Serotonin Signaling Is a Very Early Step in Patterning of the Left-Right Axis in Chick and Frog Embryos

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Supplemental Experimental Procedures

Frog Drug Screen

Xenopus embryos were dejellied in 2% cysteine 30′ after artificial fertilization and washed in 0.1× MMR (Marc's modified Ringer's solution). Batches of eggs from a single female were divided into several experimental and control groups. Each group was put into either 10 ml of 0.1× MMR (controls) or 0.1× MMR containing drugs at between 10 μM and 1 μM final concentration. Embryos were allowed to develop in the drug-containing medium until stage 16 [S1], at which point they were washed three times in 0.1× MMR and allowed to develop in 0.1× MMR + 0.1% gentamycin until stage 45. All compounds were obtained from Sigma-RBI or Tocris. The phenotype of embryos was determined by scoring the situs of the heart, stomach, and gall bladder at stage 45. Only embryos with normal dorsoanterior development (DAI = 5) and clear left-sided or right-sided organs of normal morphology were scored. Embryos with ambiguous (unscoreable) situs comprised less than 5% of each experiment. The incidences of organ situs were analyzed by the chi-square test with Pearson correction for sample size.

Chick Drug Exposure

All experimental manipulations were performed on standard pathogen-free white leghorn chick embryos obtained from Charles River Laboratories (SPAFAS). Embryos were staged according to [S2]. To perform a pharmacological screen with minimal disturbance of normal morphogenesis, we optimized a chicken in ovo culture system. A small hole was made on the top of each egg (prior to incubation), and approximately 5 ml of light albumin was removed. The experimental solution consisted of a pharmacological reagent (between 10 μM and 1 μM final concentration) in chicken light albumin and Pannett-Compton solution at a ratio of 5:1. This was replaced into the egg, and the eggs were securely wrapped with Scotch tape and incubated at 37.5°C to the desired stages [S3], at which point they were fixed for analysis of laterality markers by in situ hybridization.

Results of Pharmacological Screen in *Xenopus* Embryos

Following a strategy recently used to identify electrogenic genes involved in asymmetry [S3], we performed a drug screen examining known elements of the serotonin pathway in *Xenopus*. The screen took advantage of the large numbers of frog embryos available, the low background level of heterotaxia (1%), and the numerous well-characterized serotonergic modulators developed by neurobiologists to implicate a manageable number of specific gene products for molecular functional analysis.

Batches of at least 100 *Xenopus* embryos were exposed to various drug inhibitors (at low micromolar concentrations; see Table S1) between fertilization and stage 12; they were then washed and allowed to develop to stage 45, and the situs of the heart, gut, and gall bladder were analyzed. Embryos with ambiguous (unscoreable) situs comprised less than 5% of each experiment. Reversal of any of the three organs from their normal situs was scored. In this assay (scoring the laterality of three organs), the maximum possible incidence of laterality phenotypes that can be detected is 87.5% (because in a group of embryos in which each of three organs is fully randomized, 12.5% will have the organs in their wt positions by chance and thus would be scored as normal). The detailed data are presented below (including statistical analyses) and summarized in Figure 1.

In all cases, at the low levels of blockers utilized, the only observed effect was randomization of the visceral organs. Strictly, the phenotype we observed was heterotaxia because single-organ reversals were noted as well as complete inversions. However, a number of reagents (notably R3 blockers) most often induced complete situs inversus. No general toxicity was observed; in particular, special attention was given to avoiding reagents that caused changes in dorso-anterior character to prevent confounding results with non-specific secondary effects on the LR axis [S4]. In all of the data given in subsequent sections of the results, the heterotaxia phenotype is specific (is not accompanied by other morphological defects).

In Situ Hybridization Methods

Chick embryos were fixed in 4% paraformaldehyde at 4°C overnight. Frog embryos were fixed in MEMFA at 4°C overnight. cDNA used as template for in situ hybridization with marker probes were: *cShh* and *cNodal* [S5], *XNodal* [S6], and *Xlefty* [S7]. Histological sections were obtained by embedding embryos after in situ hybridization in

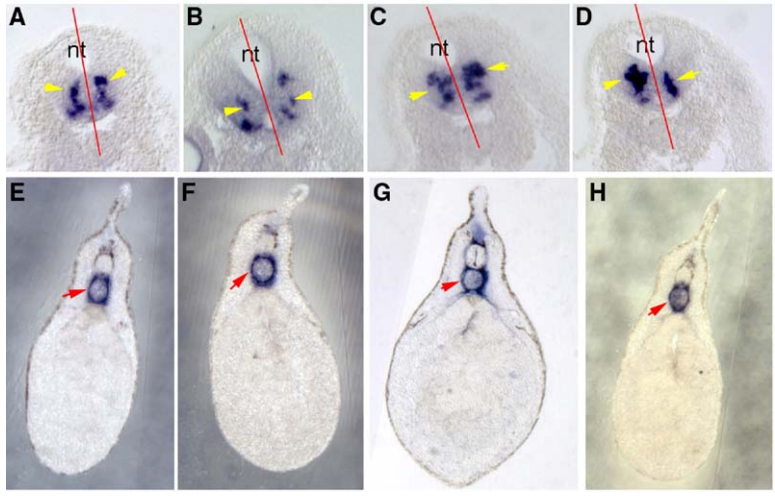


Figure S1. Midline Development Is Normal in Treated Embryos

Normal midline (notochord and neural tube) development in control embryos (A) results in two well-separated domains of expression of Tbx20 [S9] in motor neurons in the ventral neural tube. The same separation is observed in embryos treated with blockers of 5-HT-R3 (B), 5-HT-R4 (C), and MAO (D), indicating a lack of cyclopia in embryos in which elements of the 5-HT pathway have been downregulated. nt, neural tube; red line indicates axis of symmetry in the middle of the neural tube. Yellow arrowheads point to domains of Tbx20 expression. Likewise, immunohistochemistry with the antibody notochord marker Tor70 [S10] at stage 36 reveals that compared to controls (E), embryos exposed to blockers of 5-HT-R3 (F), 5-HT-R4 (G), and MAO (H) possess a notochord of normal size and position. Red arrows indicate notochord stain.

