytoskeleton component drives asymmetric localization of ion transporters, thus establishing a biased voltage gradient that results in asymmetric localization of a charged molecule that is instructive for LR identity (reviewed in (Aw and Levin, 2009;
Vandenberg and Levin, 2010)). This model is supported by molecular-genetic gain- and loss-of-function data that indicate that asymmetry is established during the first cell cleavages (Levin et al., 2002; Aw et al., 2008), and identified the neurotransmitter serotonin as the small molecule which is redistributed at cleavage stages to provide LR identity to blastomeres (Fukumoto et al., 2005a; Fukumoto et al., 2005b; Adams et al., 2006; Carneiro et al., 2011).

A recent study (Beyer et al., 2012) suggests a permissive role for serotonin in the specification of the gastrocoel roof plate (GRP, the Xenopus node) and cilia-dependent fluid flow. Thus, the two main LR asymmetry models now converge on a common molecule: the neurotransmitter serotonin. The question of timing - when serotonin is actually acting during LR patterning – is crucial not only because it impacts the plausibility of the late origin vs. early origin models of asymmetry but also because it identifies the embryonic stages that would be most sensitive to serotonergic compounds in widespread medical use today (Shuey et al., 1992; Alwan S et al., 2005; Noorlander et al., 2008; Sadler, 2011).

Here, we report the results of experiments that allowed us to resolve the role of timing and location of serotonin in LR patterning of the frog embryo. These studies were designed to experimentally distinguish two hypotheses: one that suggests a role for serotonin in right-sided ventral blastomeres during cleavage stages (i.e. the EARLY model) (Levin and Palmer, 2007), and one that requires a role for serotonin late, in the left side (Vick et al., 2009) of the dorsally-derived GRP cells (i.e. the LATE model). The results of these experiments support a role for serotonin during early cleavage stages, and exclude a role for serotonin signaling in the GRP.
Results

**Ectopic serotonin randomizes the LR axis**

The LATE model proposes that serotonin is localized throughout the embryo and acts as a competence factor for canonical Wnt signaling to specify the GRP (Beyer et al., 2012). In contrast, the EARLY model suggests that serotonin is an instructive signal asymmetrically localized to the right side of the early cleavage stage embryo (Fukumoto et al., 2005b; Vandenberg and Levin, 2010). To determine the spatial requirement for functional serotonin signaling in LR patterning, we injected 1 blastomere of 4-cell embryos with 15 or 30 ng of ectopic serotonin (+ lineage tracer to confirm targeting). We then scored animals for organ position (Fig. 1A), not pitx2 expression as in other studies (Beyer et al., 2012), because incidence of incorrect asymmetric gene expression strongly overestimates the effects of treatments on randomizing organ situs, making cilia-targeting treatments appear more penetrant than they are (Supplemental Fig. 1 and (Vandenberg, 2012)). Whereas the LATE model predicts there should be no effect from ectopic serotonin on either side, the EARLY model predicts that excess serotonin signaling, especially on the left side, will randomize the LR axis by ectopic activation of intracellular receptors (Carneiro et al., 2011). Indeed, overabundance of serotonin introduced to any blastomere significantly affected organ situs, with the greatest effects observed when injected on the left side (Fig. 1B). These results are compatible with the need for differential serotonin signaling on the L and R sides – a result predicted by the EARLY model (and data showing that endogenous serotonin is normally moved away from the left and towards the right side within the first few cell cleavages (Fukumoto et al., 2005b; Vandenberg and Levin, 2012)) and not consistent with the LATE model's view of serotonin as a competence factor whose presence above a baseline threshold simply allows ciliated cells to form.

