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Asymmetric neural development in the *C. elegans* olfactory system

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Abstract

Asymmetries in the nervous system have been observed throughout the animal kingdom. Deviations of brain asymmetries are associated with a variety of neurodevelopmental disorders; however, there has been limited progress in determining how normal asymmetry is established in vertebrates. In the *C. elegans* chemosensory system, two pairs of morphologically symmetrical neurons exhibit molecular and functional asymmetries. This review focuses on the development of antisymmetry of the pair of AWC olfactory neurons, from transcriptional regulation of general cell identity, establishment of asymmetry through neural network formation and calcium signaling, to the maintenance of asymmetry throughout the life of the animal. Many of the factors that are involved in AWC development have homologs in vertebrates, which may potentially function in the development of vertebrate brain asymmetry.

Keywords

AWC neurons; left-right asymmetry; stochasticity; gap junctions; calcium signaling; nematode

INTRODUCTION

At first glance, the human brain appears fairly symmetric across the left-right axis. However, there are several functional and anatomical asymmetries that have been observed (Sun and Walsh, 2006). A number of neurological diseases have been associated with disruption of asymmetry in the brain, including dyslexia and schizophrenia (Oertel-Knochel and Linden, 2011; Renteria, 2012). However, the mechanisms used to establish asymmetry are not very well understood due to the complexity of the vertebrate nervous system.

As in humans, the *C. elegans* nervous system is largely symmetric (Hobert *et al.*, 2002; White *et al.*, 1986), but upon closer inspection, at least two pairs of head sensory neurons of the nematode display molecular and functional asymmetries. Like other developmental asymmetries, *C. elegans* left-right neuronal asymmetries are either directional or random.

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Directional asymmetries have stereotypical asymmetric features on one particular side of an animal; while random asymmetries or antisymmetries have asymmetric features randomly distributed on either the left or right side within a population. The left and right amphid single cilliary ending (ASE) taste neurons develop a directional asymmetry in the expression patterns of chemosensory receptors, which is initiated by Notch signaling in early embryos and is established through transcriptional regulation cascades of microRNAs and transcription factors (Alqadah *et al.*, 2013; Cochella and Hobert, 2012; Hobert, 2014; Hobert *et al.*, 2002). Unlike ASE neurons, the left and right amphid wing "C" (AWC) olfactory neurons exhibit antisymmetric/stochastic expression patterns of chemosensory receptors (Fig. 1a) via a transient gap junction neural network and a downstream calcium-regulated MAPK cascade in late embryogenesis (Fig. 2,3) (Chuang and Bargmann, 2005; Chuang *et al.*, 2007; Sagasti *et al.*, 2001; Troemel *et al.*, 1999). ASE and AWC asymmetries are regulated by two completely distinct mechanisms at different stages of development. A recent study identified the zinc finger transcription factor *die-1* as a molecular link between the two completely different kinds of asymmetries (Cochella *et al.*, 2014). Both ASE and AWC asymmetries allow the animal to distinguish between different chemical cues in the environment (Ortiz *et al.*, 2009; Pierce-Shimomura *et al.*, 2001; Wes and Bargmann, 2001). To the best of our knowledge, the pairs of AWC and ASE neurons are the only examples known to date in which molecules that are asymmetrically expressed in the nervous system are clearly correlated with functional asymmetries.

The specification of asymmetric AWC terminal fates is regulated by three developmental events, including the specification of general AWC identity, asymmetric differentiation of the two distinct AWC subtypes, and the maintenance of the two AWC subtypes (Fig. 1b). In this review, we focus on the current understanding of the developmental mechanisms by which general AWC identity is specified as well as how stochastic AWC asymmetry is established and maintained.

TRANSCRIPTIONAL SPECIFICATION OF GENERAL AWC IDENTITY

Terminal selector genes encode transcription factors that control expression of genes specific to a single type of postmitotic neuron through cis-regulatory elements called terminal selector motifs (Hobert, 2008). *ceh-36*, which encodes an OTX/OTD transcription factor, has been suggested to be the terminal selector gene for AWC neurons (Kim *et al.*, 2010; Lanjuin *et al.*, 2003). *ceh-36* mutants fail to express the AWC-specific terminal identity genes including the guanylyl cyclase gene *odr-1* and lose the ability to chemotax to odors detected by AWC (Lanjuin *et al.*, 2003). In addition, a motif regulated by *ceh-36* was identified in the *odr-1* promoter region, suggesting that *ceh-36* likely activates terminal differentiation of AWC directly (Kim *et al.*, 2010).

Mutations in *mls-2*, which encodes a HMX/NKX homeodomain protein, lead to the loss of *ceh-36* expression in AWC neurons (Kim *et al.*, 2010). Furthermore, *mls-2* is transiently expressed in AWC; MLS-2 recognizes a DNA sequence in the *ceh-36* promoter that is required for *ceh-36* expression in the AWC neuron (Kim *et al.*, 2010). This suggests that *mls-2* is an inducer of the AWC terminal selector gene *ceh-36*. However, *mls-2* mutants display incomplete penetrance in losing the expression of *ceh-36* and *odr-1* in AWC

neurons, suggesting that other transcription factor(s) may be required for regulating *ceh-36* expression in the AWC neurons.

