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Serotonin Transporter Function Is an Early Step in Left-Right Patterning in Chick and Frog Embryos

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Key Words
Serotonin · 5-Hydroxytryptamine · Serotonin transporter · Vesicular monoamine transporter · Left-right asymmetry · *Xenopus* · Chick

Abstract
The neurotransmitter serotonin has been shown to regulate a number of embryonic patterning events in addition to its crucial role in the nervous system. Here, we examine the role of two serotonin transporters, the plasma membrane serotonin transporter (SERT) and the vesicular monoamine transporter (VMAT), in embryonic left-right asymmetry. Pharmacological or genetic inhibitors of either SERT or VMAT specifically randomized the laterality of the heart and viscera in *Xenopus* embryos. This effect takes place during cleavage stages, and is upstream of the left-sided gene *XNR-1.* Targeted microinjection of an SERT-dominant negative construct confirmed the necessity for SERT function in embryonic laterality and revealed that the descendants of the right ventral blastomere are the most dependant upon SERT signaling in left-right patterning. Moreover, the importance of SERT and VMAT in laterality is conserved in chick embryos, being upstream of the early left-sided gene *Shh.* Endogenous transcripts of SERT and VMAT are expressed from the initiation of the primitive streak in chick and are asymmetrically expressed in Hensen's node. Taken together our data characterize two new right-sided markers in chick gastrulation, identify a novel, early component of the left-right pathway in two vertebrate species, and reveal a new biological role for serotonin transport.

Introduction

The indoleamine serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter that regulates psychodynamic function [Lucki, 1998]. It is linked to a variety of diverse medical conditions including tumor growth [Yim et al., 2000; Meijer et al., 2003; Russo et al., 2003], circadian rhythm disorders [Morin, 1999], and cardiac disease [Frishman and Grewall, 2000]. Its crucial role in controlling neuronal activity [Lucki, 1998] has allowed it to be directly implicated in both normal cognition and a number of syndromes such as memory impairment [Buhot et al., 2000], sleep disorders [Jouvet, 1999], schizophrenia [Millan, 2000], aggression [Lesch and Merschdorf, 2000], depression [Stockmeier, 1997], and other mood disorders.

It is now appreciated that serotonin has another crucial role: control of morphogenesis during early stages, prior to the appearance of neurons [Buznikov and Shmukler,

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1981; Whitaker-Azmitia et al., 1996; Buznikov et al., 2001]. Serotonin is widely distributed in both the animal and the plant kingdoms [Pennati et al., 2001], and occurs in mammalian [Shuey et al., 1990; Wu et al., 1999] and other vertebrate and invertebrate embryos [Manukhin et al., 1981; Emanuelsson et al., 1988]. Serotonin is synthesized by embryonic stem cells [Walther, 1999], and 5-HT is likely to play a role in egg maturation [Buznikov et al., 1993], mitogen-induced vascular remodeling [Fanburg and Lee, 1997], neural crest migration [Moiseiwitsch and Lauder, 1995], control of cleavage and cell division rates, patterning of cytoskeletal structures [Emanuelsson et al., 1988; Lee et al., 1991], blastomere adhesion, gastrulation movements [Hamalainen and Kohonen, 1989; Colas et al., 1999a, b], control of gap-junctional communication [Moore and Burt, 1995; Rorig and Sutor, 1996], craniofacial patterning [Lauder and Zimmerman, 1988; Shuey et al., 1992, 1993], and the specification of neuronal identity and connectivity in the developing nervous system [Lauder et al., 1981, 1983]. Indeed, links between the use of 5-HT pathway pharmaceuticals and birth defects suggest that these roles may exist in human embryos as well, making it crucial to understand mechanisms of serotonin function in development [Lauder, 1985; Chambers et al., 1996; Moiseiwitsch, 2000].

The understanding of nonneuronal functions of serotonin is a very important yet poorly understood aspect of evolutionary developmental biology, and significant molecular advances have been made in understanding the molecular cell biology of serotonin. The serotonin pathway [Cooper et al., 1996] includes the enzymes which synthesize 5-HT from tryptophan, seven major types of membrane receptors which transduce the serotonin signal into different cellular events (5-HT R1–R7), and a system for enzymatic degradation of 5-HT [via monoamine oxidase (MAO)]. There are also two important families of serotonin transporters.

Free 5-HT cannot remain in the cytosol because it is catabolized by MAO on the outer membranes of mitochondria [Gershon et al., 1990]. To avoid degradation and provide for vesicular release, 5-HT is transported into vesicles through the action of the vesicular monoamine transporter (VMAT) [Kirk et al., 1997]. VMAT (VMAT2 in neurons, and VMAT1 in other tissues) uses proton gradients generated by the V-ATPase [Harvey, 1992] to drive transport exchanging two protons in the lumen of the vesicle for one monoamine in the cytoplasm [Knoth, 1981a, b; Kanner, 1987; Edwards, 1992]. The vesicles release 5-HT from cells through exocytosis; once in the extracellular space, 5-HT is available to diffuse and be sensed by serotonin receptors on the membrane of neighboring cells.

In contrast to VMAT, plasma membrane serotonin transporters (SERTs) use the Na^+ gradient to remove transmitter from synapses [Haase et al., 2001; Torres et al., 2003]. SERT is an extremely important regulator of nervous system activity and is thus a very popular target for pharmaceutical efforts in biomedicine [Owens and Nemeroff, 1998; Schloss and Williams, 1998; Bartlett et al., 2005; Noblett and Coccaro, 2005]; this work has led to the development of a number of selective serotonin reuptake inhibitors, of which Prozac is a leading example [Wong et al., 1995]. Interestingly, SERT function has also been implicated in embryonic morphogenesis [Shuey et al., 1992; Persico et al., 2000, 2001; Gaspar et al., 2003] and the regulation of cell proliferation [Eddahibi et al., 2001; Sari and Zhou, 2003]. In light of the fascinating yet largely unexplored roles for neurotransmitter signaling in embryogenesis, and the importance of transporters in regulating serotonin movement, it is interesting to explore possible roles of SERT and VMAT in the patterning of major body axes.

