REVIEW

Development of Left/Right Asymmetry in the Caenorhabditis elegans Nervous System: From Zygote to Postmitotic Neuron

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Summary: Despite their gross morphological symmetry, animal nervous systems can perceive and process information in a left/right asymmetric manner. How left/right asymmetric functional features develop in the context of a bilaterally symmetric structure is a very poorly understood problem, in part because very few morphological or molecular correlates of functional asymmetries have been identified so far in vertebrate or invertebrate nervous systems. One of the very few systems in which a molecular correlate for functional lateralization has been uncovered is the taste sensory system of the nematode *Caenorhabditis elegans*, which is composed of a pair of bilaterally symmetric neurons, ASE left (ASEL) and ASE right (ASER). ASEL and ASER are similar in morphology, connectivity, and molecular composition, but they express distinct members of a putative chemoreceptor gene family and respond in a fundamentally distinct manner to taste cues. Extensive forward and reverse genetic analysis has uncovered a complex gene regulatory network, composed of transcription factors, miRNAs, chromatin regulators, and intercellular signals, that instruct the asymmetric features of these two neurons. In this review, this system is described in detail, drawing a relatively complete picture of asymmetry control in a nervous system. genesis 52:528–543, 2014. © 2014 Wiley Periodicals, Inc.

Key words: brain asymmetry; gene regulatory network; miRNAs

The concept of symmetry and derivations thereof has long captured human imagination (Close, 2000; McGilchrist, 2009; McManus, 2002; Weyl, 1952). In the realm of biology and, specifically, animal body plans, anatomical symmetries appear obvious. However, there are striking deviations from symmetry, as first systematically catalogued in a detailed monograph in 1932 (Ludwig, 1932). Some of these asymmetries are more obvious than others. The asymmetric placement of visceral organs has been appreciated ever since humans displayed the curiosity to study their own anatomy. What took much longer to appreciate are the pervasive asymmetries that we now know to exist in invertebrate and vertebrate brains (Concha *et al*., 2012; Davidson and Hugdahl, 1994; Hobert *et al*., 2002; Hugdahl and Davidson, 2003; Rogers *et al*., 2013; Sun and Walsh, 2006). Among the many functional brain asymmetries, the first to be appreciated as such was language lateralization, discovered by Pierre-Paul Broca in the 19th century. The description of many other brain lateralities has followed (Rogers *et al*., 2013). Few brain asymmetries are obvious on the morphological level. As already described in Ludwig's classic monograph, the habenular nuclei of fish display striking differences in size on the left versus right side (Ludwig, 1932; Roussigne *et al*., 2012). However, apart from other relatively subtle size asymmetries, lateralities in brain function are usually not mirrored by clearly defined anatomical

Additional Supporting Information may be found in the online version of this article.

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asymmetries. In fact, there are no currently known brain structures in mammalian brains that exclusively exist on one side and not the other. Moreover, no specific molecular markers that clearly delineate functionally lateralized brain regions have been identified in mammals.

How do left/right asymmetries develop? Some brain asymmetries clearly are activity-dependent. For example, the lateralization of the visual system in chick depends on light exposure to specifically one eye (Rogers *et al*., 2013). However, the development of other lateralities, such as language lateralization, appears to be genetically hardwired by unknown means. Although genetically hardwired mechanisms of establishment of visceral organ asymmetries have been uncovered (Levin, 2005; Shiratori and Hamada, 2006; see other reviews in this issue), it is not clear how these mechanisms impinge on brain lateralities. In fact, both genetic manipulations of zebrafish and the examination of humans with situs inversus have indicated that patterning mechanisms for visceral organ asymmetries can be genetically separated from brain asymmetries (Bisgrove *et al*., 2000; Kennedy *et al*., 1999). The fortuitous discovery of asymmetrically expressed genes in the nervous system of the nematode *C. elegans* in the late 1990s (Troemel *et al*., 1999; Yu *et al*., 1997) has established this simple invertebrate as a system to understand how left/right asymmetries are genetically programmed and superimposed onto a largely bilaterally symmetric nervous system.

ASYMMETRIES IN THE NERVOUS SYSTEM OF THE NEMATODE *CAENORHABDITIS ELEGANS*

The simple and extraordinarily well-mapped nervous system of the nematode *C. elegans* provides a unique opportunity, not matched in any other model system so far, to detect and functionally dissect asymmetries in a nervous system.

Detailed lineage and anatomical studies in *C. elegans* have revealed several themes in nervous system symmetry (Sulston *et al*., 1983; White *et al*., 1986), as described in detail in a previous review (Hobert *et al*., 2002). First, like other brains, the main head and tail ganglia of the nervous system of *C. elegans* displays pervasive bilateral symmetry on an anatomical level in that most of the neurons in the major brain ganglia come as pairs of bilaterally symmetric neurons (some even come in radially symmetric groups of four or six neurons; Fig. 1; Sulston *et al*., 1983; White *et al*., 1986). The symmetric nature of these neuron pairs is evident on the level of gross anatomy (cell body placement and axo/dendritic morphology), as well as on the level of very fine grained anatomical features (e.g., synaptic connectivity patterns). Extensive expression pattern descriptions with single-neuron resolution conducted in the nervous system over the past quarter century, usually with the help of reporter genes (manually curated at www.wormbase.org), has also provided ample evidence for symmetries on the molecular level.

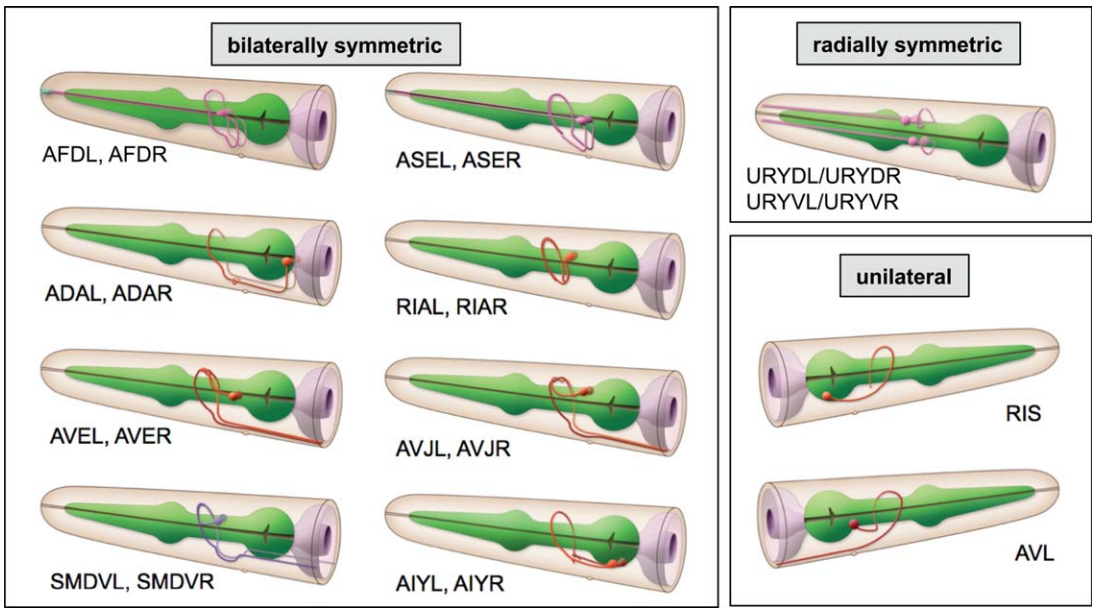

FIG. 1. Symmetric and asymmetric in the *C. elegans* nervous system. A representative sampling of neurons from the head ganglia of of *C. elegans* is shown. Most head neurons come as bilaterally symmetric pairs, a few come as radially symmetric groups that are composed of bilaterally symmetric pairs (e.g., the URY neuron class shown here) and some are unilateral neurons (e.g., the RIS and AVL neurons shown here). All images shown here are from www.wormatlas.org (Hall and Altun, 2007). For additional schematic representations of the symmetric and asymmetric features of the *C. elegans* nervous system, see Hobert *et al*., 2002.