**Loss-of-function reagents targeting serotonin signaling implicate the ventral right blastomere in LR patterning**
The LATE model suggests (Beyer et al., 2012) that loss-of-function serotonin reagents randomize asymmetry via direct effects on the flow-relevant cells of the GRP (i.e. only those on the dorsal, left-side (Vick et al., 2009)). In contrast, the EARLY model predicts that injections of such reagents into the ventral right side precursors should be most effective because endogenous serotonin signaling occurs in these blastomeres. We injected embryos with mRNA encoding a dominant negative Xenopus serotonin receptor 3A (Gunthorpe and Lummis, 2001) at the 4-cell stage, specifically targeting blastomeres that contribute to flow at the GRP (dorsal left) or blastomeres that do not (ventral right). We observed that injections into the ventral right blastomere were significantly more effective in randomizing organ situs (Fig. 1C). Such asymmetry defects from loss-of-function serotonin reagents in cells that do not contribute to the GRP are incompatible with the cilia role hypothesized by the LATE model.

Lateral plate mesoderm explants indicate that Xnr-1 is induced independent of ciliary flow

One of the key questions in this field is how biophysical events regulate asymmetric transcription of key laterality genes such as Xnr-1. We isolated explants from the left lateral plate mesoderm of embryos at st. 13 (before ciliary flow), st. 18 (after ciliary flow), and st. 22. We aged these explants to st. 23, and then examined the expression of Xnr-1, an asymmetric gene expressed normally on the left side of the embryo. The LATE model predicts that explants isolated before ciliary flow begins will not express Xnr-1, as it holds that cilia-dependent signaling is required to turn on Xnr-1 in left-side cells. In contrast, recent data suggested that Xnr-1 is instead actively suppressed by early presence of serotonin on the right (Carneiro et al., 2011); therefore the EARLY model predicts that left-side explants will express Xnr-1 at all stages regardless of when they were isolated (whether or not they participated in ciliary-dependent events). We observed strong Xnr-1 expression in explants isolated at all three stages (Fig. 1D,E). The patterns of expression were similar in shape and relative intensity to Xnr-1 expression patterns seen in whole embryos (Fig. 1D and data not shown). The expression of Xnr-1 in explants isolated at st. 13, prior to the onset of...
ciliary motion, directly refutes a requirement for ciliary flow in the induction of expression of Xnr-1.

To ensure that these results were not specific to Xnr-1 and applied to asymmetric gene expression in general, we repeated these experiments for two additional laterality genes, lefty and pitx2 (Fig. 1D). We observed that the vast majority of explanted tissue expressed these markers, regardless of when the explant was isolated (Fig. 1E), again with the strength of signal and size of expression domain observed in vivo (Hyatt et al., 1996; Cheng et al., 2000; Essner et al., 2000; Levin et al., 2002). These results are not consistent with ciliary initiation models, in which Nodal must be induced in naïve tissue on the left side by late-occurring chiral fluid flow, but are consistent with the early serotonin model, in which Nodal is on by default and does not require the presence of cilia or fluid flow in order to be expressed on the left side.

Quantitative meta-analysis reveals that ciliary parameters do not distinguish between normal and randomized embryos

In order to quantitatively assess whether cilia are functionally responsible for correct LR patterning, we performed a meta-analysis of the cilia literature and collected reported values for cilia number, cilia length, and ciliary flow rate in normal and LR-compromised conditions. Only the zebrafish literature contained enough studies to perform an analysis (sufficient numbers of measurements of the same parameters, in the same units, unfortunately do not yet exist in the literature of the mouse model). We found significant differences in reported values for cilia number, cilia length and flow rates in wildtype (untreated) embryos, even though we controlled for embryonic stage (Fig. 2). Most striking was the fact that some mutants exhibited ciliary parameter values solidly within the range of what was reported as ‘normal’ in other studies – there is no reliable way to associate any observed set of ciliary properties with a normal or abnormal laterality outcome. From this meta-analysis, we conclude that there is no support for the claim that ciliary parameters could functionally distinguish between correct and incorrect LR patterning.
Discussion