Invariant left-right (LR) asymmetry is a key feature of vertebrate morphogenesis that raises profound questions in cell, evolutionary, and developmental biology [Palmer, 2004; Levin, 2005]. It also has important implications for the biomedicine of laterality-based birth defects [Burn, 1991; Casey and Hackett, 2000; Morelli et al., 2001]. Within the last 10 years, insight at the molecular level has been gained into the mechanisms which underlie consistent laterality of the heart and viscera during embryonic development [Wood, 1997; Yost, 2001]. While the middle phase of LR patterning (the asymmetric gene cascade) is fairly well understood [Levin, 1998; Whitman and Mercola, 2001], upstream events and their conservation among species present many open questions [Burdine and Schier, 2000; Boorman and Shimeld, 2002; Levin, 2003b]. Seeking to discover epigenetic mechanisms and small molecule signals functioning upstream of the asymmetric gene cascade, we have recently shown that serotonergic signaling through receptor subtypes 3 and 4, at very early stages, was a crucial component of the LR pathway in chick and frog embryos [Fukumoto et al., 2005]. However, the possible functions of transporters – a crucial element of serotonin signaling – had remained unexplored. Thus, we sought to characterize possible roles of 5-HT transport in determination of consistent laterality. Here, we show that both SERT and VMAT are involved in LR patterning at early stages in two vertebrate species. Taken together, our data identify novel roles for serotonin

transporters, reveal a new conserved component of LR patterning, and suggest another possible teratological endpoint for the use of SERT modulators during pregnancy.

Materials and Methods

Cloning

Chick SERT. RNA was isolated from whole chicken embryos at stage 23 by TRI-Reagent extraction (Molecular Research Center). Reverse transcription of total RNA was performed with Superscript II (Invitrogen). PCR amplification of a 139-bp SERT candidate fragment was carried out using EX-Taq (Takara) with degenerate primers (5′-TTRGGITACTGYATHGGIACITC-3′ and 5′-CCACAIGGHATTTCIGTIGGTGT-3′). PCR was performed using the following cycle conditions: denaturation for 1 min at 94°C, annealing for 1 min, and extension for 1.5 min at 72°C with final extension for 15 min. The annealing temperatures of cycles 1, 2, and 3–30 were 42°C, 46°C, and 59°C, respectively. The fragments were cloned into pCR2.1 using the TOPO cloning kit (Invitrogen), 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 291–671) was amplified by PCR using specific primers: cSERT (870–): 5′-ATTAGCTGGCAATTGACCCTCTG, and cSERTEnd: 5′-TTACACGGCATTCATGCGGATGT. This fragment was used for in situ hybridization after being transferred into the pBluescript expression vector. This sequence was the same as the previously cloned GenBank sequence AY573844 [Larsen et al., 2004].

Chick VMAT. RNA was isolated from whole chicken embryos at stage 23 by TRI-Reagent extraction (Molecular Research Center). Reverse transcription of total RNA was performed with Superscript II (Invitrogen). PCR amplification of a 276-bp VMAT candidate fragment was carried out using EX-Taq (Takara) with degenerate primers (5′-ATCTGGATGATGGARACIATGTG-3′ and 5′-TAGCCCATDATIGGCATCATIGA-3′). PCR was performed using the following cycle conditions: denaturation for 1 min at 94°C, annealing for 1 min, and extension for 1 min at 72°C with final extension for 15 min. The annealing temperatures of cycles 1, 2, and 3–30 were 45°C, 48°C, and 59°C, respectively. The fragments were cloned into pCR2.1 using the TOPO cloning kit (Invitrogen), 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 263–461) was amplified by PCR using specific primers: cVMAT (785–): 5′-TGGCTCTGTTCGATGGAGCTGTT, and cVMAT (–1381): 5′-CTATAATTGTCATGAGCCATGGA. This fragment, matching VMAT2, was used for in situ hybridization after being transferred into the pBluescript expression vector. This sequence has been submitted to GenBank under the accession number AY974771.

Xenopus *Drug Exposure*

Xenopus embryos were dejellied in 2% cysteine 30 min after artificial fertilization and washed in 0.1 × Marc's modified Ringer's solution (MMR). 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 nisoxetine, fluoxetine, alaproclate, citalopram, imipramine, reserpine, and TBZOH at a final concentration of 10 μ*M*, 10 μ*M*, 10 μ*M*, 10 μ*M*, 10 μ*M*, 100 μ*M*, and 10 μ*M*, respectively. Embryos were allowed to develop in the drug-containing medium until stage 16 [Nieuwkoop and Faber, 1967], at which point they were washed three times in 0.1 × MMR and allowed to develop in 0.1 × MMR + 0.1% gentamicin until stage 45. All compounds were obtained from Sigma-RBI and Tocris.

Scoring Xenopus *Embryonic Situs*

The phenotype of embryos was determined by scoring the situs (position) of the stomach, the gallbladder, and the atrium of the heart, under a dissecting microscope using tricaine to immobilize the stage 45 embryos. Only embryos with normal dorsoanterior development (dorsoanterior index = 5) and clearly left-sided or right-sided organs of normal morphology were scored. Embryos with ambiguous (unscoreable) situs comprised less than 5% of each experiment. An embryo was considered to be affected when one or more of those three organs was/were reversed in its position. The incidences of abnormal organ situs were analyzed using the χ^2 test with Pearson correction for sample size. Statistically, if the sidedness of all three organs were independently and fully randomized, the maximum possible effect would be 87.5%, since 12.5% of the embryos would be scored as wild type (because all three organs would lie in their normal positions by chance).

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 Hamburger and Hamilton [1992]. 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 were removed. The experimental solution consisted of a pharmacological reagent (final concentration: fluoxetine 100 μ*M*, reserpine 100 μ*M*) in chicken light albumin and Pannett-Compton solution at a ratio of 5:1. This was replaced into the egg, the eggs were securely wrapped with Scotch tape, and were incubated at 37.5°C to the desired stages, at which point they were fixed for analysis of laterality markers by in situ hybridization.

In situ Hybridization

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* [Levin et al., 1995], and *XNodal* [Sampath et al., 1997]. Histological sections were obtained by embedding embryos after in situ hybridization in JB4 according to the manufacturer's directions (Polysciences). In situ hybridization was performed as previously described [Harland, 1991].

Xenopus *Blastomere Injection*

Synthetic mRNA was transcribed by the T7 polymerase from linearized pCDNA3 plasmids containing the D98G mutant of SERT. About 100 pg of the construct mRNA were mixed with 50 ng of RLD and 250 pg of mRNA encoding β-galactosidase (as lineage label) and injected into the blastomere indicated.

