UNC-4 antagonizes Wnt signaling to regulate synaptic choice in the *C. elegans* motor circuit

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**SUMMARY**

Coordinated movement depends on the creation of synapses between specific neurons in the motor circuit. In *C. elegans*, this important decision is regulated by the UNC-4 homeodomain protein. unc-4 mutants are unable to execute backward locomotion because VA motor neurons are mis-wired with inputs normally reserved for their VB sisters. We have proposed that UNC-4 functions in VAs to block expression of VB genes. This model is substantiated by the finding that ectopic expression of the VB gene ceh-12 (encoding a homolog of the homeodomain protein HB9) in unc-4 mutants results in the mis-wiring of posterior VA motor neurons with VB-like connections. Here, we show that VA expression of CEH-12 depends on a nearby source of the Wnt protein EGL-20. Our results indicate that UNC-4 prevents VAs from responding to a local EGL-20 cue by disabling a canonical Wnt signaling cascade involving the Frizzled receptors MIG-1 and MOM-5. CEH-12 expression in VA motor neurons is also opposed by a separate pathway that includes the Wnt ligand LIN-44. This work has revealed a transcriptional mechanism for modulating the sensitivity of specific neurons to diffusible Wnt ligands and thereby defines distinct patterns of synaptic connectivity. The existence of comparable Wnt gradients in the vertebrate spinal cord could reflect similar roles for Wnt signaling in vertebrate motor circuit assembly.

**KEY WORDS:** *C. elegans*, Wnt signaling, Gap junction, Motor circuit, Synaptic specificity, unc-4

**INTRODUCTION**

Nervous system function is defined by connections between specific neurons. These links include chemical synapses that utilize neurotransmitters to evoke postsynaptic responses and gap junctions that regulate ion flow between coupled neurons. Although some progress has been made towards understanding the molecular basis of chemical synaptic specificity (Sanes and Yamagata, 2009; Shen and Scheiffele, 2010), little is known about how neurons choose partners for gap junction assembly (Bennett and Zukin, 2004; Hestrin and Galarrreta, 2005). Both types of synapses are active in motor circuits that regulate body movements (Charlton and Gray, 1966; Westerfield and Frank, 1982; Van Der Giessen et al., 2008; Li et al., 2009). The key role of transcription factor codes in motor circuit neuron fate suggests that genetic programs define the specificity of these connections (Briscoe et al., 2000; Shirasaki and Pfaff, 2002). Downstream targets with roles in synaptic specificity are largely unknown but probably include a combination of diffusible cues and cell-surface proteins that regulate synaptogenic responses (Pecho-Vrieseling et al., 2009).

Wnt signaling functions as a key regulator of synaptic assembly in the brain and at the neuromuscular junction (Budnik and Salinas, 2011). For example, in cerebellar neurons, Wnt7a activates a cytoplasmic pathway that promotes local assembly of presynaptic components whereas Wnt-dependent synaptic assembly at the Drosophila neuromuscular junction can also depend on transcriptional regulation (Packard et al., 2002; Ahmad-Annuar et al., 2006; Ataman et al., 2006; Miech et al., 2008). Wnts might also function as antagonistic cues to limit synapse formation (Inaki et al., 2007; Klassen and Shen, 2007) and, in at least one case, adopt opposing roles that either promote or inhibit synaptogenesis (Davis et al., 2008). Although multiple members of the Wnt family are expressed in the developing spinal cord and have been shown to regulate axon trajectory and neuron fate, explicit roles in synaptogenesis have not been uncovered (Lyuksyutova et al., 2003; Liu et al., 2005; Agalliu et al., 2009). Here, we describe our finding that opposing Wnt signaling pathways regulate the specificity of synaptic inputs in a nematode motor circuit.

In *C. elegans*, backward movement depends on connections between AVA interneurons and VA class motor neurons, whereas forward locomotion requires AVB input to VB motor neurons (Fig. 1) (Chalfie et al., 1985; Ben Arous et al., 2010; Haspel et al., 2010). The specificity of these connections is controlled by the UNC-4 homeodomain transcription factor, which functions in VA motor neurons (Miller et al., 1992). In unc-4 mutants, AVB inputs to VAs are replaced with gap junctions from AVB and backward locomotion is disrupted. The characteristic anterior polarity of VA motor neurons is not perturbed, however, which suggests that UNC-4 regulates the specificity of synaptic inputs but not other traits that distinguish VAs from sister VB motor neurons (White et al., 1992; Miller and Niemeyer, 1995). UNC-4 functions as a transcriptional repressor with the conserved Groucho-like protein UNC-37 to block expression of VB-specific genes (Pflugrad et al., 1997; Winnier et al., 1999) (Fig. 3). We have shown that one of these VB proteins, the HB9 (MNX1) homolog CEH-12, is sufficient to rewire VA motor neurons with VB-type inputs (Von Stetina et al., 2007b). Thus, these findings revealed a regulatory switch in which differential expression...
Wnt pathways control synaptic choice

Fig. 1. Diagram of the C. elegans motor neuron circuit.
Interneurons from the head and tail extend axons into the ventral nerve cord to synapse with specific motor neurons. The forward circuit (red) includes AVB and PVC interneurons and DB (not shown) and VB motor neurons. The backward circuit (blue) includes AVE, AVD and AVE interneurons and DA (not shown) and VA motor neurons. VAs and VBs arise from a common progenitor but are connected to separate sets of interneurons (AVA and AVB shown for simplicity).