ESTABLISHMENT OF STOCHASTIC AWC ASYMMETRY

AWC neurons differentiate asymmetrically into two distinct AWC^{OFF} and AWC^{ON} subtypes in a stochastic manner

The pair of AWC neurons, like other chemosensory neurons, appears symmetric anatomically and morphologically. However, AWC neurons display both molecular and functional asymmetries. One of the AWC neurons expresses the putative chemoreceptor gene *str-2* and is defined as the AWC^{ON} neuron. The other AWC neuron in the pair that does not express *str-2* but expresses an alternative chemoreceptor gene *srsx-3* is called the AWC^{OFF} neuron (Fig. 1a). The two AWC subtypes also have different functions, as AWC^{ON} senses butanone, while AWC^{OFF} detects 2,3-pentanedione (Fig. 1a). Wild-type animals have one AWC^{ON} and one AWC^{OFF} neurons. However, AWC asymmetry has random sidedness of the AWC^{ON} and AWC^{OFF} neurons, such that the left AWC neuron becomes the AWC^{ON} subtype in 50% of the animals in a population, while the right AWC neuron becomes AWC^{ON} in the other 50%. The stochastic nature of AWC asymmetry provides a unique model for understanding the underlying mechanisms of antisymmetry, one of the common biological phenomena (such as paw preference and handedness) across the animal kingdom (Palmer, 2004).

The cell bodies of the left and right AWC neurons are distant from each other, but their axons have direct contact and form chemical synapses with each other (White *et al.*, 1986). When one of the AWC precursor cells is ablated in early embryos, the surviving contralateral AWC neuron always becomes AWC^{OFF} (Troemel *et al.*, 1999). In addition, AWC^{ON} is not specified in mutants that are defective in axon guidance (Troemel *et al.*, 1999). Furthermore, AWC asymmetry is established during the time when the two AWC neurons form synapses on each other in late embryogenesis (Chuang and Bargmann, 2005; Troemel *et al.*, 1999). Together, these findings indicate that the default AWC state is AWC^{OFF} (Fig. 2a) and that communication between the two AWC neurons is required to induce the AWC^{ON} subtype and establish AWC asymmetry (Troemel *et al.*, 1999).

AWC asymmetry is reminiscent of other developmental events in which groups of equivalent cells interact to generate distinct cell types through lateral inhibition [also referred to as lateral specification (Greenwald, 2012)]. In many systems, including *C. elegans*, lateral inhibition is mediated by the Delta-Notch signaling pathway. While the involvement of Notch in this process is still presently unclear (Troemel *et al.*, 1999), a number of alternative mechanisms have been shown to regulate AWC asymmetry. Forward genetic screens have been the major driving force of elucidating the molecular mechanisms used to establish AWC asymmetry. The neuronal symmetry (*nsy*) mutants identified from the screens have 2 AWC^{OFF} neurons (2AWC^{OFF} phenotype) or 2 AWC^{ON} neurons (2AWC^{ON} phenotype) (Chuang and Bargmann, 2005; Chuang *et al.*, 2007; Lesch and Bargmann, 2010; Lesch *et al.*, 2009; Sagasti *et al.*, 2001; Troemel *et al.*, 1999; Vanhoven *et al.*, 2006). Genetic analysis of the *nsy* mutants and molecular characterization of the *nsy* genes define a transient, embryonic gap junction neural network (Fig. 2b) and a novel

calcium-regulated Ca^{2+}/calmodulin dependent protein kinase (CaMKII)-MAP kinase pathway (Fig. 3) for the establishment of AWC asymmetry.

A calcium- and microtubule- dependent MAP kinase pathway specifies the default AWC^{OFF} identity

In the default AWC^{OFF} state, calcium influx through UNC-2/EGL-19/UNC-36 voltage-gated calcium channels activates UNC-43 (CaMKII) (Fig. 3a, right cell, steps 1,2) (Troemel *et al.*, 1999). Upon UNC-43 (CaMKII) activation, the TIR-1 (Sarm1) adaptor protein assembles a calcium-signaling complex that includes UNC-43 (CaMKII) and NSY-1 MAP kinase kinase kinase (MAPKKK) (Fig. 3a, right cell, steps 2) (Chuang and Bargmann, 2005). NSY-1/MAPKKK physically binds to and phosphorylates SEK-1/MAPKK (Tanaka-Hino *et al.*, 2002), but the MAPK involved in the signaling cascade has yet to be identified.