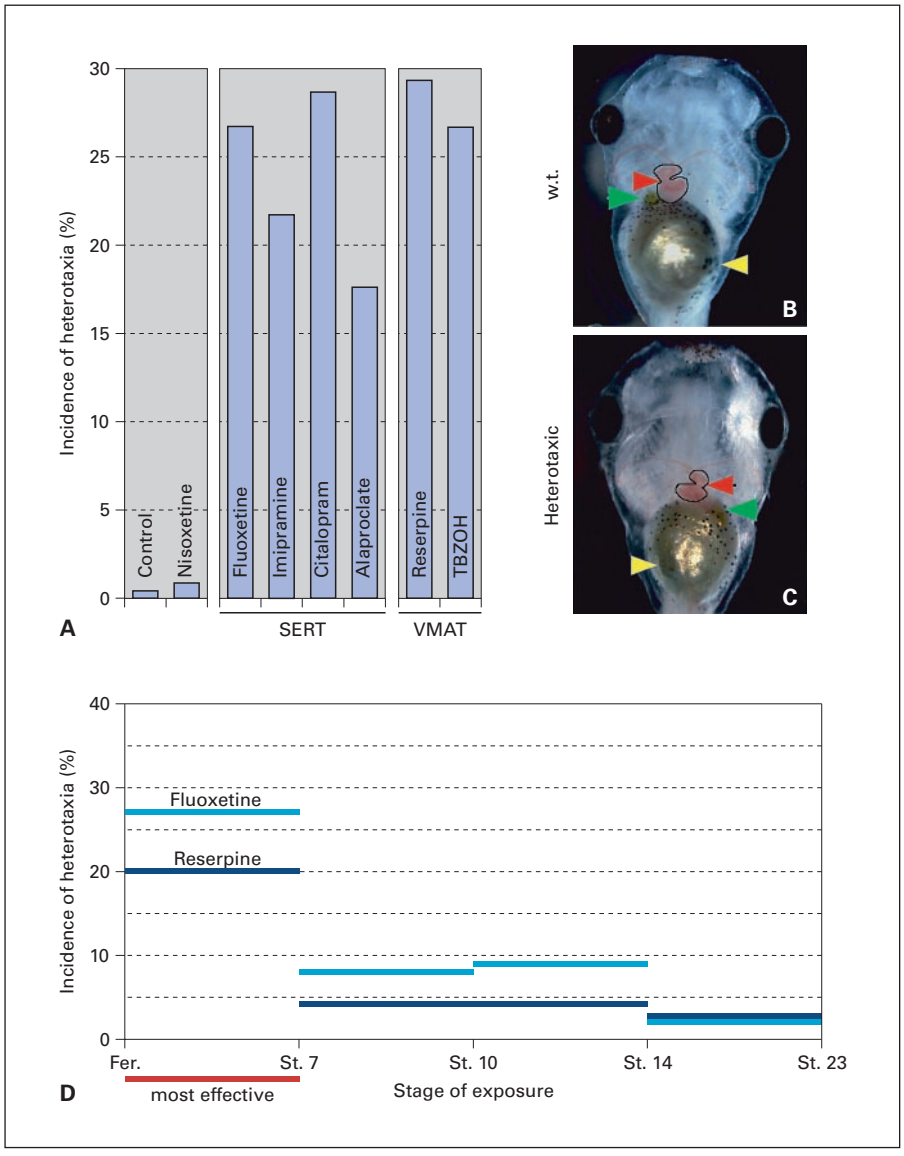


Fig. 1. Serotonin transport is required for normal LR asymmetry in *Xenopus.* **A** Exposure to specific inhibitors of SERT and VMAT from fertilization to stage 16 (0.1 × MMR containing nisoxetine, fluoxetine, alaproclate, citalopram, imipramine, reserpine, and TBZOH at a final concentration of 10 μ*M*, 10 μ*M*, 10 μ*M*, 10 μ*M*, 10 μ*M*, 100 μ*M*, and 10 μ*M*, respectively) induced significant levels of heterotaxia – independent reversal of one or more of the visceral organs (compare **B** vs. **C**, which show representative examples) in *Xenopus* embryos (see tables 1 and 2 for numerical data and statistical analysis). **D** By initiating exposure at different time points during development, it was found that the most sensitive time period is from fertilization to stage 7.

Results

Specific Inhibitors of SERT and VMAT Induce Heterotaxia in Xenopus *Embryos*

To determine whether SERT function was important for laterality, we capitalized on the highly specific and well-characterized pharmacological reagents developed by recent efforts in biomedical neuroscience [Ramasubbu, 2004]. Following the same strategy recently used to identify electrogenic [Levin et al., 2002] and serotonergic [Fukumoto et al., 2005] components in asymmetry, we sought to take advantage of the large numbers of frog embryos available, the low background level of heterotaxia, and the well-characterized and specific blockers of SERT and VMAT to implicate these targets for further molecular functional analysis.

Batches of at least 100 *Xenopus* embryos were exposed to one of the SERT blockers, i.e. fluoxetine [Wong et al., 1995], imipramine [Goulet et al., 2001], citalopram [Horschitz et al., 2001], or alaproclate [Fowler et al., 1987], or the VMAT inhibitors reserpine [Costa et al., 1977] or TBZOH [Zucker et al., 2001], from fertilization to stage 16. Embryos were then washed, allowed to develop to stage 45, and analyzed for the situs of the heart, gut, and gallbladder. The data are shown in figure 1A. The incidence of reversal of one or more of the three organs

Table 1. SERT blockers significantly randomize LR asymmetry

Reagent	Controls		Nisoxetine		Fluoxetine		Citalopram		Alaproclate		Imipramine	
Situs solitus (wild type)	263	100%	243	99%	137	73%	112	71%	178	82%	267	78%
Laterality affected	1	0%	2	1%	50	27%	45	29%	38	18%	74	22%
Situs inversus	0	0%	0	0%	26	52%	23	51%	17	45%	48	65%
Total embryos	264		245		187		157		216		341	
p value			0.95		$1.2 \cdot 10^{-17}$		$1.0 \cdot 10^{-18}$		$2.1 \cdot 10^{-11}$		$8.0 \cdot 10^{-15}$	

Control embryos or embryos exposed to nisoxetine exhibited normal asymmetry. In contrast, embryos exposed to blockers of SERT from fertilization to stage 16 and scored at stage 45 for the laterality of the heart, gut, and gallbladder exhibited between 18 and 29% incidence of heterotaxia (reversals of one or more of the heart, gut, or gallbladder).