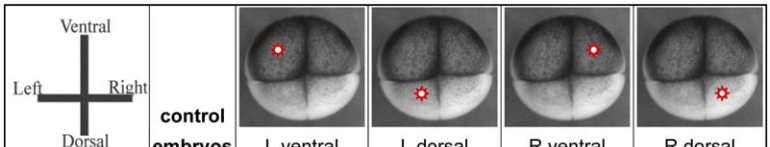
Table S1. Reagents Used

Name	Full Name	[Final]	Target	Reference
Pindol	1-(1H-Indol-4-yloxy)-3-(1-methylethyl)amino]-2-propanol	10 μM	R1	[S11]
Methysergide	[8β(S)]-9,10-Didehydro-N-[1-hydroxymethl)propyl]-1,6-dimethylergoline-8-carboxamide	1 μM	R1 and R2	[S11]
Methiothepin	1-[10,11-Dihydro-8-(methylthio)dibenzo[b.f]thiepin-10-yl]-4-methyliperazine	100 μM	R1 and R2	[S12]
Mianserin	1,2,3,4,10,14b-Hexahydro-2-methyldibenzo[c,f]pyrazino[1,2-a]azepine	1 μM	R1 and R2	[S13]
MDL72222	Tropanyl 3,5-dichlorobenzoate	1 μM	R3	[S14]
Tropisetron	endo-8-Methyl-8-azabicyclo[3.2.1]oct-3-ol Indol –3-yl-carboxylate	1 μM	R3	[S15]
GR113808	1-methyl-1*H*-indole-3-carboxylic acid, [1-[2-[(methylsulfonyl)amino] ethyl]- 4-piperidiny]methyl ester	1 μM	R4	[S16]
Ro04-6790	4-Amino-N-[2,6-bis(methylamino)-4-pyrimidinyl]-benzenesulfonamide	10 μM	R6	[S17]
DR4004	2a-[4-(4-phenyl-3,6-dihydro-2H-pyridin-1-yl)-butyl]-2a,3,4,5-tetrahydro-1H-benzo[cd]indol-2-one	10 μM	R7	[S18]
SB269970	[R]-3-[2-(2-[4-Methyl-piperidin-1-yl]ethyl)pyrrolidine-1-sulfonyl]phenol	10 μM	R7	[S19]
Meterogoline	N-CBZ-[(8β)-1,6-Dimethylergolin-8-yl]methylamine[(8β)-1,6-Dimethylergolin-8-yl]methyl]carbamic acid phenylmethyl ester	10 μM	R1, R2, R5, R6, and R7	[S20]
KYTRIL	granisetron hydrochloride	10 μM	R3	[S21]
QCN	3-(4-allyipiperazin-1-yl)-2-quinoxalinecarbonitrile maleate	1 μM	R3	[S22]
LY-278,584	1-Methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1H-indazole-3-carboxamine maleate	1 μM	R3	[S23]
SB-204070-A	1-butyl-4-piperidinylmethyl 8-amino-7-chloro-1, 4-benzodioxan-5-carboxylate hydrochloride	1 μM	R4	[S24]
SDZ-205,557	4-amino-5-chloro-2-methoxy-benzoic acid 2-(diethylamino)ethyl ester	10 μM	R4	[S25]
Harmine	7-Methoxy-1-methyl-9H-pyrido[3,4-b]indole	100 μM	MAO-A	[S26]
Clorgyline	N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy)propylamine hydrochloride	100 μM	MAO-A	[S27]
Pargyline	N-Methyl-N-(2-propynyl)benzylamine hydrochloride N-Methyl-N-propargylbenzylamine hydrochloride	100 μM	MAO-B	[S28]
Deprenyl	R(-)-N-a-Dimethyl-N-2-propynyl-benzeneethanamine hydrochloride	10 μM	MAO-B	[S29]
Furazolidone	3-(5-Nitrofurfurylideneamino)-2-oxazolidinone	100 μM	MAO-A,B	[S30]
Iproniazid	Isonicotinic acid 2-isopropylhydrazide phosphate salt	100 μM	MAO-A,B	[S31]
Nialamide	N-Isonicotinoyl-N'-[b-(N-benzylcarboxamido)ethyl]hydrazinePyridine-4-carboxylic 2-[2-(benzylcarbamoyl)ethyl]hydrazide	100 μM	MAO-A,B	[S32]
GR125487	5-Fluoro-2-methoxy-[1-[2-(methylsulfonyl)amino)ethyl]-4-piperidinyl]-1H-indole-3-methylcarboxylate sulfate	10 μM	R4	[S33]
Serotonin	5-Hydroxytryptamine hydrochloride	1 mM	R1–R7	[S34]
Melatonin	N-Acetyl-5-methoxytryptamine	100 μM	Melatonin receptors	[S35]
FMRF-amide	Phe-Met-Arg-Phe-NH_2	1 μM	Melatonin receptors	[S36]
Nisoxetine	(±)-g-(2-Methoxyphenoxy)-N-methyl-benzenepropanamine hydrochloride	10 μM	Noradre-naline pathway	[S37]
PCP	p-chlorophenylalanine	100 μM	TPH	[S38]
RS67333	1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone	10 μM	R4	[S39, S40]

JB4 according to the manufacturer's directions (Polysciences). In situ hybridization was performed as previously described [S8].

