Intercellular calcium signaling in a gap junction-coupled cell network establishes asymmetric neuronal fates in *C. elegans*

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SUMMARY

The *C. elegans* left and right AWC olfactory neurons specify asymmetric subtypes, one default AWC<sub>OFF</sub> and one induced AWC<sub>ON</sub>, through a stochastic, coordinated cell signaling event. Intercellular communication between AWCs and non-AWC neurons via a NSY-5 gap junction network coordinates AWC asymmetry. However, the nature of intercellular signaling across the network and how individual non-AWC cells in the network influence AWC asymmetry is not known. Here, we demonstrate that intercellular calcium signaling through the NSY-5 gap junction neural network coordinates a precise 1AWC<sub>ON</sub>/1AWC<sub>OFF</sub> decision. We show that NSY-5 gap junctions in *C. elegans* cells mediate small molecule passage. We expressed vertebrate calcium-buffer proteins in groups of cells in *C. elegans* cells mediating calcium waves. Vertebrate calcium-buffer proteins induce AWC<sub>OFF</sub> and promote AWC<sub>ON</sub> through NSY-5 gap junctions. Our results suggest a novel model in which calcium has dual roles within the NSY-5 network: autonomously promoting AWC<sub>OFF</sub> and non-autonomously promoting AWC<sub>ON</sub>.

KEY WORDS: Gap junctions, Calcium signaling, Stochastic left-right neuronal asymmetry, *C. elegans*

INTRODUCTION

Many cell types communicate through gap junctions during embryonic development to regulate cell-fate determination, proliferation and morphogenesis (Levin, 2007). Gap junctions form intercellular channels that assemble from head-to-head docking of hemichannels, one provided by each of two contacting cells (Bennett and Zukin, 2004). Each hemichannel consists of subunits called connexins and pannexins in vertebrates, and innexins in invertebrates. Gap junction-mediated intercellular communication allows rapid and direct transfer of small molecules and ions across a field of cells without the need for specialized ligands and receptors. Gap junction-mediated calcium waves divide the mammalian neocortex into distinct neuronal domains (Yuste et al., 1995; Bennett and Zukin, 2004). Spontaneous gap junction-dependent calcium waves are also observed in the developing retina (Kandler and Katz, 1998). In addition, gap junction communication through movement of serotonin contributes to orienting the left-right body axis in both frog and avian models (Fukumoto et al., 2005a; Fukumoto et al., 2005b; Levin et al., 2006). Importantly, most studies that defined roles for gap junctions in development relied on broad loss-of-function or inhibition of individual subunits, or global inhibition of molecules that are predicted to pass through gap junctions. Therefore, the roles that individual cells within gap junction networks may play in vivo to drive cell-fate determination or other patterning events are almost completely unknown.

We showed previously that the innexin *nsy-5* (*inx-19 – WormBase*) is required for asymmetric differentiation of the *C. elegans* AWC olfactory neuron pair (Chuang et al., 2007). The left and right AWC neurons are morphologically symmetric (White et al., 1986), but express different genes to convey the ability to sense different chemicals (Pierce-Shimomura et al., 2001; Wes and Bargmann, 2001). The two AWC neurons communicate through Notch-independent signaling during late embryogenesis to generate asymmetric cell fates: induced AWC<sub>ON</sub>, which expresses the reporter gene *str-2p::GFP*, and default AWC<sub>OFF</sub>, which does not (Troemel et al., 1999; Chuang and Bargmann, 2005). Each wild-type animal generates one AWC<sub>ON</sub> and one AWC<sub>OFF</sub>; 50% of the animals induce AWC<sub>ON</sub> on the left whereas the other 50% induce AWC<sub>ON</sub> on the right (Troemel et al., 1999). The default AWC<sub>OFF</sub> fate is executed by the cell-autonomous activity of a calcium/calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK) cascade (Sagasti et al., 2001; Chuang and Bargmann, 2005). NSY-5 gap junctions and NSY-4 claudins function in parallel to induce AWC<sub>ON</sub> by inhibiting the CaMKII cascade (Van Hoven et al., 2006; Chuang et al., 2007).

*nsy-5* is expressed during embryogenesis in both AWC neurons and in at least 17 pairs of other neurons (Chuang et al., 2007) (supplementary material Fig. S1). *nsy-5*-dependent gap junctions transiently connect AWCs and adjacent neurons in embryos. *nsy-5* functions primarily autonomously in AWCs to promote AWC<sub>ON</sub> fate. However, *nsy-5* activity in ASH, AWB and AFD neurons promotes or inhibits AWC<sub>OFF</sub> (Chuang et al., 2007). These results suggest that individual neighboring neurons in the NSY-5 network, which is defined by the 18 pairs of *nsy-5*-expressing neurons that are likely to be linked by gap junctions (Taylor et al., 2010), communicate with each other and with AWCs to fine-tune the signaling that establishes precise AWC asymmetry. However, the nature of intercellular signaling across the NSY-5 network and how
individual non-AWCs in the network influence AWC asymmetry is not known.

Components of the voltage-gated calcium channel (the pore-forming α1 subunit UNC-2 and the regulatory α2β subunit UNC-36) are also required upstream of CaMKII for communication between AWCs to ensure a precise 1AWC<sup>ON</sup>/1AWC<sup>OFF</sup> decision (Bauer Huang et al., 2007). This leads to the hypothesis that intercellular calcium signaling, through NSY-5, mediates communication between AWCs and non-AWCs, and that changes in calcium levels in neurons that are coupled with AWCs through NSY-5 influence AWC fate choice. Here, we develop a unique system to address the roles of specific cells within the NSY-5 network in AWC asymmetry by using cell type-specific expression of calcium-buffering proteins. We show that calcium in non-AWCs of the NSY-5 network is required for the induction of 1AWC<sup>ON</sup>/1AWC<sup>OFF</sup> asymmetry. In addition, calcium in AWB and ASH neurons confers different side biases of AWC<sup>ON</sup> induction. Our results support a model in which calcium within the NSY-5 network has dual roles in establishing AWC asymmetry: autonomous promotion of AWC<sup>OFF</sup> and non-autonomous promotion of AWC<sup>ON</sup>.