We utilized Xenopus, a popular model organism for the study of LR patterning, to distinguish between early and late roles of pre-nervous serotonin. These experiments were theory-neutral, allowing outcomes consistent with either or both major models, and distinguishing between two plausible roles for serotonin in LR patterning: an early instructive function whereby serotonin is asymmetrically localized to the right side of the embryo and actively suppresses Xnr-1 (Fukumoto et al., 2003; Fukumoto et al., 2005b; Carneiro et al., 2011), or a late role whereby serotonin is a permissive factor required for the specification of the GRP which in turn is necessary for cilia-dependent fluid flow (Beyer et al., 2012) (Table 1). The results of all three experiments indicate that serotonin has an endogenous role on the right side, in ventral cells that do not contribute to the GRP, and that the expression of Xnr-1, an asymmetric gene, is induced even when ciliary flow is absent. These data are consistent with previously published HPLC analyses, which indicate high levels of serotonin during early cleavage stages and low levels during gastrula/neurula (Fukumoto et al., 2005a), as well as molecular-genetic functional experiments implicating serotonin signaling during early cleavage stages on the ventral right side of the embryo (Fukumoto et al., 2003; Fukumoto et al., 2005a; Fukumoto et al., 2005b; Carneiro et al., 2011); ventral right-side serotonin-dependent events are incompatible with the LATE model because only left-dorsal cells are relevant for ciliary flow (Vick et al., 2009). Taken together, these data uniformly support a role for serotonin in LR patterning in a GRP-independent manner.

Recent experiments directed at a late role for serotonin in LR patterning (Beyer et al., 2012) did not test early roles of maternal serotonin or serotonin receptor proteins because they relied on morpholinos - maternal serotonin and serotonin receptor proteins known to be present in the cleavage-stage embryo (Fukumoto et al., 2005b) would not be targeted by these reagents. However, the report implicating serotonin in GRP specification suggested that inhibiting serotonin signaling causes serious patterning defects in treated embryos including altered specification of the superficial mesoderm (Beyer et al., 2012), a phenotype we never observed with our reagents but
one that could potentially contribute to non-specific laterality defects due to toxicity. We found that properly-titered gain- and loss-of-function reagents cleanly affected LR patterning without producing such teratogenic side-effects. Indeed this allowed us to score organ situs in perfectly-formed tadpoles, instead of the indirect assay of asymmetric gene expression, which is often used as a surrogate readout when significant other abnormalities manifest later in development and preclude assessment of actual anatomical asymmetry (Walentek et al., 2012).

Our results on early (pre-ciliary) roles of serotonergic signaling are consistent with many previous observations and functional data showing that embryos from a wide variety of phyla exhibit molecular and physiological asymmetries long before the appearance of (or without the presence of) cilia, and a number of mutant analyses have now dissected left-right relevant roles of “ciliary” proteins from functional roles of cilia themselves (reviewed in (Vandenberg and Levin, 2010)). While cilia may have a role somewhere downstream in the LR pathway (Schweickert et al., 2007), they cannot be the initiator of LR asymmetry in Xenopus (Lobikin et al., 2012).

Although ciliary proteins have earlier intracellular roles (Qiu et al., 2005; Armakolas and Klar, 2007) that are inevitably abrogated in genetic experiments (and often interpreted as evidence of ciliary roles), many studies interpreted as supporting the LATE model use ciliary structure or function as the only analysis endpoint. However, our meta-analysis of the published zebrafish literature shows that ciliary parameters are highly variable, even among unperturbed embryos: crucially, many ciliary mutants’ cilia length and quantity values lie indistinguishably among the range of normal variability found in controls (Fig. 2). Thus, it is not possible to make a quantitative link between cilia function and asymmetry outcome in the zebrafish system. Similar analyses of the mouse data are on-going.

The relative plausibility of early vs. late origins of asymmetry is further impacted by questions about the timing of action of mechanisms such as serotonin. Our data suggest that the primary role for serotonergic signaling in asymmetry is most likely to take place at very early stages. Further, the recent finding that even in mouse the early blastomeres are structurally and functionally non-equivalent with respect to LR identity (Gardner, 2010; Jefferson and Williams, 2012), and the presence of consistent
asymmetries prior to the onset of ciliary flow in both Xenopus and mouse embryos (Kramer et al., 2002; Kramer and Yost, 2002; Bunney et al., 2003; Qiu et al., 2005; Ohkawara and Niehrs, 2011; Roberts et al., 2011; Sun et al., 2011; Lobikin et al., 2012) points to the early timing of LR symmetry breaking and orientation. Some asymmetries, such as the right-sided bias of PKCγ-dependent phosphorylation of the proteoglycan syndecan-2, occur temporally between the earliest steps in the LR asymmetry pathway (ion flux and serotonin) and asymmetric gene expression (Kramer et al., 2002), and therefore may act as downstream mediators in the serotonin pathway.