Cloning and Sequence Analysis

For 5-HT-R4, RNA was isolated from whole chicken embryo at stage 23 and from a brain of adult *Xenopus* by TRI-Reagent extraction (Molecular Research Center, Inc.). Reverse transcription of total RNA was performed with Superscript II (Invitrogen). PCR amplification of a 366 bp serotonin receptor 4 candidate fragment from chicken and *Xenopus* were carried out by using Ex-Taq (Takara) with degenerate primers (5′-CCIATIATGCARGGITGGAA-3′ and 5′-GTIACRAARAA IGGIGCCCA-3′). PCR was performed with the following cycle condi-



	control embryos		L ventral		L dorsal		R ventral		R dorsal	
Situs solitus (w.t.)	194	99%	170	93%	130	96%	157	82%	166	91%
Laterality affected:	1	1%	12	7%	5	4%	35	18%	16	9%
Situs inversus:	0	0%	3	25%	2	40%	14	40%	7	44%
Total embryos:	195		182		135		192		182	
p value			$3.2 \cdot 10^{-3}$		0.087		$5.7 \cdot 10^{-9}$		$2.9 \cdot 10^{-4}$	

Figure S2. R3 Loss-of-Function: Randomized LR Asymmetry in Embryos Injected with LY-278,584, an Antagonist of 5-HT-R3 at the 4-Cell Stage

Embryos with a reversal in any one of the three organs (heart, gut, gall bladder) was scored as "laterality affected." The fourth row indicates the percentage of the randomized embryos that exhibited full situs inversus (complete reversal of all viscera). Microinjection of the 5-HT-R3 blocker LY-278,584 into the right ventral blastomere at the 4-cell stage induced the highest incidence of laterality reversals, 18%. Of the affected embryos, 40% exhibited full situs inversus.

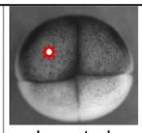
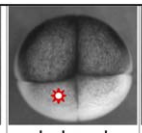
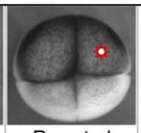
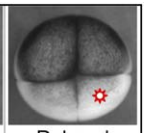
Ventral / Left / Right / Dorsal	control embryos		 L ventral		 L dorsal		 R ventral		 R dorsal	
Situs solitus (w.t.)	279	99%	120	94%	129	94%	136	80%	87	95%
Laterality affected:	3	1%	8	6%	8	6%	34	20%	5	5%
Situs inversus	1	33%	5	63%	3	38%	14	41%	2	40%
Total embryos:	282		128		137		170		92	
p value			$7.3 \cdot 10^{-3}$		$1.1 \cdot 10^{-2}$		$4.0 \cdot 10^{-12}$		$3.6 \cdot 10^{-2}$	

Figure S3. R3 Gain of Function: Randomized LR Asymmetry in Embryos Injected with a 5-HT-R3 Expression Construct at the 4-Cell Stage

Embryos with a reversal in any one of the three organs (heart, gut, gall bladder) was scored as "laterality affected." The fourth row indicates the percentage of the randomized embryos that exhibited full situs inversus. Microinjection of 5-HT-R3 mRNA into the right ventral blastomere at the 4-cell stage induced an incidence of laterality reversals 3- to 4-fold that of injections into the other blastomeres.

tions: denaturation for 1 min at 94°C, annealing for 1.5 min, and extension for 2 min at 72°C with the final extension for 8 min. The annealing temperatures of cycles 1–2 and 3–30 were 37°C and 54°C, respectively. The fragments were cloned into pCR2.1 with the TOPO cloning kit (Invitrogen) and then sequenced. The resulting sequence was used to design primers for targeting other regions. After determination of the complete cDNA sequence, a highly conserved stretch of the cDNA (aa 97 to 358) was amplified by PCR with specific primers: for chicken, cR4(289−), 5′-ACATCCCTCGATGTCTTGCT CAC-3′ and cR4(−1074), 5′-TAGCACATGAGTTGATCCGTTT-3′; for *Xenopus*, xR4(289−), 5′-ACATCACTTGACGTTCTCCTGAC-3′ and xeR4(−1074), 5′-TAATACATGAGTGGACCCATTG-3′. These fragments were used for in situ hybridization.