**MATERIALS AND METHODS**

**C. elegans strains**

Wild-type strains were *C. elegans* variety Bristol, strain N2. Strains were generated and maintained using standard methods (Brenner, 1974). Integrated transgenic lineages and mutations used in these experiments included: *kyIs140 [str-2p::GFP; lin-15(+)] I* (Troemel et al., 1999), *nsy-5::*ky634 I (Chuang et al., 2007), *lfe-2(3y326) I, kly632 [str-2p::GFP; ofm-1p::GFP] II, plc-3(tm1340) II, tph-1(mg280) II, unc-36(e251) III, itr-1(y331) IV, itr-1(y299) IV, itr-1(y291) IV, itr-1(y327) IV, itr-1(y328) IV, nsy-4(ky672) IV* (VanHoven et al., 2006), *unc-43(n498gf) X, erl-1(ns366) IV* (Nakamura et al., 2003), *unc-2 (3y505) X, kly636 [str-2p::GFP; lin-15(+)] X, vyIs18 [nsy-5p::mCherry-nsy-5 III] X.*

Transgenes maintained as extrachromosomal arrays included: *vyEx948, 499 [odr-3p::calbindin D28K; odr-1p::DsRed; ofm-1p::DsRed, vyEx699, 700 [odr-3p::parvalbumin; odr-1p::DsRed; ofm-1p::DsRed, vyEx1066, 1068, 1099 [odr-3p::calbindin D9K; odr-1p::DsRed; ofm-1p::DsRed, vyEx567, 571 [nsy-5p::calbindin D28K; odr-1p::DsRed; ofm-1p::DsRed, odr-3p::DsRed, vyEx724, 725, 726 [nsy-5p::parvalbumin; odr-1p::DsRed; ofm-1p::DsRed, odr-3p::DsRed, vyEx1063 [odr-3p::cmd-1; odr-1p::DsRed; ofm-1p::DsRed, odr-3p::DsRed, vyEx484 [nsy-5p::calbindin D28K::mCherry; ofm-1p::DsRed, vyEx992, 993, 994, 997 [odr-3p::parvCDEF/A; odr-1p::DsRed; ofm-1p::DsRed, vyEx990, 991, 996 [odr-3p::parvCDEF/A; odr-1p::DsRed; ofm-1p::DsRed, vyEx521, 522 [str-1p::calbindin D28K; odr-1p::DsRed; ofm-1p::DsRed, odr-3p::DsRed, vyEx536, 562 [sra-6p::calbindin D28K::SL2::mCherry; ofm-1p::DsRed, odr-3p::DsRed, vyEx568, 569, 570 [sra-5p::sra-6p::calbindin D28K::SL2::mCherry; ofm-1p::DsRed, vyEx1320, 1321 [str-1p::calbindin D28K::SL2::mCherry; ofm-1p::DsRed, vyEx1322, 1323 [sra-6p::SL2::mCherry; ofm-1p::DsRed].

**Plasmid construction and germline transformation**

*M. musculus* calbindin D28K cDNA from pCMV6-calbindin-28K (Origene) was subcloned to make *nsy-5p::calbindin D28K, odr-3p::calbindin D28K, nsy-5p::calbindin D28K::mCherry, str-1p::calbindin D28K* and *sra-6p::calbindin D28K::SL2::mCherry*. *M. musculus* parvalbumin cDNA from pCMV6-parvalbumin (Origene) was subcloned to make *odr-3p::parvalbumin and nsy-5p::parvalbumin*. Calbindin D9K cDNA was synthesized from mixed embryonic day (E) 14.5 and postnatal day (P) 0 mouse brain total RNA and subcloned to make *odr-3p::calbindin D9K, cmd-1 cDNA was obtained by RT-PCR from L1 stage worms and subcloned to make *odr-3p::cmd-1, odr-3p::parvCDEF/A*. This construct was made using the Quikchange II XL Kit (Stratagene). The original construct, rat ParvCDEF, contains four subunits that replace the first (D51A, D90A) and last (E62V, E101V) charged amino acids of the two EF-hand domains responsible for calcium binding (Pauls et al., 1994; John et al., 2001). We mutated the remaining charged amino acids in the second EF hand domain (K92V, D93A, D95A, K97V and E100V). To make *nsy-5IR*, antisense and sense transcripts of *nsy-5* were inserted upstream and downstream, respectively, of an 888-bp linker sequence as previously described (Chuang and Meyerowitz, 2000). This construct was subcloned to make *str-1p::nsy-5IR::SL2::mCherry.*

**Chemotaxis assays**

Chemotaxis assays were performed as previously described (Bargmann et al., 1993). Odors were diluted in ethanol and tested at standard concentrations (1:1000 for butanone and 1:10,000 for 2,3-pentanedione). About 50-200 animals were assayed for each strain and each odor in each individual assay. All assays were performed four independent times. Relative chemotaxis index was calculated by dividing each absolute chemotaxis index by the highest wild-type chemotaxis index observed for each odor for each independent trial.

**Dye transfer assays**

*C. elegans* embryonic cells were washed twice with HBS (Gibco) containing 10 mM HEPES (pH 7.35) and 5.5 mM glucose, then loaded with 3 µM NPE-HCC2/AM (plus 0.015% puroic acid in 0.015% DMSO) for 45 minutes in the dark at 22°C. Loaded cells were washed with HBS and seeded on 15-mm slides that had been precoated with peanut lectin (Sigma), in 35-mm dishes (MatTek). Seeded cells were incubated in HBS for 15 minutes to allow complete hydrolysis of acetoxyethyl (AM) esters. Localized ultraviolet (UV) uncaging of NPE-HCC2 and fluorescence imaging were performed on an inverted Zeiss Axiovert 200 with a Hamamatsu ORCA-ER cooled CCD camera and 100X oil immersion objective (NA 1.4) as previously described (Dakin et al., 2005). Images were acquired and analyzed with the Openlab integrated imaging software (Improvision). All of the five wild-type cell pairs that were analyzed showed dye transfer, whereas none of the five *nsy-5(0)II* cell pairs assayed had dye transfer.