Microtubules also play an important role in defining the AWC^{OFF} subtype. Disrupting microtubule polymerization using nocodazole causes a 2 AWC^{ON} phenotype, which recapitulates loss of function mutations in the calcium signaling proteins (Chang *et al.*, 2011). Microtubules in the AWC^{OFF} cell function to transport the UNC-43 (CaMKII)/TIR-1 (Sarm1)/NSY-1 (MAPKKK) complex to the axonal synapses via an unknown motor protein (Fig. 3b, right cell, step 3). A missense mutation within the N-terminal regulatory domain of TIR-1 in the *tir-1(ky648)* allele confers a gain of function phenotype in a microtubule-dependent manner (Chang *et al.*, 2011), suggesting an uncharacterized mechanism of regulation for the calcium signaling complex. Retrograde signaling transported from the AWC axons to the cell body is proposed to suppress the expression of *str-2* and allow the transcription of *srsx-3*, thereby defining the AWC^{OFF} subtype (Fig. 3c, right cell, steps 4,5) (Chang *et al.*, 2011). Additionally, the UNC-104/kinesin motor protein acts non-cell autonomously in the contralateral AWC neuron to promote AWC^{OFF}, indicating a role of *unc-104* in a feedback mechanism (Chang *et al.*, 2011).

Intercellular calcium signaling via a gap junction neural network coordinates AWC asymmetry

During embryogenesis, a transient neural network is formed between the cell bodies of AWC neurons and adjacent neurons via NSY-5 gap junctions (Fig. 2b) (Chuang *et al.*, 2007). The NSY-5 network is defined by the 18 pairs of *nsy-5*-expressing neurons that are likely to be linked by gap junctions (Chuang *et al.*, 2007; Schumacher *et al.*, 2012; Taylor *et al.*, 2010). Calcium in the NSY-5 network has dual roles in AWC asymmetry: 1) an autonomous role in AWC neurons in promoting the AWC^{OFF} subtype, 2) a non-autonomous role in non-AWC neurons of the NSY-5 network in promoting the induced AWC^{ON} subtype (Schumacher *et al.*, 2012). *nsy-5* activity functions primarily in AWC to promote the AWC^{ON} subtype, and *nsy-5* activity in specific non-AWC neurons of the network promotes or inhibits the AWC^{ON} induction depending on the neuron types (Chuang *et al.*, 2007). In addition, the levels of calcium in specific non-AWC neurons within the NSY-5 network influence side biases of AWC^{ON} induction in a manner dependent on NSY-5 gap junctions. A high calcium level in the right AWB neuron inhibits the right AWC neuron to become the AWC^{ON} (or promotes AWC^{OFF}) subtype and a high calcium level in the left ASH promotes the AWC^{ON} subtype on the left side (Fig. 2b) (Schumacher *et al.*, 2012). Together, these

findings indicate that intercellular calcium signaling between the two AWCs and other neurons in the NSY-5 network is required for the precise $1AWC^{ON}/1AWC^{OFF}$ decision (Schumacher *et al.*, 2012).

Neurons in *C. elegans* form chemical synapses along their axons in order to communicate with each other (White *et al.*, 1986). Proper axon guidance is required for precise contact and communication between the left and right sides of the neuron pairs in the NSY-5 gap junction network, and is thus important for the AWC^{ON} induction (Fig. 2b, 3a, left cell, step 1), as mutations in axon guidance molecule genes such as *unc-76* (plasma-membrane associated protein) and *sax-3* (ROBO) display a $2AWC^{OFF}$ phenotype (Troemel *et al.*, 1999). Differential calcium levels between the two AWC neurons establish AWC asymmetry: the AWC with a higher calcium level remains as the default AWC^{OFF} subtype, and the AWC with a lower calcium level becomes the induced AWC^{ON} subtype (Fig. 3) (Schumacher *et al.*, 2012). Several mechanisms downstream of axon outgrowth and guidance have been identified to inhibit the calcium-regulated UNC-43 (UNC-43)/TIR-1 (Sarm1)/NSY-1 (MAPKKK) signaling pathway in the future AWC^{ON} neuron (Fig. 3, left cell). 1) NSY-5 gap junctions and NSY-4 claudin-like adhesions function in parallel to inhibit voltage-gated calcium channels, which results in a low intracellular calcium concentration and repression of the UNC-43/CaMKII mediated signaling cascade (Fig. 3a, left cell, step 2) (Chuang *et al.*, 2007; Vanhoven *et al.*, 2006). 2) microRNAs have been shown to play crucial roles in various aspects of neural development, including neuronal asymmetry (Alqadah *et al.*, 2013). The microRNA *mir-71* inhibits the expression of the TIR-1/Sarm1 adaptor protein by binding to its 3′ UTR (Fig. 3b, left cell, step 3) (Hsieh *et al.*, 2012). *nsy-5* and *nsy-4* positively regulate the stabilization of mature *mir-71* via an unidentified mechanism (Fig. 3b, left cell, step 3) (Hsieh *et al.*, 2012). 3) The Raw repeat protein OLRN-1 represses UNC-43 (CaMKII) (Fig. 3c, left cell, step 4) (Bauer Huang *et al.*, 2007). Taken together, these events ensure the repression of the calcium-regulated CaMKII-MAPK signaling cascade in the future AWC^{ON} neuron, leading to the expression of the AWC^{ON} marker *str-2* and repression of the AWC^{OFF} marker *srsx-3* (Fig. 3c, left cell, step 5).