For Monoamine oxidase A/B (MAO-A/B), RNA was isolated from whole chicken embryo at stage 23 and from the brains of adult *Xenopus* by TRI-Reagent extraction (Molecular Research Center, Inc.). Reverse transcription of total RNA was performed with Superscript II (Invitrogen). PCR amplification of a 186 bp MAO candidate fragment from chicken and *Xenopus* both were carried out by using Ex-Taq. (Takara) with degenerate primers (5′-TATTAYAAIGARCCIT TCTGGAG-3′ and 5′-TCAGCCTTTCYTCTTTIGTIAGA-3′). Both PCRs were performed with the following cycle conditions: denaturation for 1 min at 94°C, annealing for 1.5 min, and extension for 2 min at 72°C with the final extension for 15 min. The annealing temperatures of cycles 1–4 and 5–30 were 40°C and 55°C, respectively. The fragments were cloned into pCR2.1 with the TOPO cloning kit (Invitrogen) and sequenced. The resulting sequence was used to design primer sets for PCR of other regions. After determination of the complete cDNA sequences, a highly conserved domain (aa 228 to 493) was amplified by PCR with specific primers: cMAO-B(681−), 5-CCGAGTGAAGCTGAAGAAACCAG-3′ and cMAO-B(−1483), 5′-GCTTTAGCAGTCCCGGTACAGAT-3′. This domain was used for in situ hybridization.

Sequences for the new clones have been submitted into GenBank under the following accession numbers: *Xenopus* R4: AY583535, Chick R4: AY583536, *Xenopus* MAO: AY675168, Chick MAO-B: AY583527. Alignment and sequence comparison of new R4 and MAO clones are shown in Table S9 and Figures S2 and S3.

Supplemental References

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Table S2. R3 Antagonists Had a Significant Randomizing Effect

Reagent	Controls		Kytril		MDL7222		QCN		LY-278,584		Tropisetron	
Situs solitus (wt)	234	98%	155	90%	139	82%	126	88%	250	79%	322	77%
Laterality affected	6	3%	18	10%	30	18%	17	12%	66	21%	98	23%
Situs inversus	3	50%	10	56%	27	90%	15	88%	60	91%	74	76%
Total Embryos	240		173		169		143		316		420	
p value			$1.5 \cdot 10^{-3}$		$2.2 \cdot 10^{-7}$		$4.3 \cdot 10^{-4}$		$3.7 \cdot 10^{-10}$		$3.5 \cdot 10^{-12}$	

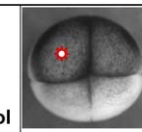
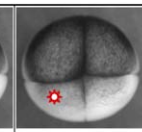
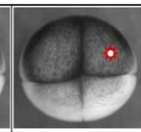
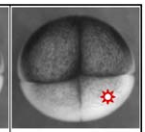
Ventral / Left / Right / Dorsal	control embryos		 L ventral		 L dorsal		 R ventral		 R dorsal	
Situs solitus (w.t.)	132	99%	148	90%	129	93%	161	80%	138	90%
Laterality affected:	2	1%	16	10%	10	7%	40	20%	16	10%
Situs inversus:	1	50%	9	56%	6	60%	20	50%	9	56%
Total embryos:	134		164		139		201		154	
p value			$6.3 \cdot 10^{-3}$		0.045		$1.5 \cdot 10^{-6}$		$4.1 \cdot 10^{-3}$	

Figure S4. Serotonin Loss of Function: Randomized LR Asymmetry in Embryos Injected with ABP, a Protein that Sequesters Serotonin

Microinjection of Amine Binding Protein, which sequesters and inactivates serotonin, into the right ventral blastomere at the 4-cell stage induced the highest incidence of laterality reversals, 20%. Of these, 50% exhibited complete situs inversus.