**Calcium imaging**

*C. elegans* embryonic cells were loaded with Fluo-4 calcium-indicator dye as previously described (Marsh, 1995). Briefly, cells were washed three times with physiological saline solution (PSS; 145 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 10 mM HEPES, pH 7.4, 10 mM glucose, plus 2 mM CaCl<sub>2</sub>) and then loaded with 1.5 µM Fluo-4, AM (plus 0.015% puroic acid in 0.015% DMSO) for 45 minutes in the dark at 22°C. Cells were washed three times with PSS (2 mM CaCl<sub>2</sub>) then recovered in the final wash for 30-60 minutes in the dark. Ionomycin (in PSS plus 10 mM CaCl<sub>2</sub>) was added with all of the cells to a final concentration of 10 µM. EGTA in PSS was added on top of cells to a final concentration of 6 mM. Optical recording was performed using an Olympus IX51 inverted microscope equipped with a Hamamatsu ORCA-AG camera. Frames were acquired at 2-second intervals for a total of 4 minutes. Images were processed using ImageJ software. At each time point, Fluoro-4 fluorescence was normalized to mCherry fluorescence.
Genetic mosaic analysis

Mosaic analysis and statistical analysis were performed as previously described (Sagasti et al., 2001; VanHoven et al., 2006; Chuang et al., 2007). For experiments using the str-1 and sra-6 promoters, bicistronic constructs were generated using the SL2 trans-splicing signal to drive both the gene of interest and mCherry, and mCherry expression determined retention of the transgenes. Previous studies showed that the nsy-5 promoter, odr-3 promoter, str-1 promoter, sra-6 promoter, odr-1p::DsRed and various coinjection markers had no effect on str-2::GFP expression (Sagasti et al., 2001; Chuang and Bargmann, 2005; VanHoven et al., 2006; Bauer Huang et al., 2007; Chuang et al., 2007). At least two independent lines of the same transgene were analyzed. Expected numbers of mosaic animals were predicted from non-mosaic animals of the same transgenic line.

RESULTS

NSY-5 gap junctions mediate small molecule transfer between C. elegans embryonic neurons

Consistent with its cell-intrinsic and network functions in AWC asymmetry, NSY-5 can form both functional hemichannels and intercellular gap junction channels in Xenopus oocytes (Chuang et al., 2007). However, the permeability properties of NSY-5 gap junctions in C. elegans have not been examined. To determine the permeability of NSY-5 gap junctions to small molecules in C. elegans embryonic cells, we used a photoactivatable (caged) fluorescent dye and the uncaging/imaging technique (Dakin et al., 2005; Dakin and Li, 2006). Embryonic neurons were isolated from nsy-5p::mCherry transgenic animals, in which neurons of the NSY-5 network were labeled with mCherry (Shaner et al., 2004; Shaner et al., 2005). Cultured neurons were loaded with the cell-permeable caged fluorophore NPE-HCCC2/AM (Zhao et al., 2004; Dakin et al., 2005). After entering the cell, the acetoxymethyl (AM) esters are hydrolyzed and the caged dye (NPE-HCCC2) becomes trapped inside the cell. When uncaged by UV light, the parent fluorophore HCCC2 is released. As the molecular weight of HCCC2 is 450 Da, well below the ~1.2 kDa molecular passage limit of typical gap junction channels (Kumar and Gilula, 1996), HCCC2 diffuses to its neighboring cells through gap junctions (Fig. 1A). We focused on pairs of nsy-5p::mCherry-expressing cells that made direct contact (Fig. 1B,C,I,J), and uncaged the fluorophore in one cell. In wild-type cell pairs, HCCC2 transferred rapidly from the uncaged cell (donor) to its neighboring cell (recipient) (Fig. 1E-H). By contrast, in cell pairs isolated from nsy-5 loss-of-function (lf) embryos, in which nsy-5-dependent gap junctions were absent (Chuang et al., 2007), fluorescent HCCC2 was only detected in the uncaged cell (cell 1) and no fluorescence was observed in its neighbors.

Fig. 1. NSY-5 gap junctions mediate small dye transfer between C. elegans embryonic neurons. (A) Schematic of the dye transfer technique. Black open circles, NPE-HCCC2; blue dots, HCCC2; red rectangles, gap junctions. (B-G) One pair of nsy-5p::mCherry cells from wild-type embryos. (B) Differential interference contrast (DIC) and (C) mCherry images. (D-G) Images of fluorescent HCCC2 in the coupled cells prior to UV (360 nm) uncaging (D), immediately after the first (E) and second (F) localized uncaging of one cell (donor), and when the transfer of fluorescent HCCC2 from donor cell to recipient cell reached equilibrium (G). (H) Time course of fluorescence intensities of HCCC2 (F_HCCC2) in the wild-type coupled cells. D, E, F and G indicate the time points at which the images in D, E, F and G, respectively, were acquired. (I-N) One pair of nsy-5p::mCherry-expressing cells from nsy-5(ky634lf) embryos. (I) DIC and (J) mCherry images. (K-N) Images of fluorescent HCCC2 before localized uncaging of cell 1 (K), immediately after uncaging (L) and 40 seconds (M) and 380 seconds (N) after uncaging. (O) Time course of fluorescence intensities of HCCC2 (F_HCCC2) in the nsy-5(lf) cell pair. K, L, M and N indicate the time points at which the images in K, L, M and N, respectively, were acquired. Scale bar: 10 μm.
neighboring cell (cell 2) (Fig. 1L-O). These results indicate that
NSY-5 gap junctions can mediate small molecule transfer between
coupled C. elegans neurons.

**Expression of vertebrate calcium buffers proteins in a subset of NSY-5 network cells disrupts AWC asymmetry**

To determine the requirements for intracellular free calcium in
specific cells within the NSY-5 network, we established transgenic
types expressing the vertebrate calcium-buffer proteins calbindin
D28K, parvalbumin or calbindin D9K in various subsets of
neurons. Calcium-buffer proteins contain EF-hand domains (four
functional domains in calbindin D28K and two each in parvalbumin and calbindin D9K) responsible for binding to and
sequestering intracellular free calcium, preventing calcium from
activating downstream targets such as CaMKII (Ikura, 1996).
There are no homologs of these calcium buffer genes in the C.