It is now necessary to examine serotonin’s role in early steps of the left-right patterning process in numerous model systems. Although it is still possible that this neurotransmitter plays some additional permissive role in the later development of ciliary flow or GRP specification, serotonin first functions in LR patterning at early stages in spatiotemporal patterns not consistent with ciliary roles. Thus, serotonin is one of the many mechanisms now known to underlie the events proximal to symmetry breaking shortly after fertilization.
Methods

Xenopus husbandry

*Xenopus laevis* embryos were fertilized *in vitro* in 0.1X Modified Marc’s Ringers (MMR) pH 7.8 according to standard protocols (Sive et al., 2000), housed at 14-18°C and staged according to (Nieuwkoop and Faber, 1967). All experiments were approved by the Animal Care and Use Committee at Tufts University and were conducted according to the Guidelines for the Care and Use for Laboratory Animals.

Construction of Dominant Negative Serotonin Receptor 3A construct

A Xenopus laevis serotonin receptor 3A cDNA was purchased from Open Biosystems (clone 4724653) and mutated as in (Gunthorpe and Lummis, 2001), using a site-directed mutagenesis kit (Agilent). The insertion of proline and point mutation of 2 other amino acids produced the sequence: 265-GPRLGFKVTVLGFSTLLLILND.

Microinjections

For injections of molecular constructs, capped synthetic mRNAs encoding a dominant negative serotonin receptor 3A and mRNAs encoding β-galactosidase, a lineage tracer, were dissolved in water. For injections of serotonin, stock solutions were prepared in distilled water and used immediately because serotonin has a very limited shelf life in solution. For all injections, droplet size was calibrated to 5-6 nl of liquid using standard methods (50-150 msec pulses with borosilicate glass needles calibrated for a bubble pressure of 50-70 kPa in water). Embryos were placed in 3% ficoll and reagents were injected into a single blastomere of 4-cell embryos.

Scoring of left-right phenotypes

At stage 45, *Xenopus* embryos were analyzed for *situs* of the heart, stomach and gall bladder according to (Levin and Mercola, 1998). Heterotaxia was defined as the reversal in position of one or more organs. Only embryos with a normal dorsoanterior index (DAI=5) were scored. Embryos were then fixed, washed, and stained with X-gal to
verify targeting of injections to specific blastomeres. Percent heterotaxia was calculated as number of heterotaxic embryos divided by total embryos. A $\chi^2$ test with Pearson correction for increased stringency was used to compare absolute counts of heterotaxic embryos. Groups were considered significantly different when $p<0.01$.

**LPM explants**

At the indicated stage, embryos were placed in 1X MMR and a sharp scalpel was used to remove the left lateral plate mesoderm (LPM) from the remainder of the embryo. These explants were maintained in 1X MMR for 1 hour to allow them to heal, and were then returned to 0.1X MMR. Uncut siblings were also placed in 1X MMR for the same period of time to allow careful staging of the explants. Explants and uncut siblings were aged to the appropriate stage for Xnr-1, lefty or pitx2 expression, fixed overnight, dehydrated, and stored at -20C for analysis.

**In situ hybridization**

Whole mount in situ hybridization was performed using standard protocols (Harland, 1991). In situ hybridization probes against Xnr-1 mRNAs (Sampath et al., 1997), lefty mRNAs (Meno et al., 1997) and pitx2 mRNAs (Campione et al., 1999) were generated in vitro from linearized template using DIG labeling mix (Roche, Branford, CT).