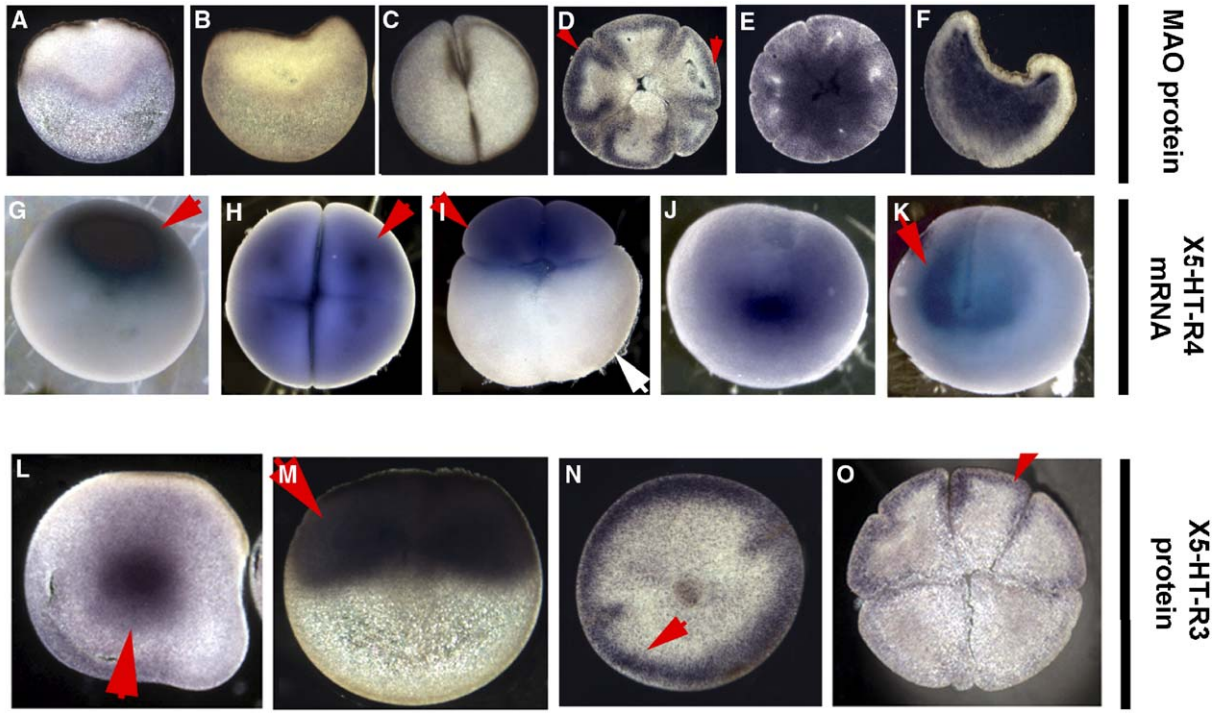


Figure S5. Localization of Serotonergic Pathway Members in Early Frog Embryos

Monoamine oxidase exhibited a very weak signal by immunohistochemistry between fertilization and 4-cell (A–C). However, it was strongly and symmetrically present in 16–32-cell-stage embryos (D and E). This timing is consistent with the drop off in 5-HT content observed during later cleavages by HPLC (Figure 1F). By gastrulation, it was very strongly localized within the endodermal cells (F). Maternal mRNA encoding *Xenopus* 5-HT-R4 was detected in the animal pole of unfertilized eggs (G, side view). At the 4-cell stage, the mRNA is symmetrically distributed in all blastomeres (H, animal pole view). At the 8-cell stage, the mRNA is present only in the animal pole blastomeres (I). During neurulation (stages 13–18), expression was detected in a pattern consistent with neural crest staining (J and K). 5-HT-R3 protein was detected as a tight spot in the center of unfertilized eggs (L). Shortly after fertilization, it was spread throughout the animal pole (M, 1-cell embryo) and then at the cell membrane of embryos during cleavage stages (N, 2-cell embryo; O, 16-cell embryo). Sections in (C), (D), (E), and (N) are perpendicular to the animal-vegetal (AV) axis. Sections in (A), (B), (F), (K), (M), and (O) are parallel to the AV axis.

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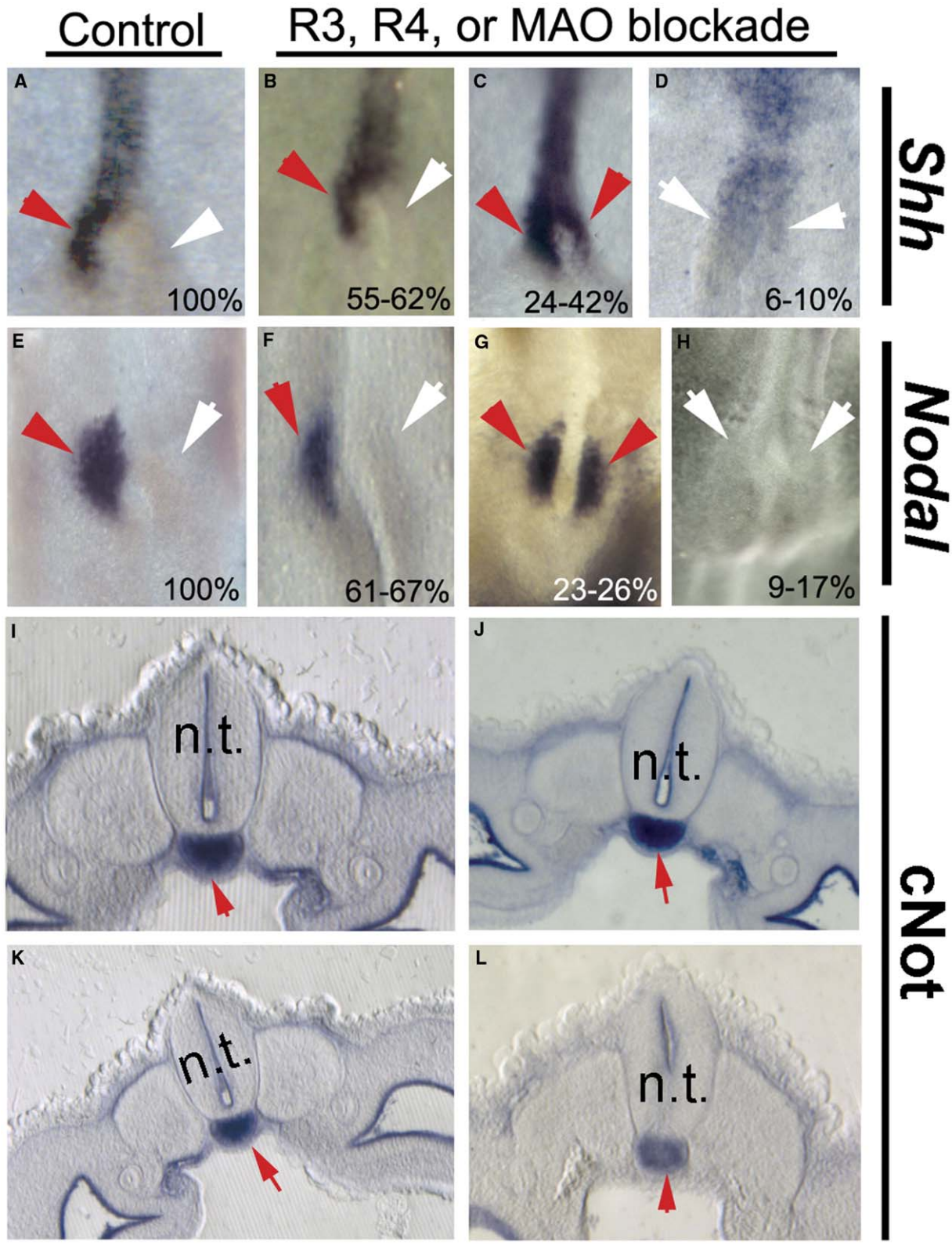