Expression of each buffer under the control of the odr-3
promoter (Roayaie et al., 1998), which is strongly expressed in
AWC and weakly in AWB neurons, led to str-2::GFP expression
in both AWC neurons (2AWC\textsuperscript{ON} phenotype) (Fig. 2D,E; supplementary material Table S1), in contrast to the
1AWCOFF/1AWCON phenotype observed in the wild-type
population (Fig. 2A,E). The 2AWCON phenotype is identical to the
loss-of-function mutant phenotype of the calcium channel \(\alpha_{2}\delta
\) subunit gene unc-36 (Troemel et al., 1999) (Fig. 2C,E). The 2AWCON phenotype is consistent with each buffer strongly inhibiting calcium signaling. Mutations of conserved charged residues at the beginning and end of the two EF-hand
calcium-binding domains of parvalbumin reduce calcium binding
in vitro (Pauls et al., 1994). To determine whether parvalbumin
generates a 2AWCON phenotype owing to its ability to bind
calcium, we mutated all charged residues in the second EF-hand
domain. This resulted in a significant reduction of 2AWCON
phenotype (\(P=0.006, n=245\)), suggesting that the 2AWCON
phenotypes are specifically due to calcium-binding activity.

\textit{nsy-5} mutants have two AWC\textsuperscript{OFF} cells (2AWCOFF phenotype) (Chuang et al., 2007) (Fig. 2B,E), consistent with the requirement of
gap junction-mediated signaling for AWC\textsuperscript{ON} induction. Calbindin
D28K or parvalbumin expression in the entire NSY-5 network
resulted in 2AWCON, but also significantly increased the percentage
of 2AWCOFF compared with expression primarily in AWCs (Fig. 2E;
supplementary material Table S1), suggesting that intracellular
calcium signals in non-AWCs of the NSY-5 network promote the
wild-type 1AWCON/1AWCOFF phenotype.

\textit{odr-3p::calbindin D28K} animals chemotaxed normally to
butanone, which is sensed by the AWC\textsuperscript{ON} cell, but did not
chemotax to 2,3-pentanedione, which is sensed by the AWC\textsuperscript{OFF} cell
(Fig. 2F). These results confirm that the AWC\textsuperscript{OFF} cell fate is lost in
\textit{odr-3p::calbindin D28K} animals, and that the AWC\textsuperscript{ON} cells are capable of performing an appropriate physiological function. Because calbindin D28K has the highest number (four) of potential
calcium-binding sites among the calcium-buffer proteins, we
focused primarily on developing this buffer as a tool to examine
the role of calcium in specific cells in the NSY-5 network.

**Calbindin D28K acts as a calcium buffer in NSY-5 network neurons**

Calcium-buffer proteins share the EF-hand calcium-binding
domain with calcium sensors, which bind to calcium and promote
signaling by activating downstream kinase cascades. Although it
is well established that calbindin D28K and parvalbumin function
as calcium buffers in vertebrate systems and transgenic fly
neurons (Chard et al., 1993; Grabarek, 2006; Harrisingh et al.,
2007), their calcium-buffering ability has not been tested in any
type of \textit{C. elegans} cells. unc-43/CaMKII gain-of-function (gf)
mutants have a 2AWCOFF phenotype (Troemel et al., 1999) (Fig.
3A; supplementary material Table S1). To confirm that calbindin
D28K does not promote calcium signaling, we compared the
abilities of calbindin D28K and \textit{C. elegans} CMD-1/calmodulin,
a calcium sensor that activates CaMKII, to induce a 2AWCOFF
phenotype. Neither protein when expressed alone in AWCs under
the \textit{odr-3} promoter strongly induced a 2AWCOFF phenotype (Fig.
3A; supplementary material Table S1). Calcium concentration
could be one of the limiting factors in allowing \textit{cmd-1} to activate
downstream targets. unc-2(zf35) is a gain-of-function allele of the neuronal P/Q type calcium channel (J.K.P. and M.J.A., unpublished), which should lead to a rise in intracellular calcium. Although unc-2(zf35gf) mutants had wild-type AWC asymmetry (Fig. 3A; supplementary material Table S1), they may provide a sensitized background for a 2AWCOFF phenotype. When the same odr-3p::calbindin D28K or odr-3p::cmd-1 transgenes were expressed in unc-2(zf35gf) homozygous mutants, odr-3p::cmd-1 generated a strong 2AWCOFF phenotype (Fig. 3A; supplementary material Table S1), indicative of promoting calcium signaling. Importantly, odr-3p::calbindin D28K did not promote 2AWCOFF in the unc-2(zf35gf) background (Fig. 3A), indicating that calbindin D28K does not activate downstream targets and supporting the role of calbindin D28K as a calcium buffer rather than a calcium sensor.

To confirm that calbindin D28K reduces the amount of intracellular free calcium, we performed calcium-imaging experiments in C. elegans primary cell culture. We targeted neurons in the NSY-5 network by expressing mCherry or calbindin D28K translational fusion protein under the control of the nsy-5 promoter. The nsy-5p::calbindin D28K::mCherry transgene allowed us to determine relative levels of calbindin D28K expression in each cell, which we broadly categorized into ‘dim’ cells, which had lower levels of calbindin D28K, and ‘bright’ cells, which had higher levels of calbindin D28K. Wild-type mCherry-expressing neurons displayed a typical increase in intracellular calcium measured by the calcium indicator Fluo-4 following ionomycin (a calcium ionophore) application (Fig. 3B,C). ‘Dim’ calbindin D28K::mCherry neurons had a similar increase to wild-type neurons (Fig. 3B,C). However, the increase in intracellular calcium was significantly reduced in ‘bright’ neurons, which expressed high levels of calbindin D28K::mCherry, indicating that calbindin D28K is able to bind and sequester intracellular calcium. Taken together, these results are consistent with calbindin D28K acting as a calcium buffer in the NSY-5 network to disrupt AWC asymmetry.

**Calcium buffers antagonize the unc-2/unc-36 calcium signaling pathway**

The 2AWCOFF phenotype caused by calcium buffer expression in AWCs resembles that seen in loss-of-function mutations of unc-2 and unc-36 calcium channels and of the downstream calcium-activated unc-43 (CaMKII)/tir-1 (Sarm1 adaptor)/nsy-1 (MAPKKK) kinase pathway (Troemel et al., 1999; Sagasti et al., 2001; Chuang and Bargmann, 2005; Chang et al., 2011) (Fig. 4A,B). This similarity suggests that the calcium buffer-induced 2AWCON phenotype could result from an inhibition of the unc-2/unc-36 calcium signaling pathway.