About half of the bilaterally symmetric neuron pairs (excluding neurons in the main feeding organ of the worm, the pharynx) are composed of individual neurons that derive from a symmetric cleavage pattern, that is, paired neurons share a similar lineage history and are only distinguished by one early cell division event along the left/right axis in the early embryo (l/r division of the ABa and ABp blastomeres; Fig. 2a and Supporting Information Table S1; Sulston *et al*., 1983). The other half of bilateral neuron pairs are composed of neurons that display nonsymmetric lineage histories. In most cases, individual neurons of these pairs derive from distinct, nonsymmetric blast cells in the embryo and converge into similar, left/right symmetric cleavage patterns only later in embryonic development (Fig. 2b and Supporting Information Table S2).

In addition to bilateral pairs, a small number of neurons, peppered throughout the head and tail ganglia, are unilateral neurons with no homologous counterpart on the contralateral side of the animal (Fig. 1; Hobert *et al*., 2002; Sulston *et al*., 1983). In these cases, the contralateral cell in the lineage is either a completely distinct type of neuron or neuroblast or a non-neuronal cell (Fig. 2a). Such striking, yet nevertheless sparsely distributed anatomical asymmetries are extraordinarily hard to detect in more complex and less well-mapped out nervous systems.

The key trigger for studying asymmetries in the context of anatomically bilateral symmetric structures were two sets of expression pattern analyses of putative chemoreceptor gene families, conducted in the 1990s (Troemel *et al*., 1999; Yu *et al*., 1997). These studies revealed gene expression asymmetries in two distinct bilateral neuron pairs, the AWC olfactory neurons and the ASE gustatory neurons. Strikingly, these asymmetries are fundamentally distinct in appearance and regulation: AWC asymmetries are stochastic and manifest themselves in the expression of olfactory-type G-protein coupled receptors (GPCRs) in either the left or right AWC neuron (Bauer Huang *et al*., 2007; Troemel *et al*., 1999). Such stochastic distributions of asymmetric features are generally referred to as "antisymmetries" (Palmer, 2004). In contrast, ASE asymmetries are directional; some receptor-type guanylyl cyclases (rGCs) are only expressed in the right ASE neuron, whereas others are only expressed in the left neuron (Ortiz *et al*., 2006; Yu *et al*., 1997). Generally, directional asymmetry is defined as an asymmetric feature being stereotypically found on one side of an animal in >95% of individuals examined (Palmer, 2004).

Although these antisymmetries and directional asymmetries are very evident in animal body plans throughout the animal kingdom (Palmer, 2004), the relative contribution of these types of asymmetries to brain asymmetries is somewhat less clear. It is very obvious that the most known brain lateralities, such as the classic cases of speech lateralization, are directionally symmetric (i.e., found in >95% of examined individuals on one side of the brain; in this case, the left side), but it is less clear how pervasive antisymmetries are in the nervous system. Perhaps the best example for antisymmetries is paw preferences in nonhuman vertebrates, which tend to be equally distributed among individuals in most vertebrate species (Rogers, 2009).

In this review, a description of directional asymmetry of the two ASE neurons is given, including their functional relevance, and developmental origin. I will not attempt to provide a historical narrative of how our understanding of ASE asymmetry evolved over the past 15 years but rather provide a succinct summary of the system. AWC antisymmetry is covered in an accompanying review in this issue (Hsieh *et al*., 2014) and will not be further considered here.

THE ASE NEURON PAIR: LINEAGE AND ANATOMY

The ASE neuron pair (Fig. 1) is composed of two seemingly bilaterally symmetric amphid neurons which project dendrites to the tip of the nose where their exposure to the environment permit sampling of water-soluble environmental cues. Their axons extend via the amphid commissure into the nerve ring where synaptic connections are made and received. Overall, the synaptic connectivity patterns of the ASE left (ASEL) and ASE right (ASER) neurons are similar (White *et al*., 1986). Their main synaptic outputs, mediated by the neurotransmitter glutamate, are to the AIY and AIB interneurons.

The only striking morphological difference between ASEL and ASER is that the cell body of the ASER neuron is about 30% larger than that of the ASER neuron (Goldsmith *et al*., 2010). This size difference is genetically programmed and under control of the same genes that also determine other asymmetric features of the ASE neuron pair (which will be described further below; Goldsmith *et al*., 2010). Why size is asymmetrically controlled is unclear, but it is striking to note that similar size differences in contralateral soma pairs are also observed in the functionally lateralized optic tectum of birds (Gunturkun, 1997; Manns and Gunturkun, 2003).

ASEL and ASER belong to the roughly one half of all neuron pairs whose lineage history is not symmetric, at least initially (Fig. 2b and Supporting Information Table S2). The ASEL neuron derives from the ABa, whereas ASER derives from the ABp blastomere. The initial positions and cleavage patterns of these precursor cells are different, but during gastrulation, descendants of the ASEL precursor, the ABalppp neuroblast, align through a highly stereotyped movement in positions that are left/right symmetric to the descendants of the ASER precursor ABpraaa (Harrell and Goldstein, 2011). The cleavage pattern of the two neuroblast lineages then become identical (Fig. 2b). Differential gastrulation