MAINTENANCE OF AWC ASYMMETRY

AWC asymmetry is established using transient signaling during embryogenesis and is maintained using distinct mechanisms throughout the life of an animal (Troemel *et al.*, 1999). Forward genetic screens identified three pathways required for the maintenance of AWC asymmetry: olfactory signaling, transcriptional regulation, and TGF-β signaling (Fig. 4) (Lesch and Bargmann, 2010; Lesch *et al.*, 2009; Troemel *et al.*, 1999). Mutations in the maintenance pathways lead to the loss of AWC asymmetry after the first larval stage.

Olfactory signaling

An olfactory cGMP transduction pathway maintains AWC^{ON} and AWC^{OFF} subtypes. Components in the olfactory signaling pathway required for the maintenance of AWC asymmetry include two receptor-type guanylyl cyclases (encoded by *odr-1* and *daf-11*), a Gα subunit (encoded by *odr-3*), a cyclic-nucleotide gated cation channel (encoded by *tax-2* and *tax-4*), and a cGMP-responsive protein kinase (encoded by *egl-4*) (Lesch and

Bargmann, 2010; Lesch *et al.*, 2009; Troemel *et al.*, 1999). *odr-1*, *daf-11*, *odr-3*, and *egl-4* are required to maintain both AWC^{ON} and AWC^{OFF} subtypes, while *tax-2* and *tax-4* only maintains the AWC^{OFF} subtype (Fig. 4) (Lesch and Bargmann, 2010; Lesch *et al.*, 2009; Troemel *et al.*, 1999).

Transcriptional regulation

Two transcription factors, NSY-7 and HMBX-1, are known to play a role in maintaining AWC asymmetry. NSY-7, a homeodomain-like transcription factor, is expressed predominantly in AWC^{ON}. NSY-7 maintains the expression of *str-2* and also functions to repress *srsx-3* in response to a transient embryonic signal from NSY-5 gap junctions in the AWC^{ON} neuron (Fig. 4, left cell) (Lesch *et al.*, 2009). HMBX-1, a homolog of mammalian HMBOX1, also functions to repress *srsx-3* expression in the AWC^{ON} neuron (Fig. 4, left cell) (Lesch and Bargmann, 2010).

TGF-β signaling

TGF-β signaling is also involved in maintaining AWC^{OFF} (Fig. 4, right cell), as mutations in a TGF-β ligand (encoded by *daf-7*) or a TGF-β type I receptor (encoded by *daf-1*) causes the loss of *srsx-3* expression in adulthood (Lesch and Bargmann, 2010). *daf-7* inhibits the formation of dauer (Lesch and Bargmann, 2010; Ren *et al.*, 1996), an arrested stage developed in adverse environments such as starvation, overcrowding, or high temperature. *daf-7* expression is inhibited by the dauer pheromone (Lesch and Bargmann, 2010; Ren *et al.*, 1996), a mixture of small molecules for entry into dauer. The dauer pheromone inhibits *srsx-3* expression through downregulating the TGF-β pathway (Lesch and Bargmann, 2010), suggesting that dynamic environmental cues can regulate the maintenance of AWC asymmetry.

BEYOND ASYMMETRY

Stochastic cell fate decisions

The AWC neuron pair acquires two mutually exclusive subtypes and distinct functions through a stochastic, coordinated cell-signaling event. Stochastic cell fate acquisition in animal development is a conserved but only partly understood phenomenon in all species (Johnston and Desplan, 2008, 2010; Losick and Desplan, 2008). For example, only one allele of the ~1300 olfactory receptor genes is expressed per neuron probably via a stochastic, random process in the mouse olfactory system (Mombaerts, 2004; Nguyen *et al.*, 2007; Serizawa *et al.*, 2003). In the human retina, the M or L cone photoreceptor cell subtypes are selected randomly through stochastic cell fate choice (Nathans, 1999; Smallwood *et al.*, 2002). In the *Drosophila* eye, two subtypes of ommatidia are specified randomly by stochastic opsin expression in R7 and R8 photoreceptors (Mikeladze-Dvali *et al.*, 2005; Wernet *et al.*, 2006). Stem cells have the ability to maintain their own populations through self-renewal and to give rise to differentiated progeny (Reya *et al.*, 2001); the balance between self-renewal and differentiation of stem cells is regulated in a stochastic manner (Blanpain and Simons, 2013). In the immune system, B lymphocytes acquire a large number of distinct fates in response to stimulation; B-cell fate choice can be stochastic, directed, inherited, or some combination of these mechanisms (Duffy *et al.*, 2012; Tarlinton,

2012). Although promoter selection and lateral signaling are shown to play roles in some of these processes, fundamental questions regarding these stochastic choice mechanisms still remain. AWC asymmetry is an excellent model to identify molecular mechanisms controlling stochastic cell fate specification.