To determine the relationship between the calcium-buffer target and other genes affecting AWC asymmetry, we crossed odr-3p::calbindin D28K or odr-3p::parvalbumin transgenic animals (2AWCON) with 2AWCOFF mutants. Loss-of-function mutations in the unc-76 axon outgrowth gene, the nsy-5 innexin gene and the nsy-4 claudin-like gene, or gain-of-function mutations in the unc-43 CaMKII gene cause a 2AWCOFF phenotype (Troemel et al., 1999; VanHoven et al., 2006; Chuang et al., 2007) (Fig. 4A,B). Calbindin D28K or parvalbumin expression strongly suppressed the 2AWCOFF phenotype of unc-76(lf), nsy-5(lf) or nsy-4(lf) mutants (Fig. 4A), indicating that calcium buffers function downstream of axon outgrowth, nsy-5 and nsy-4 (Fig. 4B). By contrast, the 2AWCON phenotype resulting from calbindin D28K or parvalbumin expression was greatly suppressed by unc-43 gain-of-function mutants (Fig. 4A), suggesting that calcium buffers act on a target predominantly upstream of unc-43 (Fig. 4B).

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**Fig. 3. Calbindin D28K acts as an intracellular calcium buffer in NSY-5 network embryonic neurons.** (A) AWC phenotypes of calcium buffer (odr-3p::calbindin D28K) and calcium sensor (odr-3p::cmd-1) expressing animals alone or in the unc-2(zf35gf) background. (B) Representative traces of calcium dynamics in cultured C. elegans embryonic neurons during addition of the stimulus (ionomycin) followed by calcium chelator (EGTA). Horizontal lines indicate the duration of ionomycin and EGTA application. $\Delta F$, the change in Fluo-4 fluorescence; $F_0$, the resting Fluo-4 fluorescence prior to ionomycin stimulation. (C) Average maximum change in Fluo-4 intensity following ionomycin application. Significance was calculated using t-test. Error bars indicate s.e.m.
These results suggest that calcium buffers affect the AWC signaling step downstream of nsy-5 and nsy-4, and upstream of unc-43. Previous genetic studies suggest that unc-2 and unc-36 calcium channels also act downstream of nsy-5 and nsy-4, and upstream of unc-43 (Troemel et al., 1999; VanHoven et al., 2006; Chuang et al., 2007). Thus, we examined the genetic interaction between the calcium-buffer target and unc-2. The unc-2(e55) mutant had a mixed 2AWC ON and 2AWCOFF phenotype due to functional redundancy of unc-2 with the additional α1 subunit egl-19 and inhibition of egl-19 activity by unc-2, respectively (Troemel et al., 1999; Bauer Huang et al., 2007) (Fig. 4A). Calbindin D28K expression significantly suppressed the 2AWCOFF phenotype of unc-2(e55) mutants (Fig. 4A). Together, these results are consistent with the antagonistic effect of calcium buffers on the unc-2/unc-36 calcium signaling pathway (Fig. 4B).

**Intracellular calcium acts cell-autonomously in AWCs to promote AWC\(^{ON}\)**

Based on previous results that calcium-signaling components (unc-43, tir-1, nsy-1) function cell-autonomously to execute the AWC\(^{OFF}\) fate (Sagasti et al., 2001; Chuang and Bargmann, 2005), we hypothesized that the calcium buffers would cell-autonomously inhibit the calcium-signaling pathway to promote AWC\(^{ON}\). To test this hypothesis, we performed genetic mosaic analysis using calbindin D28K transgenes expressed primarily in AWCs and AWBs using the odr-3 promoter. Transgenes are inherited as extrachromosomal arrays that are unstable and subject to spontaneous mitotic loss in random cell lineages. Mosaic animals were identified by loss of the co-injection marker odr-1p::DsRed, which is expressed in AWC left (AWCL), AWC right (AWCR), AWB left (AWBL) and AWB right (AWBR). These four cells represent four lineage groups within the NSY-5 network (Chuang et al., 2007) (supplementary material Fig. S1), and thus can serve as indicators of which cells within the network retain or lose calbindin D28K transgenes. Seventy percent of non-mosaic calbindin D28K animals had 2AWCON (Fig. 5B; Table 1, row d). Non-mosaic 1AWCON animals showed no side bias of AWC ON induction. In >90% of mosaic animals that retained the calcium buffer only in one AWC, the calcium-buffered AWC became AWC\(^{ON}\) and the wild-type AWC became AWC\(^{OFF}\), independent of expression of the buffer in AWB cells (Fig. 5C; Table 1, rows e,f). This suggests that intracellular calcium is required cell-autonomously to execute AWC\(^{OFF}\) cell-fate specification (Fig. 6A).