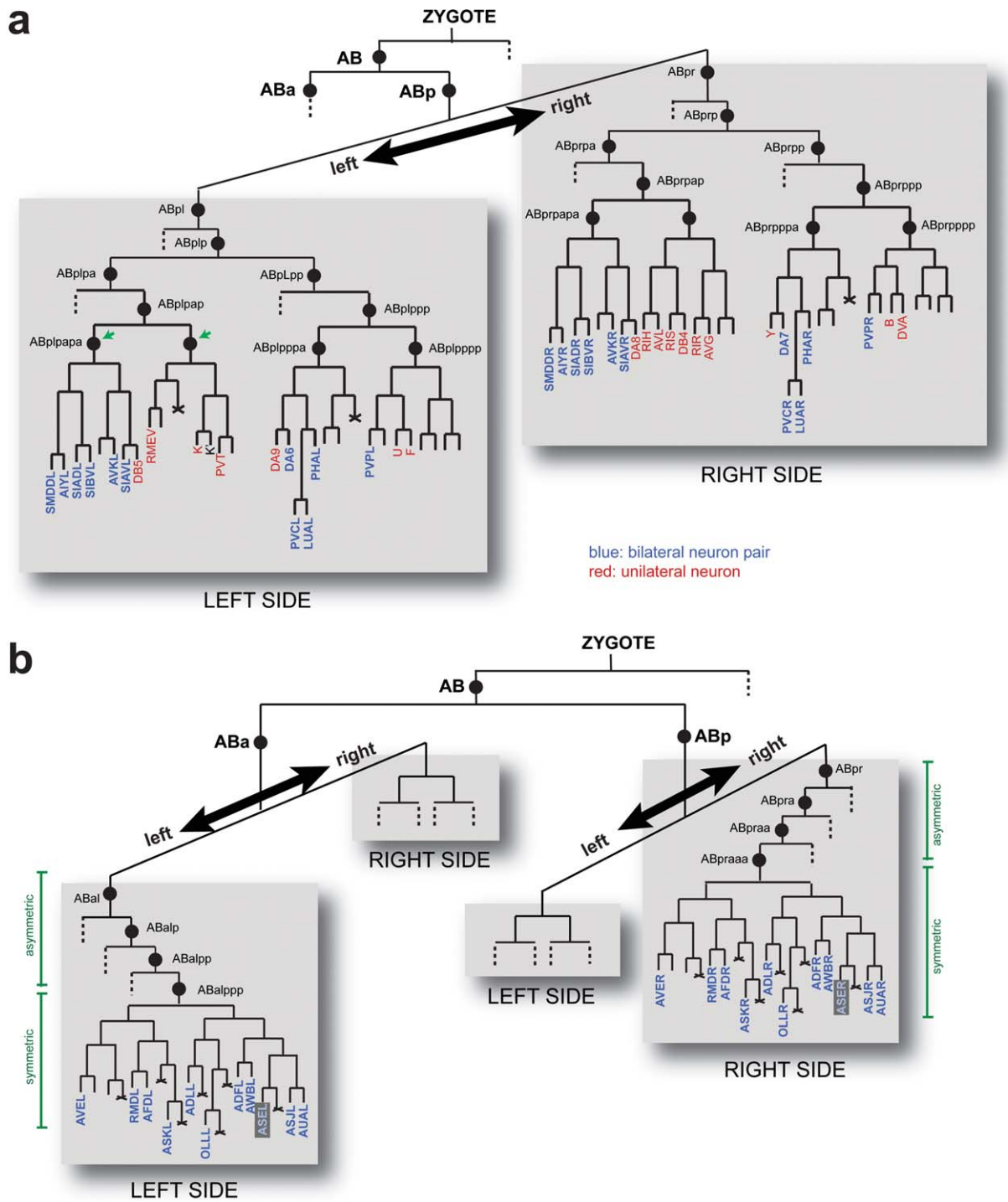

FIG. 2. Lineage history and neuronal symmetry. Lineage histories have been elucidated by Sulston *et al*. (1983). "a/p/l/r" indicate cleavages along the anterior/posterior/left/right axis. (**a**) Example of lineage branches with a bilaterally symmetric history that arise from an early division of the ABp blastomere across the left/right axis. These two branches contain bilaterally symmetric neuron pairs, indicated in blue, but they also contain unilateral neurons (3- or 4-letter code) or unilateral neuroblasts (that divide postembryonically; single-letter code), indicated in red. The small green arrows indicate that these blastomeres receive a Notch signal that may be involved in generating asymmetry within these lineage branches (see text). Supporting Information Table S1 contains a list of all bilateral neuron pairs that share a bilaterally symmetric lineage history. (**b**) Lineage branches that produce bilaterally symmetric neurons but derive from distinct blastomeres and only begin to share a bilaterally symmetric cleavage pattern after gastrulation. The branch shown here contains 11 neuron pairs, more than one quarter of the 40 neuron pairs that do not share a bilaterally symmetric lineage history (these 40 neuron pairs are listed in Supporting Information Table S2). The ASE neuron pair is shaded in gray.

movements of other, initially asymmetric neuroblasts also result in left/right symmetric positioning of specific neuroblast pairs that then go on to generate left/right symmetric neurons (Supporting Information Table S2). However, as mentioned above, in contrast to the ASE neuron pair, many other bilateral neuron pairs share a completely bilaterally symmetric lineage history (Fig. 2a and Supporting Information Table S1).

Molecular Symmetries and Asymmetries of the ASE Neurons

The morphological and lineage symmetry of the two ASE neurons that is apparent after the end of gastrulation is mirrored by a striking symmetry of gene expression in ASEL and ASER. Early patterning genes like the proneural bHLH factor *hlh-14* (Poole *et al*., 2011) and transcription factors inducing ASE differentiation, such as the nuclear hormone receptor *nhr-67* or the Zn finger transcription factor *che-1* (Etchberger *et al*., 2007; Sarin *et al*., 2009), are symmetrically expressed and so are scores of "terminal effector genes," that is, genes required for ASE neuron function. These terminal effector genes were identified through forward genetic analysis (Bargmann,) and through molecular profiling of isolated ASE neurons (Etchberger *et al*., 2007; Takayama *et al*., 2010). The symmetric gene expression profile of ASEL and ASER are controlled by a Zn finger transcription factor, *che-1*, a terminal selector-type transcription factor (Etchberger *et al*., 2007; Uchida *et al*., 2003) which binds to a sequence motif found in most of the ASEL/R expressed genes to activate their expression. Loss of *che-1* results in the failure of the ASE neurons to terminally differentiate.

However, there are some very striking exceptions to the overall theme of bilateral expression patterns in the ASEL and ASER neurons. Nine of 24 rGCs in the *C. elegans* genome were found to be asymmetrically expressed in either ASEL or ASER (Fig. 3; Ortiz *et al*., 2006; Takayama *et al*., 2010; Yu *et al*., 1997). Four of the rGC-encoding *gcy* genes are exclusively or predominantly expressed in ASEL and another five exclusively or predominantly in ASER. In addition, two *gcy* genes are expressed in both ASEL and ASER. The expansion of the rGC family appears to be a nematode-specific phenomenon; flies and vertebrates contain far fewer rGC genes (Ortiz *et al*., 2006). Most *C. elegans* rGCs are expressed in different types of sensory neurons and have been implicated in mediating a number of different sensory responses (Birnby *et al*., 2000; L'Etoile and Bargmann, 2000; Murayama *et al*., 2013; Smith *et al*., 2013a). As discussed in the next section, the ASE-expressed rGC proteins likely function as direct salt sensors.

Asymmetric Functions of the Bilateral ASEL/R Neuron Pair

Initial laser ablation studies of the ASE neurons demonstrated the importance of this neuron pair in the animal's attractive response to a number of water-soluble cues, including amino acids and salts of various ionic compositions (Bargmann and Horvitz, 1991). ASE also appears to be involved in mediating avoidance behavior to nociceptive cues (Sambongi *et al*., 1999, 2000). The observation of left/right asymmetric *gcy* gene expression in ASEL vs. ASER (described above) prompted an analysis of possibly distinct functions of these neurons (Pierce-Shimomura *et al*., 2001). The use of a combination of behavioral assays as well as the imaging of the activity of the neurons in live animals using genetically encoded calcium sensors provided the general conclusion that ASEL and ASER are strikingly different in the way they respond to salt cues (Ortiz *et al*., 2009; Pierce-Shimomura *et al*., 2001; Suzuki *et al*., 2008).

First, as assessed by measuring neuronal activity or assessing behavioral responses, ASEL and ASER can become activated separately by distinct salt ions to produce attractive chemosensory behavior (Fig. 3; Ortiz

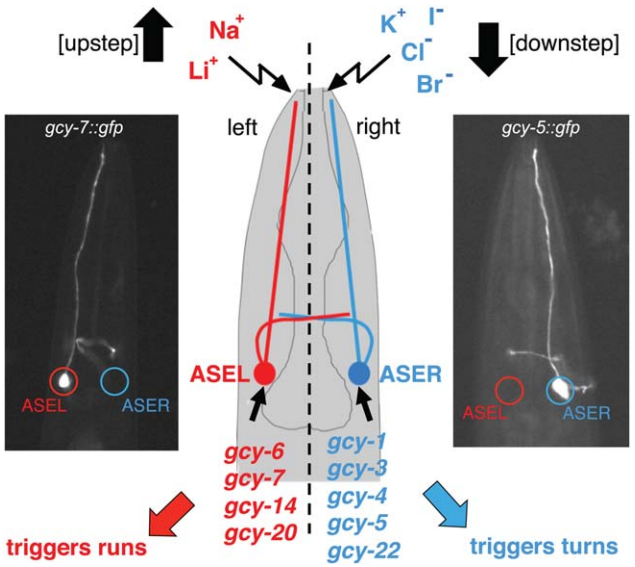

FIG. 3. Lateralized expression of receptor-type guanylyl cyclases (rGCs) and lateralized function of the ASE neurons. ASEL and ASER respond to different salt ions. If ions shown here are presented with a "neutral" counter ion, only the left or right neuron will be activated (Ortiz *et al*., 2009). If left- and right-sensed ions are paired (e.g. in NaCl), the salt will activate both ASEL and ASER (Suzuki *et al*., 2008). Differential responsiveness can be observed with genetically encoded calcium sensors in wild-type animals (Suzuki *et al*., 2008). Behavioral responses in salt gradients can be measured with wild-type animals or animals in which either ASEL or ASER is ablated (Ortiz *et al*., 2009). If, for example, ASER is ablated, animals will not be attracted to Br^-, I^-, or K^+. ASEL responds only to upsteps in the concentration of salt ions and triggers forward run behavior, whereas ASER only responds to downsteps and will trigger reversal behavior (Suzuki *et al*., 2008). rGCs are encoded by *gcy* genes. Eight of them are asymmetrically expressed (as shown), whereas others are expressed in a bilaterally symmetric manner (data not shown; Ortiz *et al*., 2006). rGCs are required for the processing of specific salt cues. For example, *gcy-6* is required for Mg^{++} sensation, *gcy-14* for Na^+ and Li^+ sensation of ASEL, *gcy-4* is required for Br^- and I^- sensation by ASER, and *gcy-1* is required for K^+ sensation by ASER (Ortiz *et al*., 2009).

