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An Innexin-Dependent Cell Network Establishes Left-Right Neuronal Asymmetry in *C. elegans*

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SUMMARY

Gap junctions are widespread in immature neuronal circuits, but their functional significance is poorly understood. We show here that a transient network formed by the innexin gap-junction protein NSY-5 coordinates left-right asymmetry in the developing nervous system of *Caenorhabditis elegans*. *nsy-5* is required for the left and right AWC olfactory neurons to establish stochastic, asymmetric patterns of gene expression during embryogenesis. *nsy-5*-dependent gap junctions in the embryo transiently connect the AWC cell bodies with those of numerous other neurons. Both AWCs and several other classes of *nsy-5*-expressing neurons participate in signaling that coordinates left-right AWC asymmetry. The right AWC can respond to *nsy-5* directly, but the left AWC requires *nsy-5* function in multiple cells of the network. NSY-5 forms hemichannels and intercellular gap-junction channels in *Xenopus* oocytes, consistent with a combination of cell-intrinsic and network functions. These results provide insight into gap-junction activity in developing circuits.

INTRODUCTION

Developing neurons and other embryonic cell types are often connected by gap junctions, intercellular channels that allow the direct transfer of electrical signals and small molecules between coupled cells (Bennett and Zukin, 2004). A gap junction is formed by aligned homotypic or heterotypic half-junctions on two adjacent cells and can be composed of either connexins, which are present only in chordates, or innexins or pannexins, which are present in all metazoa. Developing neurons in the vertebrate spinal cord, retina, and cortex are interconnected by gap junctions that fade away later in life (Kandler and Katz, 1995). The connected neurons form functional domains with coordinated patterns of spontaneous activity and intracellular calcium flux (Yuste et al., 1995). Transient gap-junction networks have been proposed to regulate proliferation, migration, cell death, contact inhibition, and synapse formation and/or elimination, but there is little direct evidence of their function. Their best understood role is in developing motor neurons, where they potentiate the synaptic refinement that leads to the selection of a single input neuron per muscle fiber (Chang et al., 1999). In addition, mutations in gap-junction genes eliminate certain chemical synapses in the *Drosophila* optic lamina, suggesting that signals for synapse formation may pass through gap junctions (Curtin et al., 2002).

Gap junctions link the earliest born nonneuronal cells in embryos and are essential for *C. elegans*, *Drosophila*, and mammalian embryogenesis (Phelan, 2005; Wei et al., 2004). The first detectable left-right asymmetry of the body axis in frog and chick embryos is generated by gap junctions (Levin and Mercola, 1999). This asymmetry predicts the laterality of the Shh and BMP signaling pathways that generate asymmetry in internal organs.

Both invariant and random left-right asymmetries are present in the nervous system of the nematode *Caenorhabditis elegans* (Hobert et al., 2002). Most left-right asymmetries are tightly coupled to the body axis, but left-right differences between the AWC olfactory neurons, which are distinguished as AWC^{ON} or AWC^{OFF} based on whether or not they express the reporter *str-2::GFP*, are stochastic. Each animal generates one AWC^{ON} neuron and one AWC^{OFF} neuron, but half of the animals express *str-2* in the right AWC neuron while the other half express *str-2* in the left AWC (Troemel et al., 1999). Cell-killing experiments suggest that AWC^{OFF} is the default state and that induction of AWC^{ON} requires an interaction between the AWC neurons. Genetic studies of symmetric mutants with two AWC^{ON} or two AWC^{OFF} neurons have defined a calcium-dependent kinase cascade that regulates AWC asymmetry near the time of synapse formation, including