Figure S6. Serotonergic Signaling Is Upstream of the Early Asymmetric Gene Expression in Chick Embryos

Chick embryos were exposed in ovo to blockers of R3, R4, and MAO (numerical data is shown in Table S10). All embryos exposed to vehicle alone exhibited correct left-sided expression of *Sonic hedgehog* (*Shh*) at stage 5 (A). In contrast, embryos exposed to blockers of 5-HT-R3, -R4, and MAO in addition to left-sided *Shh* (B) frequently exhibited bilateral expression of *Shh* (C) as well as absent expression in the node (D). The proximal domain of the downstream gene *Nodal* was always left sided in control embryos at stage 7 (E) but, in addition to left-sided expression (F), was often bilateral in treated embryos (G) as well as absent (H). Red arrows indicate expression; white arrows indicate lack of expression. Percentages indicate range of phenotype incidence from different drugs in each class. Treated embryos at stage 23 were probed with cNot, an antibody marker of notochord, to ensure that changes in the laterality of asymmetric genes were not due to lack of midline development. Compared to control embryos (I), embryos treated with blockers of 5-HT-R3 (J), 5-HT-R4 (K), and MAO (L) exhibit normal morphology of neural tube and presence of notochord. Red arrowheads indicate expression of cNot. N.t., neural tube.

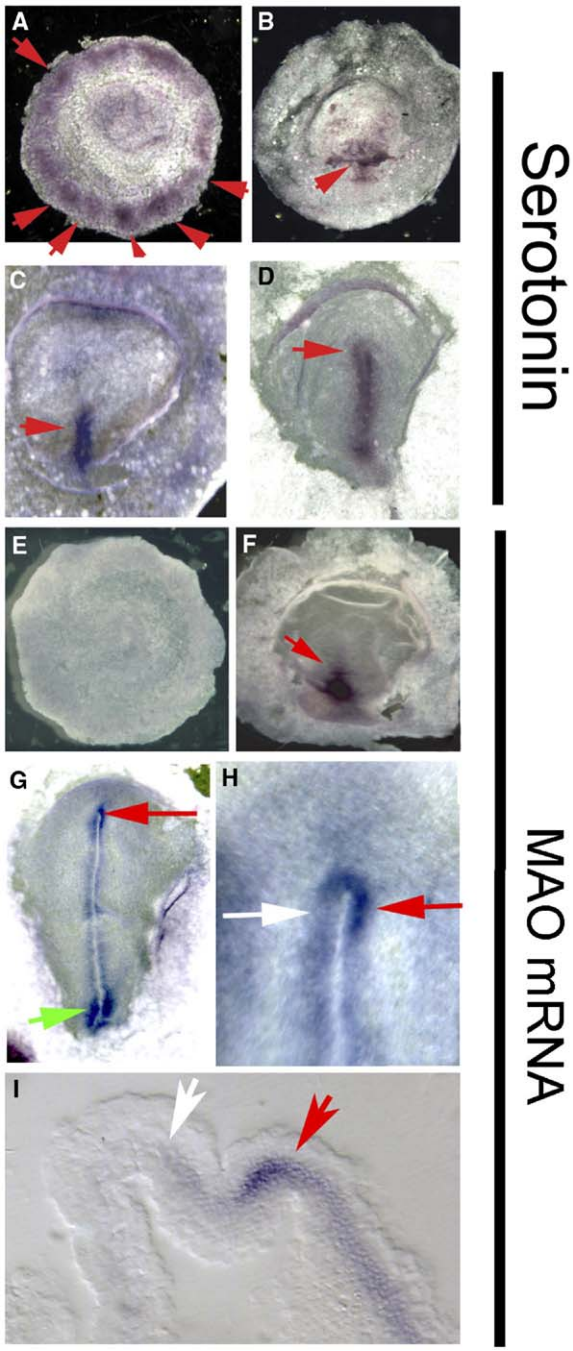

Figure S7. Serotonin and MAO Are Endogenously Expressed in Early Chick Embryos and MAO Expression Exhibits LR Asymmetry

In unincubated eggs, serotonin is detected in a radially symmetric pattern as a number of discrete spots around the periphery of the area opaca (A). At the initiation of the primitive streak, serotonin becomes specifically localized to the base of the primitive streak (B). During streak elongation, serotonin is detected in the cells of the primitive streak from stages 2–4 (C and D). Sectioning revealed that serotonin-containing cells are in the mesodermal layer at stage 3^{+} (data not shown). Monoamine oxidase transcripts are not detected in unincubated chick eggs (E). At the initiation of the primitive streak, it is expressed in nascent streak cells (F) and in the streak during elongation. At stage 4, MAO is expressed in the right side of the node as well as weakly in the primitive ridges and more strongly at the base of the primitive streak (G). The asymmetric pattern of MAO is clearly seen in the close-up view of Hensen's node (H) and confirmed in sections through the node (I). Red arrows indicate expression; white arrows indicate lack of expression. All embryos are shown from their dorsal side. The section in (I) is shown with dorsal upwards.