et al., 2009). For example, sodium, magnesium, and lithium ions are sensed primarily by ASEL, whereas bromide, iodide, and potassium ions are predominantly sensed by ASER (Ortiz *et al*., 2009). The segregated ability to sense ions through either the left or the right neuron allows the animal to discriminate left-sensed cues from right-sensed cues (Chang *et al*., 2004; Pierce-Shimomura *et al*., 2001). In other words, an ASEL-sensed cue can still be sensed in a sensory background saturated by an ASER-sensed cue and vice versa.

The above-mentioned asymmetrically expressed rGCs are likely receptors for salt ions. This notion is based on three observations (Ortiz *et al*., 2009; Smith *et al*., 2013b): (1) individual rGCs are required for processing individual salt cues; (2) individual rGCs are sufficient to confer salt sensitivity when ectopically expressed in other neurons; and (3) intracellular and extracellular domain swap experiments define the extracellular domains as the functional determinants of ion specificity. When compared with other organisms, the use of rGCs as salt receptors is unusual and may be a phenomenon specific to nematodes, which have vastly expanded their repertoire of rGC proteins (Ortiz *et al*., 2006).

ASEL and ASER are not only asymmetric on the level of sensory perception but they also evoke distinct navigational strategies in response to sensory perception (Suzuki *et al*., 2008). ASEL-sensed cues are only perceived when the concentration of the salt cue increases, and the result of the "ON" response is suppression of turns and increase in forward runs. In striking contrast, ASER-sensed cues evoke a response in ASER only on a decrease in the concentration of the sensory cue, and the result of this "OFF" response is an increase in turning frequency. Attraction to an ASER-sensed cue is therefore the result of the animal reversing its direction of movement once it is on the wrong track (salt concentration decreases).

Taken together, the asymmetric expression of the rGC proteins and their apparent direct involvement in the left/right asymmetric detection of sensory cues makes the ASE neurons the only system in any organism to date in which a functional lateralization can be correlated with directional left/right asymmetric gene expression. The system therefore provides an ideal starting point to dissect the developmental control mechanisms that initiate and maintain this asymmetry.

Cis-Regulatory Logic of Asymmetric Chemoreceptor Gene Expression in ASEL/R

One approach to understand left/right asymmetric *gcy* gene expression has been to dissect the cis-regulatory control elements in the *gcy* gene loci that instruct their left/right asymmetric expression. A mutational analysis of the *gcy* genes identified a key logical feature of asymmetric expression control. Both left- and right-expressed *gcy* loci are direct targets of the bilaterally expressed CHE-1 terminal selector-type transcription factor already mentioned above (Etchberger *et al*., 2007, 2009); their expression is lost in *che-1* mutants and they contain binding sites for CHE-1 (Fig. 4a). However, *gcy* genes are not expressed in both neurons because the bilaterally symmetric CHE-1 protein is, depending on the *gcy* locus, either not alone sufficient to induce a *gcy* gene in both cells or its activity is repressed in one of the two ASE neurons (Etchberger *et al*., 2009). Specifically, the ASEL-expressed *gcy-7* locus requires cis-regulatory motifs in addition to the CHE-1 binding site to be expressed in ASEL, whereas the ASER-expressed *gcy-5* locus contains a cis-regulatory element that represses expression in ASEL, that is, it prevents CHE-1 from activating *gcy-5* in ASEL (Fig. 4a). These findings indicate that the ASEL and ASER neurons must contain regulatory factors other than CHE-1 that direct the functional output of CHE-1 to either the left or right neuron, depending on the target gene.

An Asymmetry-Inducing Gene Regulatory Network With a Key Trigger, the *lsy-6* miRNA

Genetic screens reveal the underlying logic of left/right asymmetry control. Exploiting a key strength of the *C. elegans* model system, extensive genetic mutant screens have unveiled a number of regulatory factors that operate in ASEL or ASER to control asymmetric *gcy* expression by either promoting or repressing bilateral CHE-1 activity (summarized in Fig. 4a,b; Chang *et al*., 2003, 2004; Flowers *et al*., 2010; Johnston *et al*., 2005, 2006; O'Meara *et al*., 2010; Poole *et al*., 2011; Sarin *et al*., 2007, 2009; Zhang *et al*., 2011). A key feature of the system was revealed by the retrieval of mutants in which both ASE neurons adopt ASEL identity, that is, ASER identity is converted to ASEL identity ("Class I" or "2 ASEL" mutants), and mutants in which both neurons adopt ASER identity, that is, ASEL converted to ASER identity ("Class II" or "2 ASER" mutants; Fig. 4b). The existence of such mutants made the important point that the adoption of one identity is linked to the repression of the opposite identity and illustrated that both neurons have the potential to exist in one of the two states, but specific regulatory factors drive the system into the ASEL or ASER state.

Mutants with symmetrized ASE neurons define a complex gene regulatory network. Two antagonistically acting transcription factors lie at the core of the ASE gene regulatory network (GRN), the Zn finger transcription factor *die-1* and the homeobox gene *cog-1* (Fig. 4b). *die-1* (identified as a Class II or 2 ASER mutant) is expressed in the ASEL neuron and required for the induction of ASEL identity (Fig. 4a; Chang *et al*., 2004). After the birth of the ASE neurons, *die-1* is

initially expressed in both neurons, but becomes subsequently restricted to the ASEL neuron (Cochella *et al*., 2014). Loss of *die-1* results in a conversion of ASEL identity to ASER identity (as assessed by the expression of L/R-specific *gcy* genes). Moreover, *die-1* is continuously required to maintain ASEL identity, and its continuous presence in ASEL is ensured through both autoregulation and the activity of a late-acting homeobox gene, *ceh-36* (Cochella *et al*., 2014; O'Meara *et al*., 2010). As revealed by genetic analysis, *die-1* operates together with a number of chromatin factors that were, like *die-1*, retrieved as Class II mutants (*lsy-12*, *lsy-13*, *lsy-15*, *lsy-2*, and *lin-49*; Fig. 4b; Chang *et al*., 2003; Johnston and Hobert, 2005; O'Meara *et al*., 2010; Poole *et al*., 2011). The 2 ASER mutant phenotype of *die-1* manifests itself not only by switches in molecular markers but also by the expected behavioral changes; 2 ASER mutant animals are still attracted to ASER-sensed cues but fail to respond to ASEL-sensed cues (Chang *et al*., 2004; Ortiz *et al*., 2009).

die-1 becomes restricted to ASEL during embryonic development through the activity of the *cog-1* Nkx6-type homeobox gene, which is exclusively expressed in ASER (Fig. 4a; Chang *et al*., 2003; Cochella *et al*., 2014). *cog-1* was identified as a Class I mutant, in which ASER identity converts to ASEL identity. Two cofactors of *cog-1*, the two Groucho-like corepressors *unc-37* and *lsy-22*, were also identified as Class I mutants (Chang *et al*., 2003; Flowers *et al*., 2010). Even though continuously expressed in ASER, *cog-1* is only transiently required in the embryo to restrict *die-1* to ASEL (Cochella *et al*., 2014). The above-mentioned maintenance mechanisms