**Intracellular calcium signals in non-AWCs promote 1AWCON/1AWCOFF**

Based on our observation that expressing the buffers in the entire NSY-5 network led to a moderate 2AWCOFF phenotype (Fig. 2E),
we hypothesized that calcium within non-AWC cells is required to promote 1AWC\textsuperscript{ON}/1AWC\textsuperscript{OFF}. To test this hypothesis, we performed mosaic analysis with the \textit{ods}-3p::\textit{calbindin D28K}; \textit{odr}-1p::\textit{DsRed} transgenic extrachromosomal arrays to determine whether buffering calcium only in non-AWC neurons within the NSY-5 network was able to disrupt asymmetry. As 15 out of the 34 non-AWC cells expressing \textit{odr}-3p::\textit{DsRed} are closely related to either AWC or ASH neurons, expression of \textit{calbindin D28K} in these cells could disrupt AWC asymmetry (Table 1, row c). These results suggest that other cells in the AWB lineage of the NSY-5 network also contribute to promoting the wild-type 1AWC\textsuperscript{ON}/1AWC\textsuperscript{OFF} fate. Mosaic animals that lost \textit{ods}-3p::\textit{calbindin D28K} in both AWB\textsuperscript{ON} and ASH\textsuperscript{ON} cells did not influence AWC\textsuperscript{ON} asymmetry (Table 1, row d). These results suggest that the negative influence on AWC\textsuperscript{ON} via calcium is partly, if not completely, derived from ASH (supplementary material Fig. S2B). Control mosaic experiments showed that \textit{str}-l::\textit{mCherry} did not influence AWC\textsuperscript{ON} side bias (supplementary material Table S2). To test whether \textit{ods}-3p, like calcium, in ASH generates AWC\textsuperscript{ON} side bias, we made a tissue-specific inverted repeat (IR) RNAi construct, \textit{str}-l::\textit{nsy}-5IR. Knocking down \textit{nsy}-5 in both AWB\textsuperscript{ON} and ASH\textsuperscript{ON} cells did not disrupt AWC\textsuperscript{ON} asymmetry or generate an AWC\textsuperscript{ON} side bias (Table 1, row q). Consistent with our \textit{ods}-3p::\textit{calbindin D28K} results (Table 1, row o), 62% of \textit{awc}-\textsuperscript{ON} animals that lost \textit{str}-l::\textit{nsy}-5IR in AWB\textsuperscript{ON} had a significant bias towards AWCL\textsuperscript{ON} (Table 1, row r). These results suggest that the negative influence of AWB on AWC\textsuperscript{ON} is independent of both calcium and \textit{nsy}-5. In addition, 70% of \textit{awc}-\textsuperscript{ON} animals that lost \textit{str}-l::\textit{nsy}-5IR in AWB\textsuperscript{ON} had a significant bias towards AWCL\textsuperscript{ON} via calcium. These results suggest that the negative influence of calcium in ASH on AWC\textsuperscript{ON} is independent of both calcium and \textit{nsy}-5. In addition, 70% of \textit{awc}-\textsuperscript{ON} animals that lost \textit{str}-l::\textit{nsy}-5IR in AWB\textsuperscript{ON} had a significant bias towards AWCL\textsuperscript{ON} (Table 1, row r; supplementary material Table S1). Mosaic experiments showed that the same class of mosaic animals containing the \textit{str}-l::\textit{mCherry} did not show a bias towards AWCR\textsuperscript{ON} (Table 1, row p). These results suggest that calcium in ASH might go through NSY-5 gap junctions to negatively influence AWC\textsuperscript{ON}.

ASH neurons are derived from the same lineage as AWCs. To examine whether calcium in ASH generates AWC\textsuperscript{ON} side bias, we used the \textit{sra}-6 promoter (Troemel et al., 1995) to drive \textit{calbindin D28K} expression primarily in ASH, in addition to ASI neurons. Expression of \textit{sra}-6p::\textit{calbindin D28K} in both ASH and ASI did not disrupt AWC asymmetry nor did it generate AWC\textsuperscript{ON} side bias (Table 1, row t; supplementary material Table S1). Mosaic experiments showed that \textit{sra}-6p::\textit{mCherry} did not influence AWC\textsuperscript{ON} side bias (supplementary material Table S2). ASH was previously shown to promote AWC\textsuperscript{ON} fate via NSY-5 (Chuang et al., 2007), suggesting...
that calcium in ASHL promotes AWCLON fate (supplementary material Fig. S2C). In addition, mosaic animals that lost the sra-6p::calbindin array in ASIR had a significant bias towards AWCRON (Table 1, row w). It has not been determined whether ASI has a positive or negative influence on AWCON fate. ASIR is derived from the same lineage as AWBR and AFDR (supplementary material Fig. S1), which both inhibit AWCON (Chuang et al., 2007), so it is possible that calcium in ASIR inhibits AWCLON fate.

**IP3 and serotonin signaling are not required for AWC asymmetry**

Intercellular calcium waves can be propagated by movement of inositol trisphosphate (IP3) through gap junctions (Sáez et al., 1989). In addition, serotonin may move through gap junctions to coordinate the left-right body axis and modulate intercellular calcium signaling, and ADF neurons of the NSY-5 network synthesize serotonin (Blomstrand et al., 1999; Duerr et al., 1999; Sieburth et al., 2005).

### Table 1. Individual neurons in the NSY-5 network play specific roles in influencing AWCON fate and side bias

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Transgene array</th>
<th>Row</th>
<th>Cells with transgene array</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AWCL</td>
<td>AWCR</td>
</tr>
<tr>
<td>Wild type</td>
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<tr>
<td></td>
<td></td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>odr-3p::calbindin D28K</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
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<td>–</td>
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<td>–</td>
</tr>
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<td>+</td>
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<tr>
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<td>+</td>
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<tr>
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<td>Wild type</td>
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<td>+</td>
</tr>
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<td></td>
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</table>

The odr-3p::calbindin D28K; odr-1p::DsRed transgene analyzed in rows d-k was crossed into the nsy-5(ky634fl) mutant for the analysis in rows l, m. The str-1p::nsy-SIR::SL2::mCherry transgene was analyzed in the RNAi-sensitive strain eri-1(mg366); lin-15B(n744) (Sieburth et al., 2005). +, cells that retain the array; –, cells that lost the array; n, number of animals characterized. cbn, calbindin D28K.

Data in parentheses are the percentages or numbers of animals with AWCLON and AWCRON, calculated when the data of 2AWCOFF and 2AWCON phenotypes are excluded. Values in bold are significantly different from the expected values in the same column (row indicated by h44/4h). Significance was calculated using Z-test for two proportions.

****P<0.0001; ***P<0.001; **P<0.01 (rows h, o, v, w) or **P=0.01 (row r); *P=0.02; ns, not significant.
Ca\(^{2+}\) and neural asymmetry

Zimmermann and Walz, 1999; Sze et al., 2000; Fukumoto et al., 2005a; Fukumoto et al., 2005b; Peters et al., 2005; Levin et al., 2006). To address potential roles of IP3 and serotonin in mediating AWC asymmetry, we examined AWC\(^{ON}\) induction in mutants that disrupt components of each signaling pathway. We analyzed mutants of the following genes required for IP3 signaling: *itr-1* (a single IP3 receptor), *ife-2* (IP3 kinase), *ipp-5* (5-phosphatase) and *ploc-3* (phospholipase C) (Clandinin et al., 1998; Dal Santo et al., 1999; Sze et al., 2000; Bui and Sternberg, 2002). IP3 signaling mutants did not affect AWC asymmetry (supplementary material Table S3). *C. elegans* has a single tryptophan hydroxylase (the key enzyme for serotonin biosynthesis) gene, *tpg-1*, and *tpg-1(mg280lf)* mutants do not synthesize serotonin (Sze et al., 2000). *tpg-1(mg280lf)* mutants had wild-type AWC asymmetry (supplementary material Table S3). Together, these results suggest that IP3 and serotonin are not involved in *nsy-5*-mediated intercellular signaling for AWC asymmetry.