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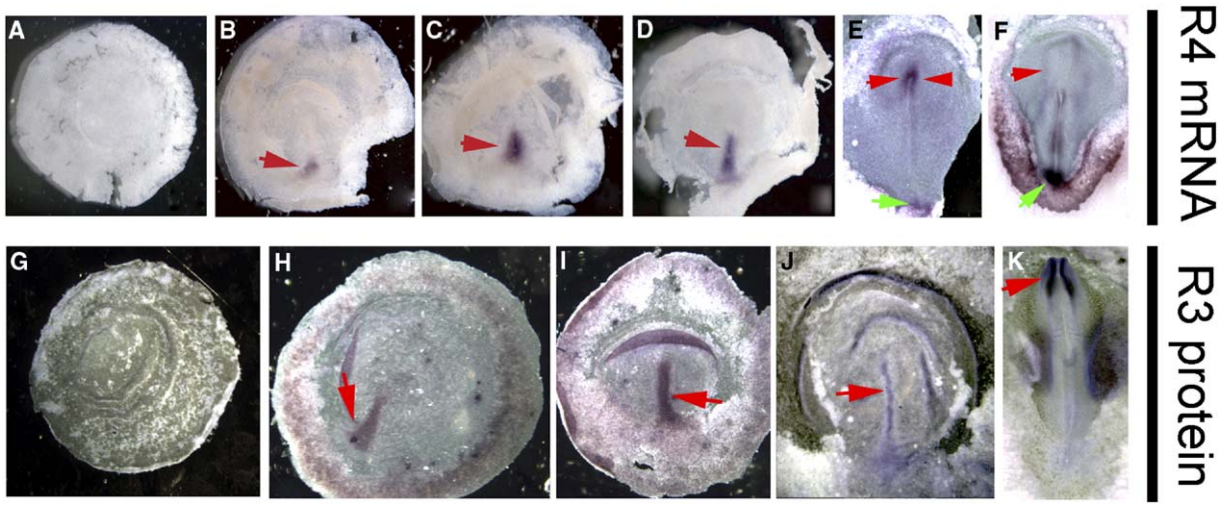

Figure S8. Localization of R3 and R4 in Early Chick Embryos

We characterized the expression of endogenous serotonergic genes in early streak embryos. Using degenerate PCR based on an alignment of mammalian genes, we cloned native chick R4 and MAO-B, which bear 90% and 77% identity to their mammalian homologs. We then performed in situ hybridization analysis. 5-HT-R4 transcripts are not detected in unincubated chick eggs (A). At the initiation of the primitive streak, R4 is expressed in nascent streak cells (B) and in the streak during elongation (C and D). 5-HT-R4 expression was detected at the base of the primitive streak and symmetrically in Hensen's node (E). At stage 7, the strong streak and node signal remains, whereas a weaker expression can be detected around the margin of the neural plate (F). 5-HT-R3 protein is not detected in unincubated eggs (G). It is strongly present in the primitive streak during elongation (H–J). In somite-stage embryos, 5-HT-R3 is present strongly in the neural folds (K). In all panels, white arrowheads indicate lack of expression, red arrowheads indicate anterior regions of expression, and green arrowheads indicate posterior expression domains.

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Table S3. R4 Antagonists Had a Significant Randomizing Effect

Reagent	Controls		SB-204070A		GR125487		GR113808		SDZ-205,557	
Situs solitus (wt)	257	100%	201	78%	223	76%	151	56%	185	86%
Laterality affected	0	0%	58	22%	71	24%	118	44%	31	14%
Situs inversus	0	0%	31	53%	53	75%	66	56%	12	39%
Total embryos	257		259		294		269		216	
p value			$2.5 \cdot 10^{-15}$		$9.3 \cdot 10^{-17}$		$6.4 \cdot 10^{-33}$		$1.1 \cdot 10^{-9}$	

Table S4. MAO Inhibitors Had a Significant Randomizing Effect

Reagent	Controls		Harmine		Clorgyline		Pargyline		Deprenyl		Furazolidone		Iproniazid		Nialamide	
MAO Isoform	—		MAO-A		MAO-A		MAO-B		MAO-B		MAO-A,B		MAO-A,B		MAO-A,B	
Situs solitus (wt)	135	100%	130	74%	125	70%	126	78%	183	72%	181	68%	272	70%	116	78%
Laterality affected	0	0%	46	26%	53	30%	36	22%	70	28%	85	32%	115	30%	32	22%
Situs inversus	0	0%	19	41%	29	55%	21	58%	42	60%	44	52%	40	35%	16	50%
Total embryos	135		176		178		162		253		266		387		148	
p value			$3.5 \cdot 10^{-10}$		$1.05 \cdot 10^{-11}$		$1.5 \cdot 10^{-8}$		$3.8 \cdot 10^{-11}$		$3.6 \cdot 10^{-13}$		$1.85 \cdot 10^{-12}$		$2.9 \cdot 10^{-8}$	

Table S5. Antagonists of R1, R2, R5, R6, and R7 Did Not Affect Laterality

Reagent	Controls		Pindol		Methysergide		Methiothepin		Mianserin		Ro04-6790		DR4004		SB269970		Metergoline	
Target	—		R1		R1 and R2		R1 and R2		R1 and R2		R6		R7		R7		R1, R2, R5, R6, and R7	
Situs solitus (wt)	192	99%	185	99%	226	99%	159	99%	261	98%	318	99%	247	100%	225	100%	254	100%
Laterality affected	1	1%	2	1%	3	1%	1	1%	4	2%	2	1%	1	0%	1	0%	1	0%
Situs inversus	1	100%	1	50%	2	67%	0	0%	3	75%	0	0%	1	100%	0	0%	0	0%
Total embryos	193		187		229		160		265		320		248		226		255	
p value			0.98		0.74		0.56		0.58		0.66		0.77		0.73		0.79	