Table 2. VMAT blocker significantly randomizes LR asymmetry

Reagent	Controls		Reserpine		TBZOH	
Situs solitus (wild type)	263	100%	299	71%	220	73%
Laterality affected	1	0%	124	29%	80	27%
Situs inversus	0	0%	58	47%	40	50%
Total embryos	264		423		300	
p value			$3.1 \cdot 10^{-21}$		$1.9 \cdot 10^{-18}$	

In contrast to controls, embryos exposed to VMAT blockers from fertilization to stage 16 and scored at stage 45 for the laterality of the heart, gut, and gallbladder exhibited 27–29% incidence of heterotaxia (reversals of one or more of the heart, gut, or gallbladder).

from their normal situs (fig. 1B) was scored (fig. 1C). In this assay (scoring the situs of three organs), the maximum possible incidence of laterality phenotypes that can be detected is 87.5% (because in a set of embryos in which each of three organs is fully randomized, 12.5% will have the organs in their wild-type positions by chance and thus be scored as normal). The detailed data are presented in tables 1 and 2.

In all cases, at the low micromolar levels of blockers utilized, the only observed effect was the independent randomization of the position of the heart and visceral organs. Strictly, the phenotype we observed was heterotaxia (an independent assortment of the organs – a loss of concordance), since single-organ reversals were observed as well as inversions of all three or any two organs. No general toxicity was observed; in particular, special attention was given to avoiding reagents which caused changes in dorsoanterior character, to prevent confounding results with nonspecific secondary effects on the LR axis [Danos and Yost, 1996]. In all of the data given in subsequent sections of the results, the heterotaxia phenotype is specific (is not accompanied by other morphological defects).

Control batches of embryos exposed to vehicle alone exhibited a background level of 1% heterotaxia; likewise, embryos exposed to nisoxetine, a selective inhibitor of norepinephrine uptake [Python et al., 1997], exhibited normal laterality (table 1). In contrast, embryos exposed to SERT or VMAT blockers exhibited a significant level of heterotaxia – an average of 24% for SERT blockers, and 28% for VMAT blockers. Based on this loss-of-function data, serotonin transport is implicated in the establishment of LR asymmetry in *Xenopus.*

SERT and VMAT Function during Early Embryogenesis in Xenopus

In order to determine what aspects of LR patterning might depend upon SERT or VMAT, we exposed embryos to specific blockers at different stages of development. We analyzed the results of exposures beginning at different time points (tables 3 and 4). We observed (fig. 1D) that exposure during cleavage stages resulted in

Table 3. Timing of SERT involvement in asymmetry

	SERT blockade							
	stages 0–7		stages 7–10		stages 10–14		stages 14–23	
Situs solitus (wild type)	99	73%	107	92%	103	91%	126	98%
Laterality affected	36	27%	9	8%	10	9%	3	2%
Total embryos	135		116		113		129	

Embryos were exposed to the SERT blocker fluoxetine at the stages indicated, then washed, and scored for the laterality of the heart and viscera at stage 45. Exposure covering fertilization to stage 7 was most effective in inducing heterotaxia (reversals of one or more of the heart, gut, or gallbladder).

Table 4. Timing of VMAT involvement in asymmetry

	VMAT blockade							
	stages 0–7		stages 7–10		stages 10–14		stages 14–23	
Situs solitus (wild type)	120	80%	118	96%	101	96%	108	97%
Laterality affected	30	20%	5	4%	4	4%	3	3%
Total embryos	150		123		105		111	

Embryos were exposed to the VMAT blocker reserpine at the stages indicated, then washed, and scored for the laterality of the heart and viscera at stage 45. Exposure covering fertilization to stage 7 was most effective in inducing heterotaxia (reversals of one or more of the heart, gut, or gallbladder).

Table 5. SERT and VMAT inhibitors symmetrize the expression of *XNR-1*

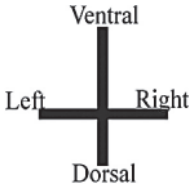
Drug	Left	Right	Bilateral	None	Total	Normal, %	Abnormal, %	χ^2	p value
Fluoxetine	96	0	34	0	130	74	26	15.5	$3.3 \cdot 10^{-114}$
Reserpine	93	0	37	0	130	72	28	17.7	$1.5 \cdot 10^{-135}$
Control	69	0	0	3	72	97	3		

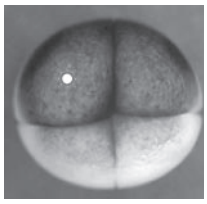
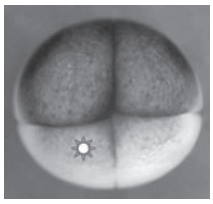
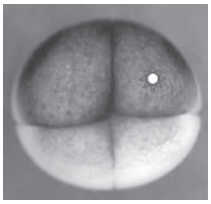
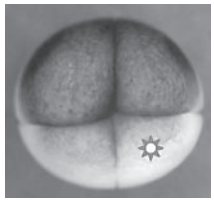
Embryos were exposed to blockers of SERT and VMAT from fertilization to stage 16 and processed for in situ hybridization with the left-sided *Nodal (XNR-1).* Inhibition of either SERT or VMAT leads to the symmetrical expression of *Nodal.*

the most effective level of randomization (20% for the VMAT inhibitor, 27% for the SERT inhibitor), while later exposures resulted in low or insignificant levels of disturbance of laterality. These data suggest that SERT and VMAT function in early steps of LR patterning, taking place between fertilization and stage 7.

To establish epistasis between serotonin transport and known LR patterning components, we assayed the expression of *XNR-1,* the earliest asymmetric marker known in *Xenopus,* in embryos exposed to SERT and VMAT blockers as above. The data and statistical analyses are presented in table 5. Control embryos exhibited the normally left-sided expression of *XNR-1,* while embryos exposed to fluoxetine and reserpine exhibited respectively 26 and 28% bilateral expression of *XNR-1.* The bilateral expression of *XNR-1* was exactly the same as previously described [Lohr et al., 1997; Levin and Mercola, 1998; Levin et al., 2002; Bunney et al., 2003;

Table 6. Microinjection of an SERT D98G mutant construct randomizes asymmetry



	Control embryos		 L ventral		 L dorsal		 R ventral		 R dorsal	
Situs solitus (wild type)	201	99%	151	94%	143	93%	146	82%	148	91%
Laterality affected	2	1%	10	6%	10	7%	33	18%	14	9%
Situs inversus	0	0%	5	50%	5	50%	20	61%	9	64%
Total embryos	203		161		153		179		162	
p value			0.013		0.01		$1.1 \cdot 10^{-8}$		$9.9 \cdot 10^{-4}$	

mRNA encoding a nonfunctional SERT mutant construct was injected into embryos at the 4-cell stage, and scored at stage 45. Embryos with a reversal of any one of the three organs (heart, gut, gallbladder) was scored as 'laterality affected' [the fourth row indicates the percentage of the randomized embryos that exhibited full situs inversus (complete reversal of all organs)]. Misexpression in the right ventral blastomere induced the highest incidence of heterotaxia (reversals of one or more of the heart, gut, or gallbladder).