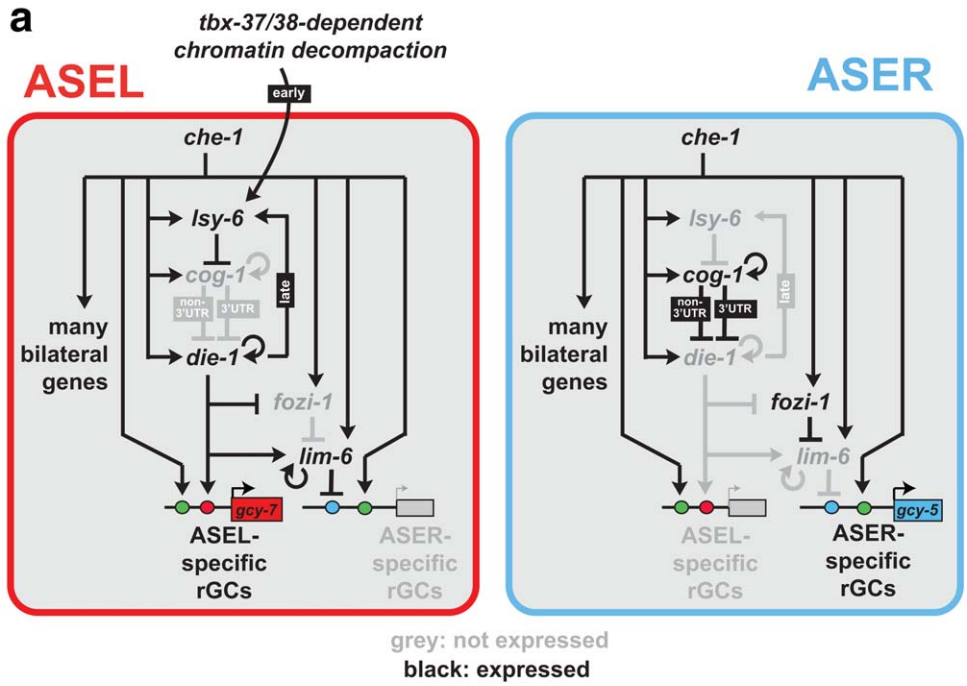

FIG. 4.

(*die-1* autoregulation and *ceh-36* maintenance) then ensure continuity of *die-1* activity in ASEL.

The effect of *cog-1* on *die-1* expression is ensured through two redundant mechanisms: The *die-1* locus contains two types of cis-regulatory elements. One is a transcriptional control region several kilobases upstream of the *die-1* start codon (Cochella *et al*., 2014), and the other one is composed of several post-transcriptional regulatory elements harbored in the 3′-UTR of *die-1* and likely controlled by several miRNAs (Chang *et al*., 2004; Didiano *et al*., 2010). The asymmetric (i.e., ASEL-specific) activity of both the transcription and post-transcriptional elements (the latter can be monitored if the 3′-UTR is attached to *gfp* driven by a bilaterally expressed promoter) genetically depends on ASER-expressed *cog-1* such that the expression of either regulatory element becomes "symmetrized," that is, derepressed in ASER, in *cog-1* mutants (Cochella *et al*., 2014; Didiano *et al*., 2010).

cog-1 expression is restricted to the ASER neuron through the activity of the ASEL-expressed *lsy-6* miRNA, which on its discovery was the first miRNA with a function in the nervous system (Johnston and Hobert, 2003). *lsy-6* is exclusively expressed in ASEL where it represses *cog-1* expression through two binding sites in the *cog-1* 3′-UTR (Didiano and Hobert, 2008; Johnston and Hobert, 2003). Failure of *cog-1* repression in ASEL, observed in either *lsy-6* mutants (Class II mutants) or in *cog-1* mutants in which the 3′-UTR is deleted (retrieved as a dominant Class II mutant from asymmetry screens; Sarin *et al*., 2007), results in the conversion of ASEL to ASER. The conversion of ASEL to ASER identity in *lsy-6* mutants can be completely suppressed by concomitant removal of *cog-1*, demonstrating that the function of this miRNA can be solely explained through its activity on a single target gene (Johnston and Hobert, 2003). Moreover, ectopic expression of *lsy-6* in ASER results in a complete switch of ASER to ASEL identity. *lsy-6* is therefore a required and sufficient trigger of ASEL identity. As expected from the only transient need for *cog-1* to establish ASE asymmetry, temporally controlled removal of *lsy-6* activity through photoactivatable antagomirs demonstrated that *lsy-6* is only transiently required in the embryo to establish ASE asymmetry (Zheng *et al*., 2011).

Taken together, a sequentially acting pathway of a miRNA and two transcription factors are key determinants of ASE asymmetry. Before discussing how the initial symmetry breaking event—manifested by the ASEL-specific expression of the miRNA *lsy-6*—is established (section below), a number of additional regulatory components and features of this pathway are discussed.

Mixed identity mutants. Genetic screens for asymmetry mutants have not only revealed mutants in which there is a full "symmetrization," that is, conversion of ASER to ASEL (2 ASEL; Class I mutants) or ASEL to ASER (2 ASER; Class II mutants), but also mutants with "mixed" fate, called Class IV mutants (Fig. 4b;

FIG. 4. The gene regulatory network (GRN) that controls ASE asymmetry. (**a**) GRN acting in postmitotic ASE neurons. Aside from the terminal selector *che-1*, only asymmetrically expressed genes are shown. This schematic also encapsulates the cis-regulatory analysis of *gcy* genes which revealed the existence of bilaterally activated cis-regulatory motifs (CHE-1 binding site = "ASE motif") as well as cis-regulatory motifs that are either required in conjunction with the ASE motif for gene activation or that counteract activation via the ASE motif. Note that in ASEL, the *che-1* input in the ASEL-expressed rGC genes (exemplified by *gcy-7*) is not sufficient, and an additional, *die-1*-dependent input is required (which is perhaps mediated by *ceh-36*, a bilaterally expressed gene, which is not shown here); in contrast, the *che-1* input is sufficient to activate ASER-expressed rGC genes (exemplified by *gcy-5*), but this input is inhibited in ASEL via a repressor element that is *lim-6*-dependent. *cog-1* regulates *die-1* through two distinct mechanisms, a post-transcriptional, 3′-UTR-mediated and a transcriptional mechanism (Cochella *et al*., 2014; Didiano *et al*., 2010). Not shown here are two neuropeptide-encoding genes, *flp-4* and *flp-20* whose expression is biased to ASEL and which are positively regulated by *lim-6* (Johnston *et al*., 2005). Note that many arrows in this diagram are "genetic arrows" and do not necessarily indicate biochemical interactions; for example, it is not clear whether *lim-6* or *die-1* regulate *gcy* gene expression directly. However, most if not all of the downstream targets of *che-1* are direct targets, as evidenced by the presence of functionally required CHE-1 binding sites in many if not all of the genes shown here. In two cases (*lsy-6* and *cog*-1), regulatory alleles have been identified from genetic screens in which the CHE-1 binding site is mutated (O'Meara *et al*., 2009; Sarin *et al*., 2007). This schematic also does not incorporate temporal aspects of regulation. For example, *lsy-27* is only transiently required after the time of ASE birth (Zhang *et al*., 2011), whereas other factors such as the *ceh-36* gene are required to maintain ASE asymmetry (Cochella *et al*., 2014). Feedback regulation from *lim-6* back into the bistable loop (via control of *lsy-6* and/or *die-1* expression) is also not shown. Curiously, this feedback regulation appears much more pronounced under conditions of starvation (unpublished data). Previous representations of this gene regulatory network included additional arrows from *die-1* to terminal genes such as *gcy-7* and *gcy-5*, which were inferred from *die-1* mutant phenotypes being more expressive than the effects of Class IV mutant (e.g., *gcy-5* was initially thought to be more strongly derepressed in ASEL of *die-1* mutant than in *lim-6* mutants; Johnston *et al*., 2006). As different experimenters have scored expressivity differently and as such quantification may not represent an accurate enough reflection of endogenous gene expression, the existence of parallel *die-1* activities is currently questionable. We also explicitly tested whether *lim-6* and *fozi-1* act in a double-negative manner, that is, whether *lim-6* is also required for asymmetric *fozi-1* expression. However, we find this not to be the case (unpublished data). Table S3 in the Supplementary Information lists currently unanswered questions that concern this regulatory network. (**b**) Schematic phenotype of Class I, II, III, and IV mutants retrieved from genetic mutant screens using left- or right-specific *gfp* reporter strains (Sarin *et al*., 2007). Asymmetrically expressed genes are boxed in gray. Nonboxed genes are expressed in both ASEL and ASER and act permissively. The "*" at *mir-273* indicates that ASER-expressed *mir-273* is sufficient to drive ASER fate on ectopic expression in ASEL (Chang *et al*., 2004) but *mir-273* mutants have no phenotype (Didiano *et al*., 2010). ZnF, zinc finger; NHR, nuclear hormone receptors; HAT, histone acetyltransferase; HMT, histone methyltransferase. Note that *nhr-67* is the only gene in this network that is known to act at two different steps: it activates *che-1* expression in both ASEL and ASER and therefore the bilaterally symmetric differentiation program of the ASE neurons, and it acts together with *che-1* to activate expression of *cog-1* in ASER (Sarin *et al*., 2009).