Lateral inhibition

The asymmetry observed in AWC neurons reflects a novel example of lateral inhibition independent of Notch. A classic example of Notch-mediated lateral inhibition is observed in the *C. elegans* gonad primordium, in which two cells have equal potential to become either an anchor cell (AC) or a ventral uterine cell (VU). The two initially equivalent cells interact via the LIN-12 (Notch) receptor and the LAG-2 (Delta) ligand. During this interaction, positive and negative feedback loops amplify a small difference in Notch activity, so that the cell with lower Notch activity adopts the AC fate and the cell with higher Notch activity becomes VU (Greenwald, 2012). In the adult peripheral nervous system of *Drosophila*, interactions among a small proneural cluster of equivalent cells via Notch and Delta ensure that the cell with lower Notch activity becomes the sense organ precursor and the cells with higher Notch activity become epidermal (Hartenstein and Posakony, 1990; Simpson, 1990), which is analogous to the *C. elegans* AC/VU decision. In the developing vertebrate nervous system, neurogenesis is also controlled by Notch-dependent lateral inhibition (Formosa-Jordan *et al.*, 2013; Kageyama *et al.*, 2008; Kiernan, 2013).

Although AWC asymmetry could possibly be independent of Notch (Troemel *et al.*, 1999), similarities between the Notch signaling system of lateral inhibition and that of AWC are observed. First, as in Notch signaling, cell-cell interaction between the two AWC neurons and other neurons is important for the specification of the two AWC subtypes (Chuang *et al.*, 2007; Troemel *et al.*, 1999). Second, in Notch lateral inhibition, high levels of Notch receptor lead to a particular fate versus another. Similarly, differential levels of calcium determine AWC asymmetry. High levels of calcium in an AWC neuron specify an AWC^{OFF} fate, whereas low calcium levels in an AWC neuron results in induction of AWC^{ON} (Schumacher *et al.*, 2012). Third, similar to Notch signaling, AWC lateral inhibition also displays feedback mechanisms (Chang *et al.*, 2011; Chuang *et al.*, 2007). AWC feedback is mediated via a network of neurons, which are connected by gap junctions to ensure precise AWC^{ON}/AWC^{OFF} fate specification perhaps by sensing the levels of calcium (Chuang *et al.*, 2007; Schumacher *et al.*, 2012).

Homologs of AWC asymmetry genes and their relevant functions in vertebrates

Calcium is an important signaling molecule for AWC asymmetry. In vertebrates, calcium is also required for left-right patterning and neuronal development. Transient calcium release in the left side of vertebrates initiates asymmetric expression of the TGF-β ligand Nodal to regulate visceral and brain asymmetry (Bisgrove *et al.*, 2003; Liang *et al.*, 2000; McGrath and Brueckner, 2003). Spontaneous patterns of calcium spikes during embryonic CNS development are critical for the specification of transmitter expression (Borodinsky *et al.*, 2004).

Vertebrate homologs of AWC asymmetry genes have been implicated in left-right patterning as well as nervous system development and function: 1) Claudin tight junction proteins play a role in left-right patterning of internal organs in chick and frogs (Brizuela *et al.*, 2001; Simard *et al.*, 2006). 2) Connexin gap junctions play critical roles in early left-right patterning in frog embryos (Levin and Mercola, 1998). Gap junctions are also involved in the differentiation of P19 embryonic carcinoma cells into neurons (Bani-Yaghoub *et al.*, 1999). Furthermore, gap junction adhesions are required for glial-guided neuronal migration in the mouse embryonic cerebral cortex (Elias *et al.*, 2007). 3) UNC-2/UNC-36 is an N/P-type voltage-gated calcium channel. In vertebrates, N/P-type calcium channels are important for initiation of synaptic transmission at fast synapses (Catterall, 2011). 4) The vertebrate TIR domain adaptor protein Sarm1, which is also called MyD88-5, is a homolog of *C. elegans* TIR-1 (Kim *et al.*, 2007; Mink *et al.*, 2001). Sarm1 is required for cell death of hippocampal neurons during deprivation of oxygen and glucose (Kim *et al.*, 2007) and for activation of an injury-induced axon death pathway (Osterloh *et al.*, 2012). The functions of Sarm1 in cell death and axon degeneration are consistent with the role of *tir-1* in promoting a cell death program in *C. elegans* linker cells (Blum *et al.*, 2012). In addition, Sarm1 controls syndecan-2 dependent dendritic arborization (Chen *et al.*, 2011). 5) CaMKII is important for dendritic development, memory, and plasticity (Wayman *et al.*, 2008). CaMKII directly phosphorylates NeuroD, a proneural transcription factor of the basic helix loop helix class, to regulate dendrite morphogenesis (Gaudilliere *et al.*, 2004). 6) MAPK signaling is required for fear conditioning in mice and CaMKII is activated in the same hippocampal region after learning (Atkins *et al.*, 1998).