Fukumoto et al., 2005]. Thus, we conclude that the functions of SERT and VMAT in the LR pathway are upstream of the asymmetric gene cascade as it is known in *Xenopus* embryos.

Molecular Loss of Function of SERT Reveals an Asymmetry among Blastomeres

The pharmacological reagents are useful for implicating targets for molecular analysis, and for testing the timing of serotonin transport in embryonic patterning. We next sought to use a specific molecular reagent to confirm the involvement of SERT in *Xenopus* embryogenesis. Because SERT proteins must form homomultimers [Chen et al., 1997a, b, 1998; Smicun et al., 1999; Chen and Rudnick, 2000; Sitte et al., 2004], a nonfunctional SERT mutant which reaches the cell surface but does not perform serotonin transport would serve as a dominant negative mutant by titrating out wild-type SERT complexes [Barker et al., 1999; Ramsey and DeFelice, 2002]. We thus utilized the D98G mutant, which has been tested in *Xenopus* oocytes, to explore the dominant negative effects of SERT [Barker et al., 1999; Ramsey and DeFelice, 2002].

We microinjected synthetic, capped mRNA encoding the SERT D98G mutant into each blastomere of frog embryos at the 4-cell stage. The data and statistical analyses are presented in table 6. Misexpression of this dominant negative form of SERT induced 18% randomization of the organs when injected into the right ventral cell. Targeting the other cells produced less effect; the embryos injected in the right dorsal side exhibited half (9%) as much heterotaxia, while the left dorsal and ventral cells exhibited 6–7% heterotaxia. These data confirm the importance of SERT function in early embryogenesis, and reveal that the descendants of the right ventral blastomere are likely to be the major locus of SERT function in the LR pathway.

SERT and VMAT Are Expressed in the Primitive Streak in Early Chick Embryos

In order to test the evolutionary conservation of this novel mechanism, and explore its properties in a model system with a radically different gastrulation architecture, we sought to ask whether SERT and VMAT may be present endogenously in chick embryos prior to the expression of *Shh* (before the development of serotonergic neurons). To test this prediction and characterize the involvement of SERT and VMAT in LR-relevant processes, we cloned and examined the expression of native chick SERT and VMAT during gastrulation.

Using degenerate primers based on alignment of mammalian sequences, we cloned chick SERT and VMAT, which respectively had 91 and 84% homology to the mouse homologs at the amino acid level. In situ hybridization with a probe antisense to cVMAT revealed no expression prior to incubation (fig. 2A); expression began at the initiation of the primitive streak (fig. 2B) and continued throughout the streak during its elongation