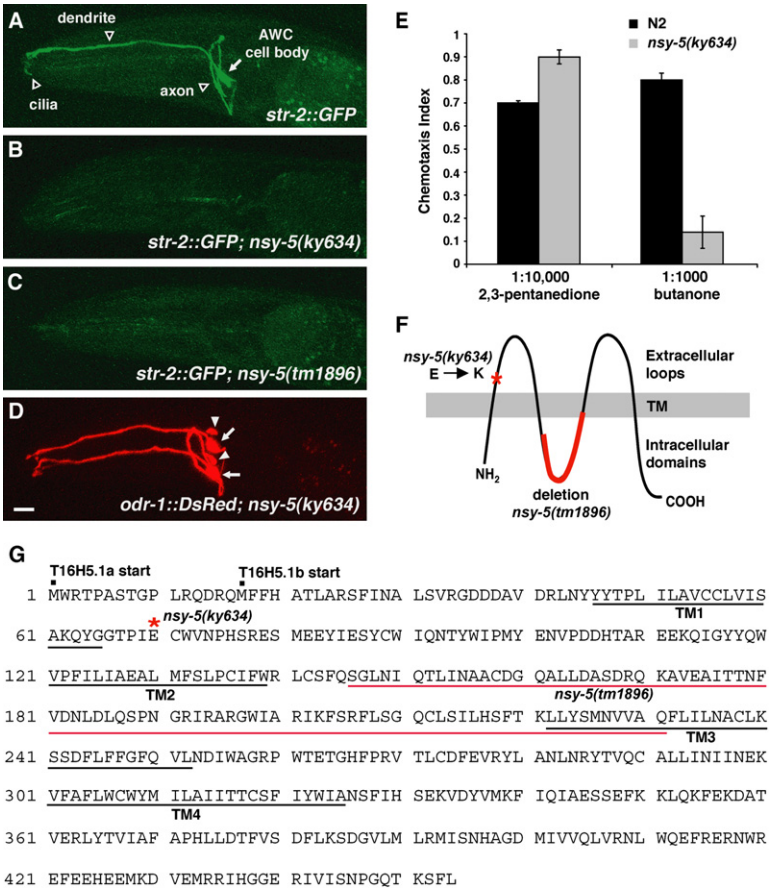

Figure 1. *nsy-5* Encodes an Innexin Gap-Junction Protein

(A–C) Expression of *str-2::GFP* in wild-type and *nsy-5* mutant animals.

(D) Expression of *odr-1::DsRed* in a *nsy-5(ky634)* mutant. Arrows indicate the AWC cell body; arrowheads indicate the smaller AWB cell body. All images are confocal projections. Anterior is at left; ventral is down. Scale bar = 10 μm.

(E) Chemotaxis assays. A chemotaxis index of 1 represents 100% of animals approaching the odor; a chemotaxis index of 0 represents random behavior. Error bars indicate standard error of the mean.

(F) Predicted structure of NSY-5 protein encoded by T16H5.1a.

(G) Amino acid sequence of NSY-5, indicating location of transmembrane domains (TM1–4), the G→A (E70K) change in the *nsy-5(ky634)* allele, and the in-frame deletion in the *nsy-5(tm1896)* allele.

a voltage-activated calcium channel, the calcium-dependent kinase CaMKII, and a MAP kinase cassette (Chuang and Bargmann, 2005; Sagasti et al., 2001; Tanaka-Hino et al., 2002; Troemel et al., 1999). The earliest signaling molecule in this cascade is NSY-4, a transmembrane protein related to vertebrate claudin adhesion proteins and regulatory γ subunits of voltage-activated calcium channels (VanHoven et al., 2006). Axon guidance mutants disrupt AWC asymmetry, but classical synaptic communication is not essential, suggesting that a different kind of cell communication is involved (Troemel et al., 1999).

Here we analyze the signaling between AWC neurons by characterizing an AWC asymmetry gene, *nsy-5*, that encodes a member of the innexin/pannexin family of gap-junction proteins. We show that AWC neurons belong to a transient neuronal network connected by *nsy-5*-dependent junctions and that this network coordinates communication between AWC^{OFF}, AWC^{ON}, and other neurons to generate left-right asymmetry.

RESULTS

nsy-5 Encodes an Innexin Homolog Required for AWC Asymmetry

nsy-5(ky634) was identified in a genetic screen for mutants that do not express *str-2::GFP* in either AWC cell (Figures 1A and 1B; Figure 2A). This 2 AWC^{OFF} phenotype can be caused by mutations that affect general AWC fate, axon guidance, signaling between AWC neurons, or activity-dependent maintenance of the *str-2::GFP* reporter (Troemel et al., 1999). AWC fate appeared to be normal in *nsy-5(ky634)* mutants based on the bilateral expression of the AWC marker *odr-1::DsRed*, and the same marker revealed apparently normal AWC axons, dendrites, and cilia (Figure 1D). The *str-2::GFP* expression defect was observed at all developmental stages, unlike the late-onset defect in the maintenance mutants (Troemel et al., 1999). These results suggest that *nsy-5* affects the establishment of left-right asymmetry of AWC neurons.

nsy-5 animals did not chemotax to the odorant 2-butanone, which is sensed by AWC^{ON}, but responded normally to the odorant 2,3-pentanedione, which is sensed by AWC^{OFF} (Wes and Bargmann, 2001) (Figure 1E). These results support the idea that *nsy-5* mutants have one or more functional AWC^{OFF} neurons but no functional AWC^{ON} neurons.

nsy-5(ky634) was mapped to a small interval on the first chromosome, and the mutant phenotype was rescued with an 18 kb PCR product of genomic DNA containing only one full-length open reading frame, T16H5.1/*inx-19* (Figure 3, row 1). T16H5.1 encodes two innexin-related