CONCLUSIONS

In late embryogenesis, the *C. elegans* left and right AWC olfactory neurons communicate through intercellular calcium signaling across a gap junction neural network to differentiate into two distinct subtypes: default AWC^{OFF} and induced AWC^{ON}. AWC^{ON} and AWC^{OFF} express different odorant receptors and sense different odors. Asymmetrical differentiation of the two AWC neurons diversifies sensory repertoire of the animal. The AWC^{OFF} subtype is specified by a calcium- and microtubule-dependent CaMKII-MAPK cascade. Lateral signaling resulting from cell-cell interaction (mediated by gap junctions and claudin-like adhesions) inhibits the calcium-CaMKII-MAPK pathway in the AWC cell that becomes AWC^{ON}. AWC asymmetry is maintained throughout the life of the animal by three distinct mechanisms: olfactory transduction, transcriptional regulation, and TGF-β signaling.

C. elegans has emerged as a powerful organism to investigate the molecular mechanism involved in establishing asymmetry in the nervous system. The organism's genetic amenability combined with the ability to identify mutants in an unbiased manner using forward genetic screens has provided insight into the mechanisms on how asymmetry of the nervous system occurs and the rationale for evolutionary advantage in developing asymmetry. However, much remains to be elucidated on how AWC asymmetry is established. One of the most important questions that remain unaddressed is how stochastic AWC asymmetry is initiated. In addition, there are some gaps in the calcium-regulated CaMKII-MAPK pathway required for AWC asymmetry. In AWC^{OFF} cell specification, there are several unanswered questions including the identification the microtubule-based

motor protein(s) required for the transport of the TIR-1 calcium-signaling complex to the AWC synapses, the identification of downstream signaling components (such as MAPK and transcription factors) downstream of SEK-1 (MAPKK), and the retrograde signal that leads to the expression of *srsx-3* and the suppression of *str-2* expression. In the AWC^{ON} cell, it is still unknown how gap junctions and claudin-like adhesions result in the inhibition of voltage-gated calcium channels and the transcription factor(s) that initiate *str-2* expression. Furthermore, it is possible that molecular and functional asymmetries may extend to other neuronal pairs of the NSY-5 network and this hypothesis is worth further investigation.

Many of the genes involved in AWC asymmetry are evolutionarily conserved and vertebrate homologs of these genes are required for left-right patterning and many facets of nervous system development and function. The knowledge gained from AWC asymmetry may shed light on various aspects of developmental asymmetries and neuronal development in vertebrates. Furthermore, AWC asymmetry is a unique form of lateral inhibition, which leads to stochastic cell fate specification. Pioneering work using *C. elegans* and *Drosophila* was instrumental to elucidating the Notch signaling system, which proved to be conserved in vertebrates. The novel type of AWC lateral inhibition may prove to be common in developmental decisions in other systems.

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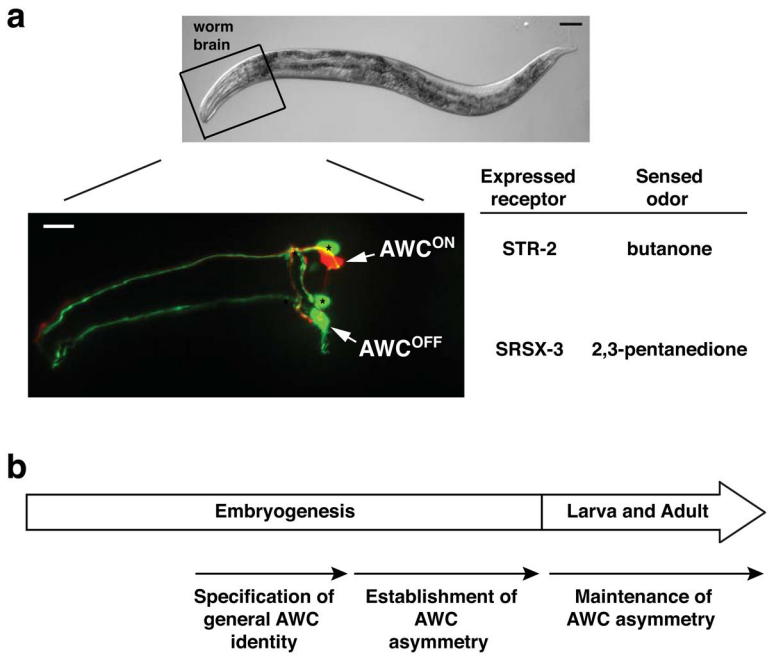

FIG. 1. The *C. elegans* left and right AWC olfactory neurons differentiate asymmetrically at molecular and functional levels

(**a**) Top panel: DIC image of an adult *C. elegans* with anterior to the left and dorsal to the top. Scale bar, 50 μm. Bottom panel: Fluorescent micrograph image of the AWC^{ON} neuron expressing *str-2p::TagRFP* and the AWC^{OFF} neuron expressing *srsx-3p::GFP*, taken from the head region outlined in the top panel. Arrows indicate the cell body of AWC neurons. Asterisks indicate AWB neurons. Scale bar, 10 μm.