**DISCUSSION**

Discovering the nature of intercellular signaling is an important step towards understanding how groups of cells coupled by gap junctions coordinately communicate to diversify cell fates. We have taken advantage of well-characterized vertebrate calcium-buffer proteins and powerful *C. elegans* genetic tools to manipulate intracellular calcium levels selectively in individual cells or small groups of cells within the NSY-5 gap junction network to determine the non-cell-autonomous role of calcium in AWC fate diversification across the left-right axis. The genetic mosaic results revealed that calcium in non-AWC neurons of the NSY-5 network promotes wild-type AWC\(^{ON}/AWC^{OFF}\) cell fate decision. In addition, calcium in AWB, ASH and ASI cells influences AWC\(^{ON}\) side biases. To our knowledge, this is the first in vivo study that directly reveals novel roles for intercellular calcium signaling within a gap junction network and dissects roles for specific cells in the network in stochastic cell-fate choice. In addition, the calcium-buffer transgenic lines represent a unique system in which to rigorously test the actual role of intracellular calcium in various neurons in directing cell-fate choice, rather than simply correlating calcium dynamics with cell-fate outcomes.

Based on the mosaic results, we propose a model in which calcium regulates AWC asymmetry by providing both cell-autonomous inputs from AWCs and non-cell-autonomous inputs from other neurons in the NSY-5 network (Fig. 6). Prior to their interaction, the two AWC neurons have spontaneous activity in order to maintain voltage-gated calcium channel-mediated signaling and the default AWC\(^{OFF}\) fate (Troemel et al., 1999; Sagasti et al., 2001; Tanaka-Hino et al., 2002; Chuang and Bargmann, 2005; VanHoven et al., 2006; Bauer Huang et al., 2007; Chuang et al., 2007) (Fig. 6A). Once the AWC cells sense each other’s presence through intercellular calcium signaling between AWCs and other neurons within the NSY-5 network, calcium channels in the two AWC cells may be differentially regulated by an initial stochastic signaling event that breaks AWC symmetry. Voltage-gated calcium channels generate both calcium and voltage signals, and are subject to both calcium- and voltage-dependent activation and inactivation (Catterall, 2000). As calcium-activated signaling pathways are very sensitive to the temporal pattern of calcium signals (Thomas et al., 1996; West et al., 2001), transient differences in calcium influx between two cells can potentially generate sustained differences in calcium-regulated signaling outputs through positive- and negative-feedback mechanisms. Our results support the model that NSY-5 gap junctions transmit or modify calcium signals across the neural network, leading to differential regulation of calcium channel activity in the two AWC cells and the subsequent asymmetric differentiation of AWC cells (Fig. 6B). This extends the previous model of NSY-5 function by identifying calcium as a major mediator of intercellular communication in the NSY-5 network.

Gap junction-dependent intercellular calcium signaling has been observed in a number of different cell types and organ systems, including the neocortex, retina, airway epithelia, hepatocytes and osteoblastic cells (Sáez et al., 1989; Botiano et al., 1992; Yuste et al., 1992; Lee et al., 1994; Yuste et al., 1995; Kandler and Katz, 1998; Singer et al., 2001). One mechanism for transduction of these calcium waves is the spread of the second messenger IP3 through gap junctions, which then triggers the release of intracellular calcium stores in neighboring cells. Our results suggest that AWC asymmetry does not rely on IP3 signaling, implying an alternative mechanism for propagation of calcium signaling in the NSY-5 network. One possibility is that calcium itself travels through gap junctions to mediate intercellular communication. This type of communication is found in coupled hepatocytes, in which calcium diffusion through gap junctions may help to equalize hormonal...
responses across a field of cells (Sáez et al., 1989). In addition, membrane potential is a possible signal that mediates NSY-5 gap junction intercellular communication. In support of this notion, SLO-1 and EGL-2, which represent two different classes of voltage-regulated potassium channels, also affect AWC asymmetry (Troemel et al., 1999; Sagasti et al., 2001; Davies et al., 2003).

It is clear from these results that AWC neurons integrate signals from a number of different cells both to induce AWCON and to influence AWCON side bias. In particular, the mosaic analysis in nsy-3(II) mutants revealed that the calcium input from AWBR requires the presence of NSY-5 gap junctions to inhibit AWCON, suggesting that there is a flow of information from AWBR to AWC through gap junctions. AWBs do not form direct gap junctions with AWCs (Chuang et al., 2007), thus the influence from AWBs on AWCs might go through other cells in the NSY-5 network. However, it is possible that AWCR or AWCN receive inputs from additional neurons that were not included in this analysis. AWC can respond to nsy-5 directly but AWCL requires nsy-5 function in multiple cells of the network (Chuang et al., 2007), suggesting that AWCL requires more inputs from neighboring cells to become AWCON. The result that AWCN is promoted by inputs from AWBR and ASHL, whereas AWCN has only the cell-autonomous input, is consistent with this.

Side-biased calcium-wave dynamics have been implicated in establishing the left-right axis in vertebrate systems. Intracellular calcium elevation on the left side of the mouse embryonic node, Hensen’s node in chick, or zebrafish Kupffer’s vesicle is the earliest asymmetric molecular event that is functionally linked to organ lateralization (McGrath et al., 2003; Sarmah et al., 2005; Garic-Stankovic et al., 2008). Calcium may move through gap junctions to reach left side target cells, leading to activation of downstream transcription (Levin and Mercola, 1999; Hatler et al., 2009). In zebrafish, left-sided elevation of intracellular calcium transiently activates CaMKII phosphorylation, which is required for proper left-right organ placement (Francescatto et al., 2010). A similar situation might occur in AWC asymmetry. It is possible that side biases of calcium dynamics in non-AWC cells of the NSY-5 network cause elevation of calcium in one AWC and reduction of calcium in another AWC, leading to increased and decreased CaMKII activity in the future AWCOFF cell and the future AWCON, respectively.