**Analysis of ciliary parameters**

Data was collected from 16 published zebrafish studies (Oishi et al., 2006; Shu et al., 2007; Okabe et al., 2008; Ferrante et al., 2009; Hatler et al., 2009; Lin and Xu, 2009; Neugebauer et al., 2009; Francescatto et al., 2010; Gao et al., 2010; Lopes et al., 2010; Kim et al., 2011; Liu et al., 2011; Wang et al., 2011; Bisgrove et al., 2012; Caron et al., 2012; Chen et al., 2012) and analyzed using Graphpad version 5. Only studies examining cilia number, cilia length, and ciliary flow in wildtype fish, and including means, standard error/standard deviations and sample sizes could be used for analysis. For cilia number and cilia length, analysis was limited to studies examining fish at the 10ss stage. One-way ANOVA and, when appropriate, Bonferroni posthoc analyses
were performed to determine if there were significant differences between groups. Bartlett’s test for equality of variance was performed as well.
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Financial or Competing Interests Disclosure

The authors have nothing to disclose.

Author Contributions

LNV and ML conceived and designed the experiments. LNV performed experiments. JML contributed reagents. LNV and ML analyzed the data. LNV, JML and ML wrote and edited the manuscript.
References


Figure 1: Theory neutral experiments indicate that serotonin has early, non-ciliary roles in left-right patterning.

A) For experiments where gain-of-function or loss-of-function reagents were examined, organ situs was examined to determine the effects of treatments on LR patterning. In st. 45 tadpoles, the position of three organs was examined: the heart, normally positioned on the animal’s left with the apex on the animal’s right (red arrowhead); the gall bladder, normally positioned on the animal’s right (green arrowhead); and the stomach, normally coiled toward the animal’s left (yellow arrowhead). Inversions of one or more of these organs were scored as heterotaxia.

B) Ectopic serotonin induces heterotaxia, with the greatest effects observed when injections were targeted to left blastomeres. Serotonin (15 or 30ng) was injected into one blastomere at the 4-cell stage, together with a lineage label to verify blastomere targeting. At the lower dose of serotonin, only injections in the ventral left (VL) or dorsal left (DL) blastomere induced significant amounts of heterotaxia; at the higher dose, injections into any blastomere including the ventral right (VR) or dorsal right (DR) induced heterotaxia, but left-sided injections were most effective. * p<0.01 compared to uninjected controls (2.5% heterotaxia, not shown). # p<0.01 comparing heterotaxia incidence between treatment groups. For all treatments, at least 100 embryos were included for analysis.

C) Expression of dominant negative (DN) serotonin receptor 3A (R3A) mRNA induces heterotaxia. Injection of this construct into either the dorsal left (DL) or ventral right (VR) blastomeres was sufficient to induce significant levels of heterotaxia, but expression in the VR blastomere produced more than twice as many heterotaxic embryos compared to DL expression. * p<0.01, **p<0.001 compared to embryos injected with β-gal lineage label alone. # p<0.01 comparing heterotaxia incidence between treatment groups. For all treatments, at least 75 embryos were included for analysis.

D) Left lateral plate mesoderm (LPM) explants were isolated prior to ciliary flow (st 13), after ciliary flow (st 18), or at the time that asymmetric genes (Xnr-1, lefty, pitx2) begin to be expressed asymmetrically in a robust manner (st 22-23). Explants and
unmanipulated siblings were developed to st. 23 (Xnr-1), st. 25/26 (lefty) or st. 29/30 (pitx2), fixed, and probed for expression of the appropriate mRNA. Arrows indicate areas with positive expression of the indicated mRNA.

E) Quantification of expression of laterality markers in left LPM explants. For all three markers assessed, the vast majority of explants were positive for expression of each of the 3 left-side marker genes.

**Figure 2: Analysis of cilia number, cilia length, and ciliary flow in published studies indicates large variability, lack of consistency in these parameters.**

To analyze the consistency of ciliary parameters in an *in vivo* model, data on ciliary length, cilia number, and ciliary flow parameters were collected from published studies (Oishi et al., 2006; Shu et al., 2007; Okabe et al., 2008; Ferrante et al., 2009; Hatler et al., 2009; Lin and Xu, 2009; Neugebauer et al., 2009; Francescatto et al., 2010; Gao et al., 2010; Lopes et al., 2010; Kim et al., 2011; Liu et al., 2011; Wang et al., 2011; Bisgrove et al., 2012; Caron et al., 2012; Chen et al., 2012). Zebrafish studies were selected because, in contrast to *Xenopus*, a significant number have measured the same parameters and reported data in a consistent manner (including means, standard error/standard deviations, and sample sizes).