(**b**) Developmental timeline of AWC asymmetry.

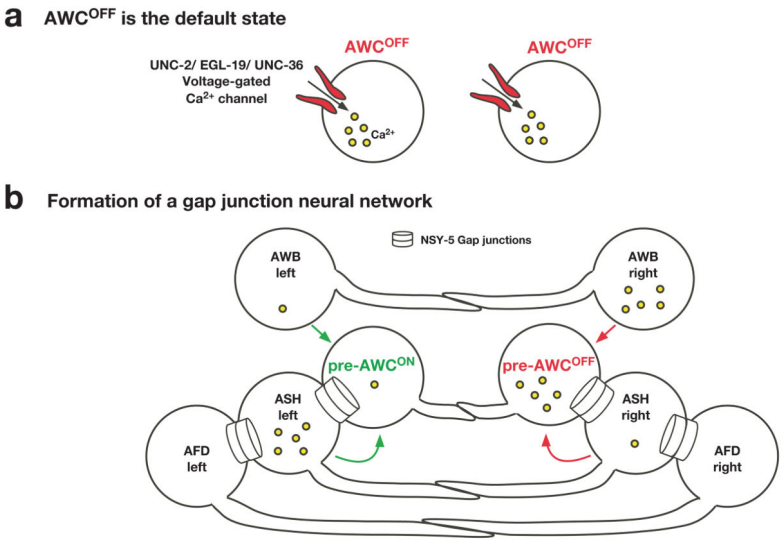

FIG. 2. A transient embryonic gap junction neural network coordinates stochastic AWC asymmetry

(**a**) Prior to cell-cell communication, both AWC neurons have high intracellular calcium levels and symmetrically exist in the default AWC^{OFF} state.

(**b**) AWC, ASH, AFD, and AWB sensory neurons are part of a transient embryonic neural network connected by NSY-5 gap junctions and contribute to the decision making of stochastic AWC asymmetry. Differences in calcium levels between left and right sides of neuronal pairs promote the AWC^{ON} or AWC^{OFF} subtype, depending on the cellular context. AWC asymmetry is stochastic, and this figure illustrates the case when AWC^{ON} is on the left and AWC^{OFF} is on the right.

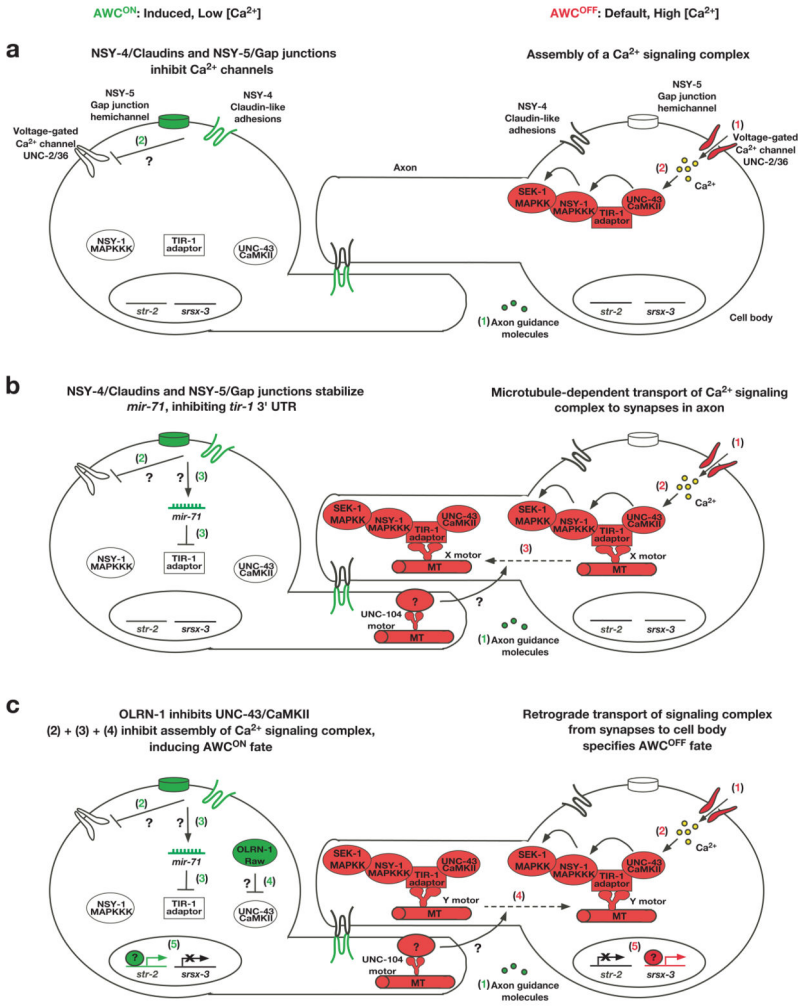

FIG. 3. Establishment of AWC asymmetry

(**a–c**) AWC asymmetry is stochastic, and this figure illustrates the case when AWC^{ON} is on the left and AWC^{OFF} is on the right. Molecules in red represent AWC^{OFF} promoting, those in green represent AWC^{ON} promoting, and those in white indicate inactive or less active molecules. Question marks represent steps in which the molecular mechanism is unknown. Steps in the AWC^{OFF} neuron are proposed to take place sequentially, however the steps in AWC^{ON} may not occur in the sequence proposed in the illustration, as the sequence of events has not yet been determined.