Sarin *et al*., 2007). In one set of these mutants, the ASER neuron is unaffected, but ASEL adopts both ASEL and ASER features or vice versa the ASER neuron is unaffected, but the ASEL neuron now adopts both ASEL and ASER features. Class IV mutants affect two transcription factors, the ASEL-expressed *lim-6* LIM homeobox gene (Hobert *et al*., 1999) and the ASER-expressed *fozi-1* Zn finger transcription factor (Johnston *et al*., 2006). In *fozi-1* mutants, ASEL identity is unaffected, but ASEL-specific *gcy* genes become derepressed in ASER, whereas ASER-specific *gcy* genes remain unaffected. ASER-specific expression of *fozi-1* is ensured by the ASEL-specific *die-1* gene described above, which represses *fozi-1* in ASEL (Fig. 4a). In *lim-6* mutants, ASER identity is unaffected, but ASER-specific *gcy* genes become derepressed in ASEL, whereas ASEL-specific genes remain unaffected. ASEL-specific expression of *lim-6* is ensured by the ASEL-specific *die-1* gene described above as well as the ASER-specific *fozi-1* gene which represses *lim-6* in ASER (Fig. 4a; Johnston *et al*., 2006).

lim-6 and *fozi-1* can be considered as "executors" of specific subfunctions of the *die-1* transcription factor. What makes the Class IV mutant phenotype, particularly the *lim-6* mutant phenotype, specifically informative is that it allowed the testing of the hypothesis that left/right asymmetric segregation of chemosensory capacities is essential for discrimination of sensory cues. This is because the gain of ASER identity features in ASEL and the unaffected ASEL identity features in *lim-6* mutants go hand-in-hand with a gain of normally ASER-restricted chloride responsiveness of ASEL. This results in a situation where ASEL can now sense both ASEL cues (Na^+) and ASER cues (Cl^-). Under this circumstance, animals are unable to discriminate between Na^+ and Cl^- (Pierce-Shimomura *et al*., 2001). This finding underscores the fundamental logic of lateralization: the segregation of chemosensory functions into distinct neurons ensures sensory discrimination such that one sensory cue can be detected in the presence of another cue. Such discriminatory features are key features of most sensory systems.

Apart from controlling rGC genes, *lim-6* also positively regulates the expression of two neuropeptide-encoding genes, *flp-4* and *flp-20* which are asymmetrically expressed in ASEL, but not (or much less) in ASER (Johnston *et al*., 2005). Their function is currently not known.

Developmental Programming of ASE Asymmetry: Notch and Chromatin Decompaction

The asymmetric initiation of *lsy-6* expression in ASEL, the key trigger of asymmetry is the result of the distinct lineage histories of ASEL and ASER. As mentioned above, these two neurons derive from two distinct blastomeres in the four-cell stage embryo, ABa (−> ASEL) and ABp (−> ASER) (Fig. 5a). Apart from giving rise to ASEL and ASER, these two blastomeres produce very different body parts of *C. elegans*. For example, ABa (but not ABp) produces large parts of the pharynx (Sulston *et al*., 1983). The distinct fates of the ABa vs. ABp descendants are controlled via activation of the Notch GLP-1 receptor in ABp, but not ABa. This activation is achieved by a Notch ligand, APX-1, produced by the P2 blastomere, which only neighbors ABp, but not ABa (Fig. 5a; Priess, 2005; Priess *et al*., 1987; Schnabel and Priess, 1997). Notch signaling in ABp results in downregulation of two redundantly acting T-box genes, *tbx-37* and *tbx-38*, in ABp descendants (Good *et al*., 2004). Not having received the Notch signal, ABa descendants will produce TBX-37/38. Postmitotic ASE asymmetry is controlled by these early patterning events. Loss of this specific Notch signal (and resulting upregulation of *tbx-37/38* in ABp descendants) results in ASER transformation to ASEL, whereas ASEL converts to ASER in *tbx-37/38* mutants (Poole and Hobert, 2006).

tbx-37/38 are only transiently expressed for one cell division (Good *et al*., 2004).

The link from transient activation of *tbx-37/38* to full activation of *lsy-6* in ASEL five cell divisions later lies in a "prime and boost" mechanism (Fig. 5b; Cochella and Hobert, 2012). Through a cis-regulatory control element located downstream of the *lsy-6* locus, TBX-37/38 trigger very low level expression of *lsy-6* in ABa descendants. After TBX-37/38 disappearance, the "primed" state of the *lsy-6* locus becomes evident—and is maintained through several cell divisions—in the form of a decompacted chromatin state of the *lsy-6* locus (Cochella and Hobert, 2012). This decompaction is only apparent in the lineage that produces ASEL and not in the ASER-producing lineage in which *tbx-37/38* is downregulated via the Notch signal (Fig. 5a,b).

Decompaction of the *lsy-6* locus in the left lineage is essential for the ensuing "boosting" phase, characterized by strong upregulation of *lsy-6* in ASEL at around the time the neuron is born, and is coincident with the onset of *che-1* expression. The boosting phase depends on the CHE-1 transcription factor that directly binds to and activates the *lsy-6* locus (Fig. 5a,b).

How the decompacted state of the *lsy-6* locus is maintained through several cell divisions after it has been established by transient TBX-37/38 is entirely unclear at the moment. Extensive genetic screens, including a genome-wide RNAi screen (Poole *et al*., 2011), have so far failed to reveal intermediary factors. Although such screens have identified several components of the COMPASS complex, a H3K4 methyltransferase (Poole *et al*., 2011), and a MYST-type histone acetyltransferase complex (O'Meara *et al*., 2010) as regulators of *lsy-6* expression, these chromatin factors do