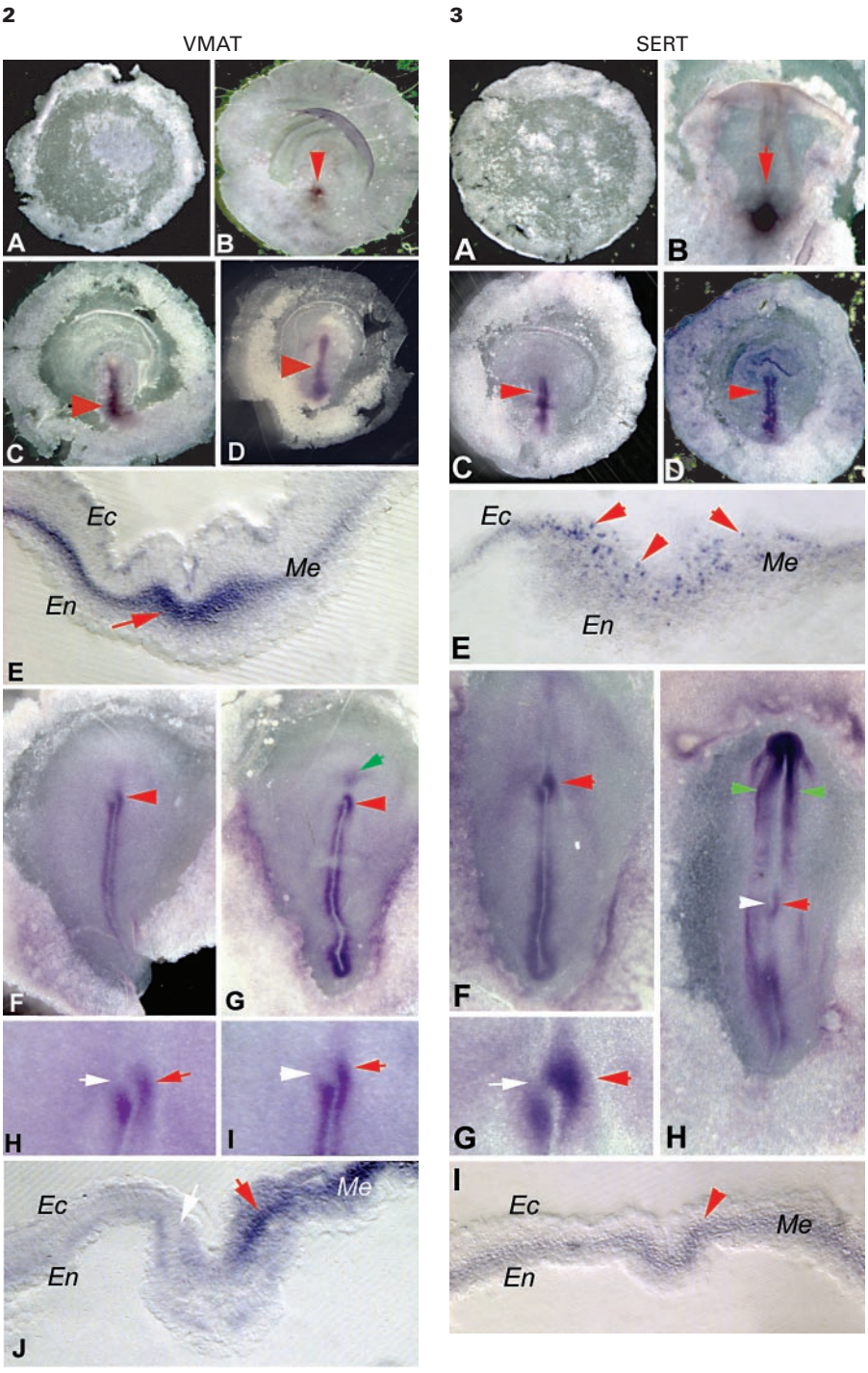


Fig. 2. Expression of VMAT in chick embryos. **A** Embryos prior to incubation exhibited no expression of VMAT. **B** At stage 1, VMAT transcripts can be detected at the base of the primitive streak. At stages 2 (**C**) to 3 (**D**), VMAT is expressed throughout the streak. Sectioning reveals that the expression is mesodermal (**E**). At stages 4 (**F**) and 5 (**G**), VMAT is expressed in the primitive ridges and more strongly expressed on the right side of the node. The asymmetry is shown in close-ups of Hensen's node in **H** (stage 4) and **I** (stage 5). Sectioning confirms the asymmetry in the node (**J**). Ec = Ectoderm; En = endoderm; Me = mesoderm. Red arrowhead indicates streak expression; green arrowhead indicates expression at the nascent notochord cells leaving the node; white arrowhead indicates lack of expression.

Fig. 3. Expression of SERT in chick embryos. **A** Embryos prior to incubation exhibited no expression of SERT. **B** At stage 1, abundant SERT transcripts can be detected at the base of the primitive streak. At stages 2 (**C**) to 3 (**D**), SERT expression can be detected throughout the streak. Sectioning reveals that a punctate subpopulation of cells express SERT; these cells are mainly mesodermal and ectodermal (**E**). At stage 4+, SERT is expressed in Hensen's node (**F**, red arrowhead) and in the primitive ridges (**F**, close-up in **G**). At stage 7+, expression can be detected in the neural folds, and SERT signal can be more strongly seen on the right side of Hensen's node (**H**). Sectioning reveals that at stage 4+, expression is mesodermal (**I**). Ec = Ectoderm; En = endoderm; Me = mesoderm. Red arrowhead indicates streak expression; green arrowhead indicates continuing expression at the base of the primitive streak; white arrowhead indicates lack of expression.

(fig. 2C, D). Sectioning revealed that it was the mesodermal cells which expressed VMAT, and that cells somewhat lateral to the streak also expressed VMAT (fig. 2E). At stages 4–5, expression was observed in the primitive ridges (fig. 2F, G), and expression could be observed in the notochord cells leaving the node (fig. 2G, green arrow). In Hensen's node, stronger VMAT expression was observed on the right side (fig. 2F, G, close-ups in fig. 2H, I, section in fig. 2J), revealing VMAT as a new right-sided asymmetric marker.

In situ hybridization revealed a similar profile for cSERT; it was not detected before incubation (fig. 3A),

Table 7. Effects of SERT and VMAT inhibition on expression of asymmetric genes in chick

Drug	Gene	Left	Right	Bilateral	None	Total	Wrong	p value
Fluoxetine	*Shh*	34	0	15	4	53	36%	0.01
		64%	0%	28%	8%			
	Nodal	37	0	10	4	51	27%	0.01
		73%	0%	20%	8%			
Reserpine	*Shh*	33	0	17	3	53	38%	0.007
		62%	0%	32%	6%			
	Nodal	34	0	11	5	50	32%	0.004
		68%	0%	22%	10%			
Control	*Shh*	17	0	0	0	17	0%	
		100%	0%	0%	0%			
	Nodal	25	0	0	0	25	0%	
		100%	0%	0%	0%			

Embryos were treated in ovo from just prior to incubation to stage 4+ with inhibitors of SERT (fluoxetine) and VMAT (reserpine), and processed for in situ hybridization with the left-sided genes *Shh* and *Nodal.* Inhibition of either SERT or VMAT effectively randomizes the expression of both markers.

but became strongly expressed at the base of the primitive streak (fig. 3B) and was detected throughout the streak during its early elongation (fig. 3C, D). Interestingly, sectioning (fig. 3E) revealed that unlike VMAT, which was expressed throughout the mesoderm cells in the streak, SERT was expressed in a punctate pattern in the ectoderm and mesoderm, indicating that only a subpopulation of cells expressed SERT. At stages 4–5 (fig. 3F, G), Hensen's node expressed SERT more strongly on the right side; expression was detected in the primitive ridges in the rostral two thirds of the streak. At stage 7+ (fig. 3H), the neural folds expressed SERT (consistent with the appearance of serotonergic neurons) and the right-sided asymmetry in Hensen's node became more pronounced (compare fig. 3G, H, red vs. white arrowhead). Sectioning (fig. 3I) revealed mesodermal expression, but only a weak lateralization, consistent with a very narrow region of asymmetry being present (fig. 3H). These expression data confirm the existence of endogenous targets of fluoxetine and reserpine in the chick embryo during the stages at which exposure to these blockers randomize, reveal that SERT and VMAT are expressed in the organizing center of the chick embryo in which many LR patterning events are occurring, and identify a novel right-sided asymmetric marker. Taken together, our data strongly suggest that SERT and VMAT are involved in early steps that pattern the LR axis of frog and chick embryos.

Specific Inhibitors of SERT and VMAT Randomize Asymmetry in Chick Embryos

In light of the expression data, we next tested the hypothesis that SERT and/or VMAT function in chick embryos [Mortensen et al., 2001; Larsen et al., 2004] was an important endogenous component of LR patterning. The data and statistical analyses are shown in table 7. Chick embryos exposed to fluoxetine and reserpine between the start of incubation and fixing at stage 4+ exhibited a significant randomization of the expression of the early left-sided marker *Shh* and the downstream marker *Nodal* (compare fig. 4A to fig. 4B, and fig. 4D to fig. 4E). The incidence of incorrect *Shh* sidedness was 36 and 38% for SERT and VMAT, respectively, consistent with the magnitude of laterality defects induced by SERT/VMAT inhibition in frog. These data (fig. 4C, F) support the necessity for SERT and VMAT function upstream of the consistent left-sided *Shh* expression, and suggest that SERT and VMAT must function between stages 1 and 4.