Default AWC^{OFF} (right): (**a**) 1. Calcium enters the cell through voltage-gated calcium channels. 2. Calcium influx stimulates UNC-43 (CaMKII), allowing the assembly of a calcium-signaling complex consisting of UNC-43 (CaMKII), the TIR-1 (Sarm1) adaptor protein, and NSY-1 (MAPKKK). The assembly of the calcium-signaling complex brings these molecules in close proximity of each other, so that UNC-43 (CaMKII) phosphorylates NSY-1 (MAPKKK) and then NSY-1 (MAPKKK) phosphorylates SEK-1 (MAPKK). (**b**) 3. Microtubules transport the UNC-43 (CaMKII)/TIR-1 (Sarm1)/NSY-1 (MAPKKK) calcium-signaling complex to synapses in AWC axons via an unidentified “X” motor protein. The UNC-104 kinesin motor protein in the contralateral AWC transports an unknown molecule,

which is required for the transport of the TIR-1 signaling complex in the AWC^{OFF} cell to specify the AWC^{OFF} subtype. (**c**) 4. Proposed retrograde signaling, mediated by an unidentified "Y" motor protein, may convey the lateral signaling between the two AWC cells from the synapses to regulate gene expression in the cell body. 5. The AWC^{OFF} marker *srsx-3* is transcribed, and the expression of the AWC^{ON} marker *str-2* is suppressed. *Induced* AWC^{ON} (left): (**a**) 1. Axon guidance molecules contribute to AWC axon outgrowth, allowing chemical synapse formation and communication between the two cells. 2. NSY-5 gap junctions and NSY-4 claudin-like adhesions act in parallel to inhibit voltage-gated calcium channels, resulting in a low level of intracellular calcium. (**b**) 3. NSY-5 and NSY-4 stabilize mature *mir-71* miRNA, which inhibits calcium signaling through targeting the 3′ TUR of *tir-1*/Sarm1. (**c**) 4. OLRN-1 Raw repeat protein inhibits UNC-43 (CaMKII). 5. The AWC^{ON} marker *str-2* is expressed, and the AWC^{OFF} marker *srsx-3* is inhibited.

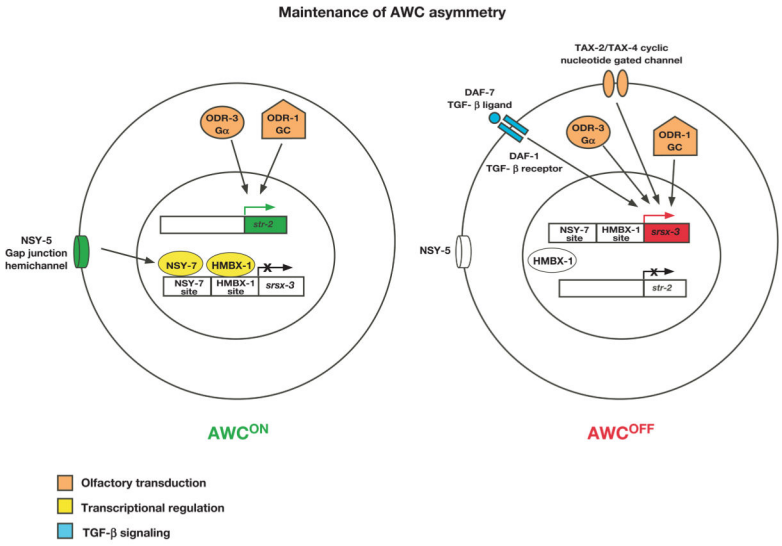

FIG. 4. Maintenance of AWC asymmetry

AWC asymmetry is stochastic, and this figure illustrates the case when AWC^{ON} is on the left and AWC^{OFF} is on the right. Molecules in red represent AWC^{OFF} promoting, those in green represent AWC^{ON} promoting, and those in white indicate inactive or less active molecules. Molecules in orange, yellow, and blue represent the three distinct mechanisms used for the maintenance of AWC asymmetry. GC, guanylyl cyclase. *Left*: The AWC^{ON} subtype is maintained using two mechanisms: olfactory transduction (ODR-1 and ODR-3 represented in orange maintain *str-2* expression), and transcriptional regulation (HMBX-1 and NSY-7 represented in yellow repress *srsx-3* expression). *Right*: The AWC^{OFF} subtype (*srsx-3* expression) is maintained using olfactory transduction molecules (TAX-4, ODR-1, and ODR-3 represented in orange) and TGF-β signaling (DAF-7 and DAF-1 represented in blue).