Table S6. Manipulation of Other Pathways Did Not Affect Laterality

Reagent	Controls		Melatonin		FMRF		Nisoxetine	
Target	—						(Noradrenaline)	
Situs solitus (wt)	368	100%	122	94%	81	100%	243	91%
Laterality affected	1	0%	8	6%	0	0%	2	1%
Total	132		130		81		245	
p value			0.031		0.40		0.84	

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Table S7. Early Blockade of R3 and R4 Signaling Randomizes Laterality

	R3 Blockade Stages 0–7		R3 Blockade Stages 7–12		R4 Blockade Stages 0–7		R4 Blockade Stages 7–12	
Situs solitus (wt)	93	74%	134	96%	80	62%	115	93%
Laterality affected	32	26%	5	4%	50	38%	9	7%
Total embryos	125		139		130		124	
p value	$6.9 \cdot 10^{-7}$				$9.6 \cdot 10^{-9}$			

Exposure of embryos to R3 and R4 blockers (see Table S1) between stages 0 and 7 induces significant heterotaxia, whereas exposure after stage 7 does not.

Table S8. Effect of GJC Blockade between 4 Cell and Stage 6^+

	Controls		GJC Block between 4 Cell and Stage 6^+	
Situs solitus (wt)	79	99%	83	81%
Laterality affected	1	1%	19	19%
Total embryos	80		102	
p value	$4.98 \cdot 10^{-4}$			

Because our data implicate the necessity of GJC for asymmetry during stages at which serotonin is localized and because previous studies of GJC timing in *Xenopus* did not specifically examine the period from 4-cell stage and stage 6^+, we tested the role for GJC during that time period. Embryos exposed to the GJC blockers heptanol, carbenoxolone, and lindane as in [S41] exhibited 19% heterotaxia when scored at stage 45.

Table S9. Effects of 5-HT Modulation on Expression of Asymmetric Genes in Frog Embryos

Drug	Gene	Left	Right	Bilateral	None	Total	% Situs Solitus	% Laterality Defects	χ^2	p Value
Tropisetron	Nodal	98 64%	9 6%	23 15%	23 15%	153	64%	**36%**	49.23	$2.27 \cdot 10^{-12}$
	Lefty	146 84%	4 2%	9 5%	15 9%	174	84%	**16%**	28.51	$9.28 \cdot 10^{-8}$
GR113808	Nodal	85 57%	15 10%	39 26%	11 7%	150	57%	**43%**	64.08	$1.19 \cdot 10^{-15}$
	Lefty	141 86%	5 3%	4 2%	14 9%	164	86%	**14%**	23.42	$1.30 \cdot 10^{-6}$
Iproniazid	Nodal	77 57%	13 10%	28 21%	17 13%	135	57%	**43%**	62.08	$3.29 \cdot 10^{-15}$
	Lefty	162 85%	3 2%	7 4%	19 10%	191	85%	**15%**	26.72	$2.35 \cdot 10^{-7}$
Control	Nodal	17 100%	0 0%	0 0%	0 0%	17	100%	0%		
	Lefty	25 100%	0 0%	0 0%	0 0%	25	100%	0%		

Inhibition of R3, R4, and MAO randomize the laterality of the normally left-sided markers, *Nodal* (*XNR-1*) and *Lefty*. All of the reagents were used at levels indicated in Table S1 (identical to dosage used in initial pharmacological screen in Figure 1). Bold text indicates a significant effect.

Table S10. Effects of 5-HT Modulation on Expression of Asymmetric Genes in Chick Embryos

Drug:	Gene	Left	Right	Bilateral	None	Total	% Correct	% Wrong	χ^2	p Value
5-HT	Shh	28 55%	0 0%	18 35%	5 10%	51	55%	**45%**	9.658	0.002
	Nodal	35 65%	0 0%	13 24%	6 11%	54	65%	**35%**	9.736	0.002
5-HTP	Shh	30 58%	0 0%	19 37%	3 6%	52	58%	**42%**	8.701	0.003
	Nodal	35 61%	0 0%	13 23%	9 16%	57	61%	**39%**	11.29	0.001
Tropisetron	Shh	32 62%	0 0%	16 31%	4 8%	52	62%	**38%**	7.433	0.006
	Nodal	34 67%	0 0%	12 24%	5 10%	51	67%	**33%**	8.901	0.003
GR113808	Shh	29 55%	0 0%	19 36%	5 9%	53	55%	**45%**	9.791	0.002
	Nodal	35 65%	0 0%	14 26%	5 9%	54	65%	**35%**	9.736	0.002
Iproniazid	Shh	29 55%	0 0%	22 42%	2 4%	53	55%	**45%**	9.791	0.002
	Nodal	36 67%	0 0%	9 17%	9 17%	54	67%	**33%**	8.981	0.003
Control	Shh	17 100%	0 0%	0 0%	0 0%	17	100%	0%		
	Nodal	25 100%	0 0%	0 0%	0 0%	25	100%	0%		

Embryos were treated in ovo from just prior to incubation to stage 4$^-$. Exogenous serotonin as well as inhibition of R3, R4, and MAO randomize the laterality of the normally left-sided markers, *Sonic Hedgehog* and *Nodal*. Bold text indicates a significant effect.