Discussion

Because the serotonin system includes machinery for manufacturing serotonin (a source), 5-HT degradation (a sink), and sensing of 5-HT levels (via receptors), this system is an ideal candidate for a morphogen-like signal. Indeed, the serotonergic synapse has been suggested to have evolved from far more primitive signaling events

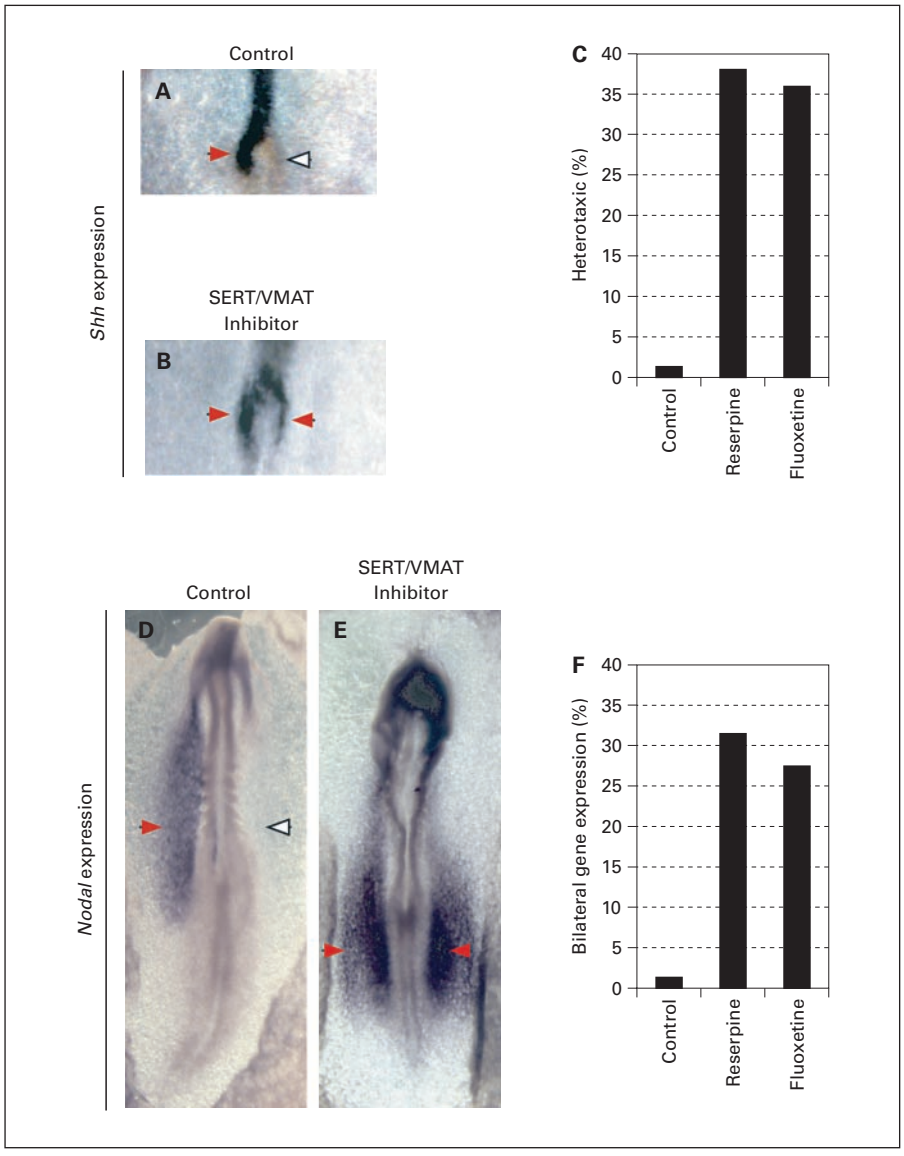


Fig. 4. Serotonin transport is required for normal LR asymmetry in chick embryos. Expression of the early left-sided gene *Shh* (**A**) was made bilateral (**B**) by exposure to inhibitors of SERT or VMAT prior to incubation (**C**, see numerical data and statistical analysis in table 6). The expression of the downstream marker *Nodal,* which is normally left-sided (**D**), was also made bilateral (**E**) by exposure to the SERT and VMAT blockers (**F**, see numerical data and statistical analysis in table 6).

between embryonic blastomeres [Buznikov and Shmukler, 1981; Buznikov, 1984; Buznikov et al., 1996]. We have recently demonstrated that in both chick and frog embryos, serotonin exists long before the appearance of neurons, and that 5-HT signaling through the R3 and R4 receptor subtypes is an obligate aspect of the patterning of the LR axis [Fukumoto et al., 2005]. However, the possible role of serotonin transporters was not tested in that study. Since both the availability of serotonin to intracellular signaling mechanisms, and its concentration outside of neighboring cells can be controlled by SERT and VMAT function, any understanding of 5-HT signaling in a patterning context will have to take into account the possible involvement of transport.

Several different SERT inhibitors with different mechanisms of action specifically randomized asymmetry, while a multitude of compounds targeting different ion transporters [Levin et al., 2002; Levin, 2003a] and blockade of a transporter of a different neurotransmitter (nisoxetine) had no effect. While drug experiments cannot conclusively prove the involvement of a target, our pharmacological data (utilizing low micromolar doses, somewhat higher than the IC_{50} in mammals, to compensate for possible structural divergence between frog and mammalian transporters) strongly support the involvement of SERT and VMAT in LR patterning since a strong and specific heterotaxia phenotype was observed. The pharmacological implication of SERT is further strengthened by mo-

lecular data showing that LR randomization was induced by misexpression of a nonfunctional SERT mutant to titrate out native functional SERT complexes. The importance of 5-HT transporters is consistent with the existence of serotonin in prenervous chick and frog embryos; indeed, 5-HT is endogenously localized in the primitive streak and node in the chick [Emanuelsson et al., 1988; Fukumoto et al., 2005], and is thus available to be moved across membranes by SERT and VMAT proteins expressed in these important organizing centers.

We analyzed the timing of the involvement of these new components in the LR pathway. Because washout of drugs is extremely difficult to demonstrate, instead of exposures which end at particular time points, we analyzed the results of exposures beginning at different times; this strategy previously allowed us to ascertain the timing of function of gap-junctional communication [Levin and Mercola, 1998] and H,K-ATPase [Levin et al., 2002] in asymmetry. Blockade of SERT or VMAT taking place after stage 7 had little effect on laterality, while earlier blockade randomized effectively. Injections of mutant SERT mRNA at the 4-cell stage can result in 18% heterotaxia, which is close to the average of the heterotaxia incidence induced by pharmacological blockade between stage 1 and stage 16, suggesting that there is not an important component to SERT function prior to the first few cell cleavages. Since protein from injected constructs will not be translated and functional for about an hour [unpubl. observations], this suggests that SERT probably functions in the LR pathway between the 16/32-cell stage and stage 7. The randomization of *XNR-1* expression by SERT and VMAT blockers is consistent with an early role for serotonin transport upstream of asymmetric gene expression.