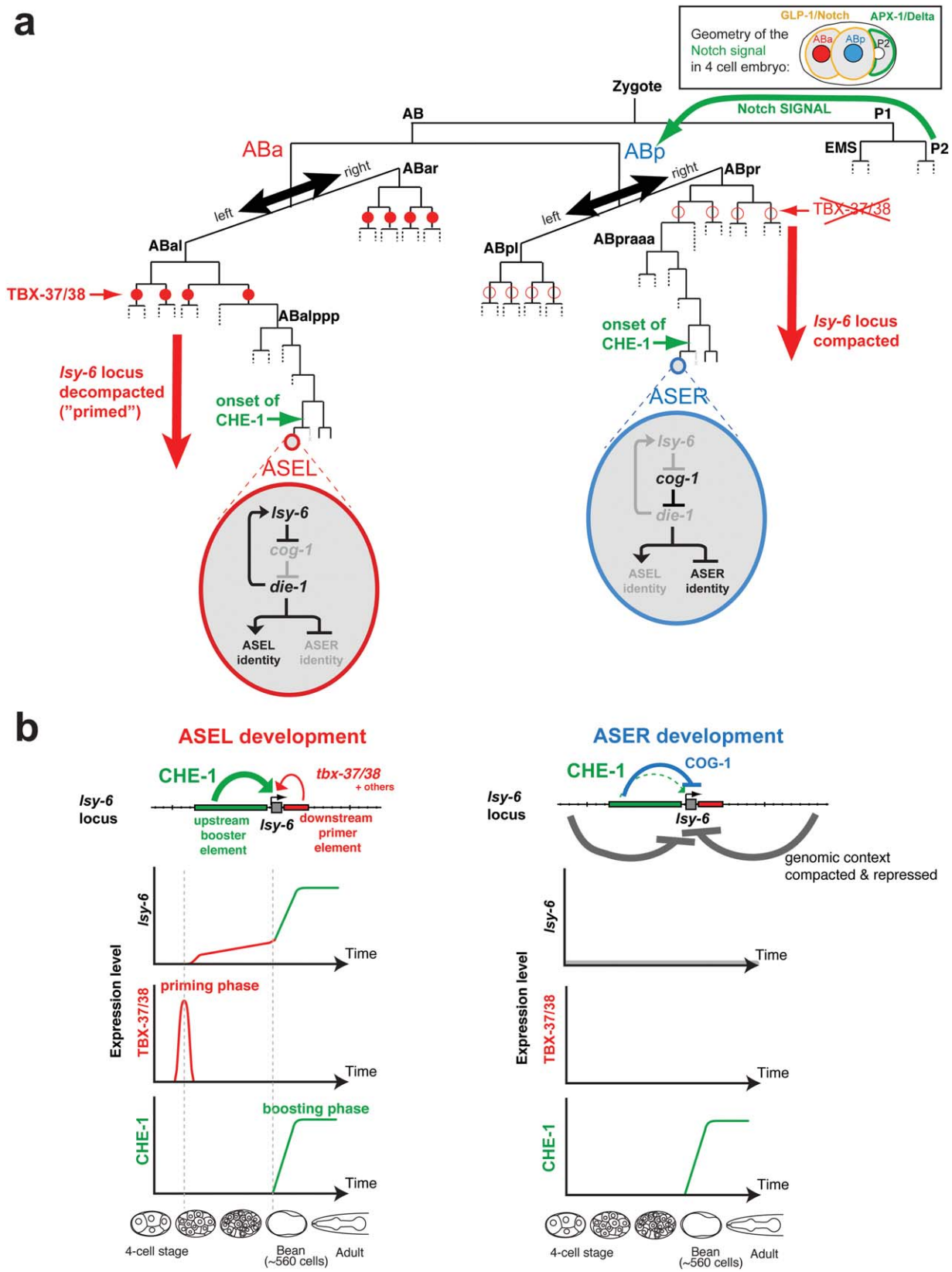

FIG. 5. Postmitotic ASE asymmetry is preprogrammed in the embryo. (**a**) An early Notch signal prepatterns postmitotic *lsy-6* asymmetry through repression of *tbx-37/38*. TBX-37/38, whose transient expression for one cell cycle is indicated with filled red circles (no expression in ABp derivates is indicated with empty red circles) are required to decompact ("prime") the *lsy-6* locus which then allows for *che-1*-mediated "boosting" of *lsy-6* expression several cell divisions later. Note that other bilaterally pairs of neurons in the lineage branches that produce ASEL and ASER (shown in Fig. 2B) may have also used TBX-37/38 to prime other loci that may introduce asymmetries in these neuron pairs. For more details, see Cochella and Hobert, 2012. (**b**) An alternative representation of the prime and boost mechanism that ensures ASEL-specific expression of *lsy-6* (Cochella and Hobert, 2012).

not affect the state of compaction of the *lsy-6* locus (our unpublished data).

Taken together, establishment of postmitotically observed functional ASE asymmetry can be traced back to a Notch signal at the four-cell stage. As mentioned above, specificity is achieved by the Notch ligand APX-1 being in physical proximity only to one of the two blastomeres that produce ASEL and ASER (Fig. 5a). The specificity of expression of APX-1 in the P2 blastomere from where it signals to ABp is in turn explained by asymmetric segregation of cell fate determinants upon the very first cell division that produces the AB and P0 cell. The basis for this polarity lies in the site of sperm entry (Gonczy and Rose, 2005).

A Distillation of the Most Notable Features of ASE Asymmetry Control

A number of core principles can be extracted from the establishment of ASE asymmetry in *C. elegans*. Several of these principles (e.g., prepatterns, ground states, and temporal progression) may bear relevance for the establishment of asymmetry in other systems, whereas other principles (feedforward/feedback control and 3′-UTR regulation) provide even more general lessons about the structure of GRNs.

Transforming morphological asymmetries into neuronal asymmetries. One key feature of asymmetry establishment in the ASE neuron pair is that the ASEL and ASER neurons derive from distinct lineages and, therefore, have been exposed to distinct cellular environments, enabling only one of the two precursors to receive a specific signal (see inset in Fig. 5a). This distinct signal exposure is "memorized" via *tbx-37/38*-dependent chromatin reconfiguration. Apart from the ASE pair, a number of additional neuron pairs, many of them sensory neurons, share the same lineage histories as the ASEL and ASER neurons (Fig. 2b), and it is conceivable that the left neuron of these neuron pairs may also use the *tbx-37/38*-dependent priming signal to perhaps decompact other gene regulatory loci that could be used to generate asymmetry in these other neuron pairs. In principle, one can imagine such a lateralization mechanism to apply to any neuron pairs whose constituents have distinct lineage histories and therefore may have been differentially exposed to an asymmetry-inducing signal in their developmental history (Supporting Information Table S2). One of the key goals of future analysis in the *C. elegans* nervous system should be the systematic analysis of asymmetric gene expression patterns in these neuron pairs.

Asymmetric prepatterns and temporal progression through distinct regulatory states. Postmitotic ASE asymmetry depends on a prepattern established early in the embryo, but manifested only at the time of the birth of the neurons (prime and boost mechanism). From the perspective of the *lsy-6* locus, the system passes through two regulatory states, a primed state (chromatin decompacted and low-level expression) and a boosted state (high expression of *lsy-6*; Fig. 5b).

The progression through distinct regulatory states can also be observed for other factors in the GRN. *die-1* is initially expressed in both ASE neurons, but becomes restricted to ASEL via transiently acting *cog-1* (Cochella *et al*., 2014). Once asymmetrically expressed, *die-1* maintains its asymmetric expression through autoregulation and the help of additional regulatory factors (Cochella *et al*., 2014). If maintained *die-1* expression is disrupted, asymmetry of rGC sensory receptor expression is lost (O'Meara *et al*., 2010).

Another transiently acting regulatory factor is the very broadly expressed zinc finger transcription factor *lsy-27*, which is genetically required to assist *die-1* in turning on *lim-6* expression (Zhang *et al*., 2011). Once initiated, *lim-6* expression is maintained via autoregulation and likely also by *die-1*, which also autoregulates.