A) Cilia length was reported in 13 studies examining wildtype zebrafish at somite stage 10 (control samples C1-C13). ANOVA and Bartlett values indicate significant differences between groups and significantly different variances. Posthoc analyses were not performed because of the large number of groups examined. Additionally, six treatments (mutations, knockdowns via morpholinos, etc.) that were shown to affect cilia length (T1-T6), either by decreasing or increasing the length of cilia, are also shown on the graph. Clearly, these treated groups cannot be statistically distinguished from wildtype groups in at least one other study.

B) Cilia number was reported in 8 studies examining wildtype zebrafish at somite stage 10 (control samples C1-C8). ANOVA values indicate significant differences between groups; Bartlett’s values indicate no significant differences in variance between groups. Additionally, three conditions/treatments that were shown to affect cilia number
(T1-T3) are displayed. Again, these treated groups cannot be statistically distinguished from one or more wildtype control group.

C) Ciliary flow rate was reported in six zebrafish studies (C1-C6). ANOVA and Bartlett’s tests indicate significant differences between groups and significant differences in variance. Because there were fewer studies included in this measure, Bonferroni posthoc analyses were performed. Lowercase letters on the graph indicate significant differences between groups of controls. For all three graphs, data shown are means +/- standard deviations as collected from original publications, or calculated from reported standard errors and sample sizes.

A similar analysis was attempted of the mouse literature. However, this analysis was hindered by the small number of studies that report means, standard error/standard deviation and sample sizes for wildtype populations. There are a few examples where large differences in reported measures suggest that ciliary parameters are not consistent in the mouse literature, similar to what we found in the zebrafish literature (above). For example, while one study reports the number of node cilia in the range of 60-80 (Shinohara et al., 2012), another reports the number in the range of 130-210 (a large range in itself) (McGrath et al., 2003). In addition, two studies report nodal flow rates in wildtype embryos in the range of 2-5 µm/sec (Tanaka et al., 2005; Shinohara et al., 2012), while others report flow in the range of 12-17 µm/sec (Nonaka et al., 2002; Buceta et al., 2005), and a final study reports flow in the range of 20-50 µm/sec (Okada et al., 1999). We conclude that given the large divergence and overlap between measurements from normal controls and LR-impaired animals in the published literature, it is impossible to conclude what measured values for these ciliary functions correspond to normal vs. abnormal states or consequences for asymmetry.
Clinical issue

All normal human embryos exhibit consistently asymmetric (sided) placement or anatomy of the heart, visceral organs, and brain. Disturbances in this process result in isomerism (lack of asymmetry), situs inversus (reversal of asymmetry), or heterotaxia (randomization of asymmetry among the organs). Such errors of laterality form a relatively common class of birth defects. Thus, understanding the molecular mechanisms that establish consistent and correct left-right (LR) asymmetry during embryogenesis is crucial. Work in chick and frog embryos has revealed a novel role for the important neurotransmitter serotonin in LR patterning; dissecting the molecular mechanism and timing of its involvement is important for assessing the impact of serotonergic pathway modulator drugs on fetal development.

Two main models for serotonin function during LR patterning exist. The EARLY model views serotonin as an instructive signal, because it is asymmetrically localized during early cleavage stages and initiates a cascade of asymmetric gene expression on the right side via interaction with epigenetic control machinery. The driving forces for serotonin’s rightward movement is a L-R voltage gradient established by very early intracellular (cytoskeletal) chirality present immediately after fertilization. In contrast, the LATE model proposes that serotonin is a permissive factor needed for the induction of ciliated tissue during neurulation stages (and holds that asymmetry is first generated by the vertical action of these cilia fairly late in development). A role for cilia as symmetry-breaking (initial) mechanism requires that no prior asymmetrical signaling exist.