The microinjections of a nonfunctional SERT mutant allowed us to test the spatial properties of serotonin transport in the LR pathway. We observed that the descendants of the right ventral blastomere were most sensitive to titration of SERT complex by nonfunctional SERT proteins, suggesting a spatially patterned role for serotonin transport. The right ventral blastomere and its descendants have previously been implicated as a crucial locus for LR patterning with respect to serotonergic signaling [Fukumoto et al., 2005] and H^+/K^+ flux [Levin et al., 2002]. The endogenous distribution of SERT and VMAT proteins among the descendants of the right ventral cells is not yet known; antibodies specific to *Xenopus* SERT/VMAT proteins will have to be developed and used in immunohistochemistry on early embryos, since mRNA expression analysis is insufficient in the *Xenopus* system (which contains a significant maternal protein component at cleavage stages).

Since the conservation of early LR mechanisms of asymmetry among phyla is currently controversial [Burdine and Schier, 2000; Essner et al., 2002; Levin, 2003b], we examined the role of SERT and VMAT in chick embryos. Serotonin has previously been found in the streak stage chick embryo [Lauder et al., 1981; Emanuelsson et al., 1988], and our functional data have recently implicated MAO, R3, and R4 in chick asymmetry prior to neurulation [Fukumoto et al., 2005]. The expression of endogenous SERT and VMAT in chick embryos was localized in the primitive streak – an important organizing center – and is consistent with an early role, since endogenous 5-HT and MAO are also localized in the streak and node. The asymmetric expression of VMAT in Hensen's node identifies it as a new right-sided marker, and suggests that its function is able to provide differential serotonergic signals to the right and left sides of the chick organizer. Crucially, the randomization of the early asymmetric marker *Shh* by SERT and VMAT blockade during early streak stages suggests that serotonin transport is a crucial component of the 5-HT pathway in chick embryos.

Serotonin has been implicated in aspects of axial patterning in other species. Auxin (a very close analog to serotonin) is involved in the establishment of bilateral symmetry in plants [Liu et al., 1993], while misexpression of a 5-HT receptor in sea urchins causes extra spicules properly patterned with respect to animal/vegetal axis but without asymmetry on the oral/aboral axis [Cameron et al., 1994]. Moreover, the 5-HT-R1a receptor has recently been implicated in the patterning of the dorsoventral axis in *Xenopus* [Kim and Han, 1999]. The involvement of SERT and VMAT in mouse asymmetry has not yet been explored; however, since serotonin reuptake inhibitors are known to influence ciliary motion [Uhler et al., 2000], and cilia appear to be a component of LR patterning in mammals [Essner et al., 2002; Tabin and Vogan, 2003], it is possible that future studies will uncover a role for serotonin transport in mammalian asymmetry. Wide conservation of serotonergic signaling in axial patterning would suggest that the use of selective serotonin reuptake inhibitors during pregnancy might lead to laterality-specific teratologies in human beings, although this has not yet been reported [Pastuszak et al., 1993].

How might serotonin transport link to known LR patterning mechanisms? Unfortunately, given the small size of serotonin, current technology does not permit labeling it in such a way as to allow in vivo analysis of its move-

ment. All available tags which are compatible with detection in living cells alter the molecular weight of serotonin by an order of magnitude, likely significantly altering its interaction with endogenous binding partners, as well as the rate and extent of its movement. Future advances in labeling and in vivo imaging may allow tracking the movement of 5-HT in living embryos, which will be crucial to gaining a detailed, molecular understanding of how its intracellular movement controls asymmetry. Nevertheless, a number of hypotheses may reasonably be proposed in light of available data.

First, SERT and VMAT may function primarily by controlling the localization of 5-HT. Expression of transporters in chick is asymmetric, and thus may be expected to differentially regulate the availability of intracellular and extracellular 5-HT on the left versus right sides of Hensen's node. Since storage of serotonin in vesicles prevents catabolism by MAO prior to synaptic release [Kirk et al., 1997], VMAT activity may interact with the right-sided MAO expression [Fukumoto et al., 2005] to precisely control 5-HT levels. The function of SERT can increase cytoplasmic 5-HT but also reduce the availability of serotonin to neighboring cells. Serotonin levels are known to be important for asymmetry [Fukumoto et al., 2005], and future work will determine whether this occurs by virtue of an interaction of 5-HT with extracellular R3 and R4, or with cytoplasmic serotonin-binding proteins [Jimenez Del Rio, 1992, 1993; Del Rio et al., 1995], or both.

Another possibility is that SERT participates in LR asymmetry by virtue of its interaction with 14-3-3 proteins. We have recently shown that 14-3-3 proteins are an important component of LR patterning in frog embryos during early cleavage stages [Bunney et al., 2003]. The reported interaction of SERT with 14-3-3 isoforms [Haase et al., 2001] suggests that SERT might function in the LR pathway through regulation of 14-3-3 protein signaling.

A final hypothesis concerns the known importance for ion flux in LR patterning of a number of species; both membrane voltage level and pH conditions are important for normal laterality [Levin et al., 2002; Levin, 2003a; McGrath et al., 2003; Raya et al., 2004]. VMAT moves monoamines from the cytoplasm into secretory and synaptic vesicles, and this substrate transport is thermodynamically connected to the outward movement of H^+ ions across membrane [Schuldiner, 1994; Liu and Edwards, 1997; Amara and Sonders, 1998]. Thus, one important component of VMAT function is through its coupling to cytoplasmic pH gradients. SERT is usually not electrogenic, but displays at least 4 conducting states; 3 of these states are permeable to Na^+ and the other to protons [Lester et al., 1996; Cao et al., 1997, 1998; Mager et al., 1998]. However, SERT can be electrogenic, such as by association with syntaxin 1A [Quick, 2003], because an unbalanced number of charges can be translocated in each transport cycle. Interestingly, 5-HT-induced currents have been shown to be suppressed by the D98G mutant [Ramsey and DeFelice, 2002]. Thus, it is possible that serotonin regulates LR asymmetry by virtue of SERT- and VMAT-mediated changes in ion flows in early cells.

Taken together, our data reveal a new biological role for serotonin transporters – guidance of important morphogenetic events well before neuronal development – and identify 5-HT movement across membranes as a novel, early component of the LR pathway in two vertebrate species. Future studies will elucidate the molecular details of serotonin movement in early embryos, and reveal novel aspects of the developmental biology of this versatile signaling molecule.

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