A bilateral ground state?. Although the initial notion of a so-called hybrid precursor state, in which multiple components of the ASE GRN are expressed in both neurons before they become restricted to one neuron (Johnston *et al*., 2005), could not be confirmed with improved, that is, fosmid-based reporter gene reagents (Cochella *et al*., 2014), the ASE system nevertheless could be viewed as being derived from a bilateral ground state. In the case of *die-1*, this is evident by the bilateral expression of the gene in both ASEL and ASER, before it becomes restricted to ASEL (Cochella *et al*., 2014). In the case of *lsy-6*, *cog-1*, and terminal effector genes such as *gcy-5*, which are only ever expressed in an asymmetric manner, this bilateral ground state is evident by these loci all harboring functionally required response elements (ASE motifs) activated by the bilaterally expressed CHE-1 terminal selector; their functional necessity was revealed by the retrieval of mutant alleles of *cog-1* and *lsy-6* in which the ASE motif harbored point mutations (O'Meara *et al*., 2009; Sarin *et al*., 2007). This allows, in principle, to have both genes be activated in both ASEL and ASER; however, this activation is inhibited by "superimposed" control mechanisms that restrict *cog-1* or *lsy-6* to either the left or right neuron. In the case of *cog-1*, the superimposed control mechanism consists of post-transcriptional regulation by *lsy-6*; in the case of *lsy-6*, the superimposed control mechanism that restricts CHE-1 activity relates to distinct chromatin states of the *lsy-6* locus.

Abundant feedback and feedforward mechanisms. There is abundant feedback control in the ASE GRN. *cog-1*, *die-1*, and *lim-6* autoregulate their expression (Cochella *et al*., 2014; Johnston *et al*., 2005). *die-1* autoregulation can easily be understood because, as mentioned above, continuous *die-1* activity is required to maintain ASE asymmetry. *lim-6* may autoregulate for the same reason. However, why *cog-1* autoregulates is

not clear because, as mentioned above, it is only transiently required during embryogenesis (Sarin *et al*., 2007). Perhaps this autoregulation is only required right after initiation of *cog-1* expression to boost its expression above a critical threshold.

Apart from autoregulation of individual loop components, there is also feedback control from *cog-1* and *die-1* on *lsy-6* expression. The precise nature and raison d'etre of this feedback is not clear. Early studies had revealed that a cis-regulatory element upstream of the *lsy-6* locus is positively controlled by *die-1* in ASEL and repressed by *cog-1* in ASER (Johnston et al., 2005); however, a fosmid-based *lsy-6* reporter that contains more cis-regulatory information is not derepressed in ASER of *cog-1* mutants and the effect of *die-1* loss on the fosmid-based *lsy-6* reporter manifests itself only during adulthood (our unpublished data). Other feedback connections that link more distal parts of the regulatory network are also restricted to the adult stage. The absence of *lim-6* results in late, adult-stage loss of *lsy-6* expression (Johnston *et al*., 2005). The reason for all these feedbacks is not clear given that *lsy-6* is apparently only transiently required in the embryo (Zheng *et al*., 2011). Perhaps there are as yet unknown conditions under which *lsy-6* may in fact be required during postdevelopmental stages.

In addition to feedback control, there are a number of feedforward control mechanisms built into the ASE GRN. Feedforward loops (FFLs) are characterized by the basic regulatory structure of "Gene A" regulating "Gene B" and Gene A plus B jointly regulating "Gene C." The most prominent FFLs in the ASE GRN show a complex nested structure, in which "Gene A" is *che-1*, "Gene B" are the intermediary regulators *lsy-6*, *die-1*, and *cog-1*, and "Gene C" are the battery of terminal *gcy* genes. Another FFL is the control of *che-1* expression by the bilaterally expressed *nhr-67* gene, which also cooperates with *che-1* to turn on *cog-1* expression (Sarin *et al*., 2009). FFLs are generally thought to define specific dynamic and temporal properties of a GRN (Alon, 2007); however, the logic of complex interwovenness of the various FFLs in the ASE GRN makes its properties harder to predict.

Distinct types of 3′-UTR control mechanisms. Another notable feature of the ASE GRN is not just the use of 3′-UTR control, but the substantially distinct way in which 3′-UTR control manifests itself. In the case of *cog-1* 3′-UTR regulation via the miRNA *lsy-6*, the regulatory control mechanism operates as a required and sufficient switch (Johnston and Hobert, 2003). In striking contrast, 3′-UTR regulation of *die-1* operates in a redundant manner with transcriptional control of *die-1* (Cochella *et al*., 2014; Didiano *et al*., 2010). The benefits of such redundant control are not clear but may ensure robustness of asymmetric *die-1* expression.

OPEN QUESTIONS IN THE ASE GENE REGULATORY NETWORK

Even though previous studies have provided, in aggregate, a somewhat satisfying picture of how neuronal asymmetry is genetically programmed, a number of open questions, some small, some bigger, remain. Several questions relate to specific mechanistic aspects of the ASE GRN but a number of them relate to the application of lessons learned from the ASE GRN to other potential and yet to be discovered asymmetries in the *C. elegans* nervous system. A collection of open question is provided in Supporting Information Table S3.

Signals That May Induce Other Neuronal Asymmetries in *C. elegans*

Exposure of blast cells to another asymmetrically positioned Notch signal also generates other asymmetries in the nervous system of *C. elegans*. These asymmetries are different than ASE asymmetry and are of two types: first, they concern an asymmetry in the gene expression programs of pairs of neurons (SMDD, AIY, SIA, and SIB) that originate—unlike ASE—from left/right symmetric lineage branches (Fig. 2a; Bertrand *et al*., 2011), and second, this particular Notch signal may also be responsible for the generation of unilaterally positioned neurons (Fig. 2a), a currently unproven notion based on the effect of loss of Notch signaling on other cells in lineage branches that produce unilateral neurons (Lambie and Kimble, 1991). This Notch signal derives in the form of expression of the LAG-2 Delta-like ligand in the mesodermal MSap descendants (Moskowitz and Rothman, 1996; Priess, 2005). The asymmetric position of the LAG-2 signal is the result of a skewed cell division axis that asymmetrically positions a LAG-2-expressing signaling cell relative to receptor-expressing AB-derived neuroblasts. Bilaterally symmetric AB-derived neuroblasts therefore differentially receive a Notch signal (Hermann *et al*., 2000). LAG-2 expression in MSap descendants is controlled by a noncanonical Wnt system that acts as a global coordinate system during anterior/posterior cell divisions in the developing embryo (Lin *et al*., 1998; Mizumoto and Sawa, 2007).

This noncanonical Wnt signaling system may also play a role in other neuronal asymmetries. There are a number of unilateral neurons in the pharynx. Some of these unilaterally positioned pharyngeal neurons are embedded in left/right symmetric lineage branches, and the contralateral homologs of these neurons (specifically, MI and I5) are non-neuronal cells. In one case, the MI neuron, recent studies have pinpointed the involvement of chromatin structure (Nakano *et al*., 2010, 2011), but the origins of this laterality are still unclear. They may lie in a noncanonical Wnt signaling event that occurs in all a/p divisions in the embryo (Kaletta *et al*., 1997; Lin *et al*., 1998) and which may act in the a/p division of the

ABaraap blast cells, which generates two blast cells, ABaraapa and ABaraapp from which MI and its non-neuronal contralateral lineage homolog, e3D, arise.

Outlook

With a limited number of neurons in the nervous system it makes a lot of sense to diversify neuronal functionality across the left/right axis. It is easily conceivable that many more asymmetric gene expression programs in otherwise bilaterally symmetric *C. elegans* neuron pairs have so far escaped detection, not the least because neuronal expression patterns are not often rigorously assessed for their symmetry. Good candidates for lateralized neuron pairs are those where the individual constituents of a neuron pair have distinct lineage histories, as is the case in the ASE neuron pair (Supporting Information Table S3). More systematic expression analysis approaches are required to detect more lateralities and to determine whether they are imposed by similar or distinct mechanisms as those described here.

Looking beyond *C. elegans*, it will be fascinating to examine which of the principles described in this simple model will apply to neuronal asymmetries in higher organisms. As illustrated by the studies on the ASE system, it will be of key importance to first identify genes expressed in an asymmetric manner in vertebrate systems. Such genes can then serve as molecular entry points to investigate the functional relevance and developmental establishment of asymmetry. The establishment of more fine-grained gene expression atlases in vertebrate brains should be the first step into this direction.

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