Stochastic cell-fate acquisition in the nervous system is a conserved but only partly understood phenomenon in all species (Johnston and Desplan, 2008; Losick and Desplan, 2008; Johnston and Desplan, 2010). This study suggests a novel mechanism by which stochastic neuronal diversification is established. We propose that stochastic AWC asymmetry is coordinated through competition of different AWCON side biases generated by intracellular calcium signals in non-AWCs of the NSY-5 network, in addition to previously identified intrinsic opposite biases of nsy-4 and nsy-5 activity in AWCs (Chuang et al., 2007).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.083428/-/DC1

References


**Fig. S1. Lineage groups in the NSY-5 gap junction-dependent neural network.** *nsy-5* is expressed in both AWC neurons and at least 17 pairs of other sensory neurons and interneurons in the head and tail (Chuang et al., 2007). AWBL, AWCL, AWBR and AWCR cells represent four lineage groups within the NSY-5 network and express the co-injection mosaic marker *odr-1p::DsRed*. Fifteen out of the 34 non-AWC cells expressing *nsy-5* are closely related to either AWB by lineage. Asterisks indicate cells analyzed for generation of AWC<sup>ON</sup> side biases.

<table>
<thead>
<tr>
<th>AWBL*</th>
<th>AWCL</th>
<th>AWBR*</th>
<th>AWCR</th>
</tr>
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<tbody>
<tr>
<td>AFDL</td>
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<td>AFDR</td>
<td>ASHR*</td>
</tr>
<tr>
<td>ASKL</td>
<td>ASIL*</td>
<td>ASIR*</td>
<td>AIMR</td>
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*Fig. S1. Lineage groups in the NSY-5 gap junction-dependent neural network. *nsy-5* is expressed in both AWC neurons and at least 17 pairs of other sensory neurons and interneurons in the head and tail (Chuang et al., 2007). AWBL, AWCL, AWBR and AWCR cells represent four lineage groups within the NSY-5 network and express the co-injection mosaic marker *odr-1p::DsRed*. Fifteen out of the 34 non-AWC cells expressing *nsy-5* are closely related to either AWB by lineage. Asterisks indicate cells analyzed for generation of AWC<sup>ON</sup> side biases.*
Fig. S2. Schematic of key mosaic results. (A) Intracellular calcium in non-AWCs of the NSY-5 network is required for AWCON induction. Mosaic animals that retain the *nsy-5p::calbindin D28K* array in both AWB lineages but neither AWC lineage have buffered calcium in about half of the population of non-AWCs in the NSY-5 network, leading to defective intercellular calcium signaling within the network required for AWCON induction. In the absence of proper calcium-based intercellular communication in the NSY-5 network, both AWC cells maintain endogenous high calcium levels via calcium influx through voltage-gated calcium channels and thus stay as the default AWCON fate (see Table 1, row c). (B, C) Intracellular calcium levels in non-AWCs of the NSY-5 network generate side biases of AWCON induction by communicating with AWC via NSY-5 gap junctions. (B) Mosaic animals that retain the *str-1p::calbindin D28K* array in AWBL have higher calcium in AWBR, which inhibits AWCR from becoming AWCON (see Table 1, row o). (C) Mosaic animals that retain the *sra-6p::calbindin D28K::SL2::mCherry* array in ASHR have higher calcium in ASHL, which promotes AWCL to become AWCON (see Table 1, row v). AWB cells express *nsy-5*, but do not directly contact AWC. The influence from AWBR on AWCR may go through other cells in the NSY-5 network. Red cells indicate retention of calcium buffer transgenes. Blue cylinders indicate NSY-5 gap junctions.
**Table S1. Effects of mutants and transgenes on Ca\(^{2+}\) level/signaling and AWC terminal fate**

<table>
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<th>Genetic background</th>
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<th>Cells expressing transgene</th>
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<tr>
<td></td>
<td>odr-3p::calbindin D28K</td>
<td>AWC</td>
<td>–</td>
<td>1 AWC(^{ON})</td>
</tr>
<tr>
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<td>AWC, non-AWCs(^{*})</td>
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<td>unc-43(n498gf)</td>
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+, positive effect; –, negative effect.

\(^{*}\)Non-AWCs are other 34 neurons that express nsy-5 as listed in supplementary material Fig. S1.

**Table S2. mCherry expression in AWB and ASH does not affect AWC asymmetry**

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<td></td>
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Table S3. IP3 and serotonin signaling mutants have wild-type AWC asymmetry

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<thead>
<tr>
<th>Genetic background</th>
<th>2 AWC\textsuperscript{OFF} (%)</th>
<th>1 AWC\textsuperscript{OFF}</th>
<th>1 AWC\textsuperscript{ON} (%)</th>
<th>2 AWC\textsuperscript{ON} (%)</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1</td>
<td>99</td>
<td>0</td>
<td>0</td>
<td>110</td>
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<tr>
<td>IP3 signaling mutants</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IP3 receptor / itr-1(sy331lf)*</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>127</td>
</tr>
<tr>
<td>IP3 kinase / lfe-2(sy326lf)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>143</td>
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<tr>
<td>5-phosphatase / ipp-5(sy605lf)</td>
<td>1</td>
<td>99</td>
<td>0</td>
<td>99</td>
<td>139</td>
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<tr>
<td>phospholipase C / plc-3(tm1340lf)</td>
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<td>99</td>
<td>0</td>
<td>99</td>
<td>117</td>
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<tr>
<td>Serotonin mutants</td>
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</tr>
<tr>
<td>tph-1(mg280lf)</td>
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<td>99</td>
<td>0</td>
<td>99</td>
<td>244</td>
</tr>
</tbody>
</table>

*Like itr-1(sy331) mutants, itr-1(sy290), itr-1(sy291), itr-1(sy327) and itr-1(sy328) mutants had wild-type AWC asymmetry.