Results

We used theory-neutral experiments, specifically designed to distinguish between two competing hypotheses, to determine both the timing (early versus late) and the location of serotonin’s role in LR patterning. Our gain-of-function experiment, injecting ectopic serotonin into targeted blastomeres, revealed that excess serotonin on the left side randomized asymmetry. Our loss-of-function experiment, injecting a molecular construct that would disrupt serotonin signaling, revealed that serotonin is
required in the ventral right cells. Our final experiment demonstrated that asymmetric genes become expressed on the left side of the embryo even when ciliary flow is not present. Importantly, a meta-analysis of ciliary parameters in the literature revealed that the variability in cilia length and number, even in unperturbed animals, is such that many ciliary function mutants fall indistinguishably in the range of “normal” ciliary measurements, revealing that quantification of cilia properties does not reliably distinguish between animals that have correct and incorrect left-right patterning.

Implications and Future Directions

The results of these experiments uniformly support the EARLY model. Moreover, our meta-analysis refutes the oft-cited link between parameters of cilia function and left-right asymmetry. Together with the recent discovery that even in mouse the early blastomeres are not functionally LR-equivalent, these data support the view that very early mechanisms of LR symmetry breaking should be considered, and that cilia are unlikely to be a well-conserved initiating step. Important future directions include the investigation of early (pre-node) mechanisms of serotonergic signaling in the mouse, and a dissection of the epigenetic steps that lead from intracellular serotonin redistribution to the differential gene expression that drives asymmetric organ morphogenesis. A better understanding of instructive patterning roles for this important neurotransmitter will significantly impact our understanding of neuropharmacology and the etiology of a wide range of birth defects.
Vandenberg et al. Figure 2
Table 1. Theory neutral experiments: predictions and results

<table>
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<th>LATE model’s prediction</th>
<th>Results</th>
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<tbody>
<tr>
<td>Ectopic serotonin injected at 4-cell</td>
<td>Greatest effects on left side</td>
<td>No effect, regardless of where injected</td>
<td>Consistent with EARLY</td>
</tr>
<tr>
<td>Injected DN serotonin receptor</td>
<td>Greatest effects on ventral right</td>
<td>Effects only on dorsal left</td>
<td>Consistent with EARLY</td>
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<tr>
<td>Left LPM explants (timing)</td>
<td>Explants express Xnr-1, lefty and pitx2 regardless of when isolated</td>
<td>Explants express Xnr-1, lefty and pitx2 only after onset of ciliary flow (stage 18)</td>
<td>Consistent with EARLY</td>
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Fig. S1. Regression analysis of abnormal pitx2 expression and improper organ laterality indicates that asymmetric gene expression overestimates effects on LR patterning. To determine how predictive pitx2 expression is of asymmetric organ position, we analysed published Xenopus studies that measured both for treatments that affect LR patterning (Chen et al., 2004; Hatayama et al., 2011; Kramer and Yost, 2002; Levin et al., 2002; Maisonneuve et al., 2009; Mogi et al., 2003; Sakano et al., 2010; Schweickert et al., 2007; Vick et al., 2009; Yasuhiko et al., 2001) and performed a regression analysis. Similar to what has been reported previously for the relationship between asymmetric expression of Xnr-1 and organ position (Vandenberg, 2012), abnormal localization of pitx2 overestimates the effects of treatments on organ laterality. This is demonstrated by the regression line with a Y-intercept of 18.5, indicating that a significant amount of abnormal pitx2 expression would have little to no effect on asymmetric organ position. Additionally, we examined the effects of three treatments we have studied including vibrations of 7Hz and 15Hz from 1-cell to st. 19 (Vandenberg et al., 2011) and injections with serotonin in the dorsal left blastomere at the 4-cell stage. This regression analysis indicated a Y-intercept of 24.9, again suggesting that pitx2 overestimates the effects of treatments on organ laterality. These results indicate that pitx2 expression should not be used as an accurate substitute for examining the effects of treatments on organ situs, the LR patterning event that is most important for health outcomes.