of the transcription factors, UNC-4 versus CEH-12, in VAs results in alternate sets of presynaptic inputs. This mechanism, however, shows regional specificity along the length of the ventral nerve cord. Ectopic expression of ceh-12 in unc-4 mutants is limited to posterior VA motor neurons and VA input specificity in this location depends on ceh-12. These findings suggest that UNC-4 might regulate multiple targets that function in parallel to specify inputs to selected VAs motor neurons (Von Stetina et al., 2007b). Here, we report the discovery that ceh-12 expression in posterior VA motor neurons is activated by a specific Wnt protein, EGL-20, that is secreted from adjacent cells in this region. We propose that UNC-4 normally prevents VAs from responding to EGL-20 by antagonizing a canonical Wnt signaling pathway utilizing the Frizzled (Frz) receptors MOM-5 and MIG-1. We have also identified a separate Wnt pathway, involving the Frz receptor LIN-17 and the Wnt ligands LIN-44 and CWN-1, that preserves VA inputs by blocking CEH-12 expression in anterior VAs. Our results have uncovered a key role for the UNC-4 transcription factor in modulating the relative strengths of Wnt signaling pathways with opposing roles in synaptic choice. The widespread occurrence of regional Wnt signaling cues in the developing spinal cord could be indicative of similar functions for transcription factors in regulating synaptic specificity in the vertebrate motor circuit.

MATERIALS AND METHODS

Nematode strains and genetics
Nematodes were cultured as described (Brenner, 1974). Mutants were obtained from the Caenorhabditis Genetics Center (CGC) or by generous donations from other laboratories (supplementary material Tables S1, S2). Transgenic strains and primer sequences used for building constructs are listed in supplementary material Table S3.

Molecular biology
Punc-4::ANT-BAR-1 was generated by overlap PCR from plasmid pHCK19 (supplementary material Table S3) (Gleason et al., 2002), microinjected (Fire et al., 1991) with pH86 (dpy-20(+)) to produce NC1847 [wdEx636[Punc-4::ANT-BAR-1, dpy-20(+)]) and crossed into wdIs85 (see below). Punc-4::MIG-1::YFP was generated by overlap PCR (supplementary material Table S3) and microinjected to produce NC1870 [wdEx639[Punc-4::MIG-1::YFP, dpy-20(+)])]

ceh-12::GFP expression
A spontaneous integrant, wdIs85 (LGIII), of wdEx310 was used to assay ceh-12::GFP expression. L2 larval VAs and VBs were scored for the presence or absence of ceh-12::GFP expression (n>10 for each neuron).

Animals were anesthetized with either 0.25% tricaine/0.025% tetramisole or 10 mM levamisole. Supplementary material Table S6 shows results used in pie charts (supplementary material Fig. S8).

Detecting AVB gap junctions with ventral cord motor neurons
The AVB gap junction marker strain NC1694 [wdIs54[Punc-7::UNC-7S::GFP, col-19::GFP] unc-7(e5)] was integrated by γ irradiation (4000 Rads) of EH578 (Starich et al., 2009) and backcrossed into wild type ten times. AVB gap junctions in the ventral cord were detected by anti-GFP immunostaining in L4 larvae and specific motor neurons were identified as described (Von Stetina et al., 2007b). n>10 for each neuron. Supplementary material Table S7 shows values used in pie charts (supplementary material Fig. S8).

Single molecule mRNA FISH
In situ hybridization assays were performed (Raj et al., 2008; Harterink et al., 2011) in wild type, unc-4(e120) and unc-37(e262) with unc-4::GFP to mark DA and VA neurons. Synchronized worms were fixed in 4% formaldehyde, 70% ethanol. Oligonucleotide probes (www.singlemoleculefish.com) were coupled to Alexa 594 (mig-1) or Cy5 (mom-5). Nuclei were stained with DAPI. z-stacks (0.5 μm per slice) were collected using a Leica DM6000 microscope with 100× objective and Tx2 (Alexa594) or Y5 (Cy5) filter cube. 1024×1024 images were subjected to 2×2 binning. Each VA neuron was identified by its position in the ventral nerve cord and its cell soma was delineated by the outside edge of unc-4::GFP staining (Leica AF Lite). Individual fluorescent puncta (mRNA) from this region were counted by direct inspection of the z-stack. n=5 for each neuron.

GRASP assay of AVA to VA10 synapses
The GFP reconstitution across synaptic partners (GRASP) marker wdIs65 was integrated by γ irradiation (4000 Rads) of wEx1845 (Feinberg et al., 2008) and used to label AVA to A-class motor neuron synapses. z-stacks were collected with identical microscope settings. Line scans in the GFP channel were collected in the VA10 to DA7 interval (Feinberg et al., 2008). An equivalent length scan was obtained from the posterior dorsal nerve cord (devoid of GFP puncta) to obtain an average background signal in the GFP channel for each animal. The intensity score for each experimental sample was calculated from the percentage of measurements from the VA10 to DA7 interval that exceeded this background signal. n≥10 for each neuron.

Microscopy
wdIs85(ceh-12::GFP) and wdIs54(Punc-7::UNC-7S::GFP) were scored with a 100× objective in a Zeiss Axioplan microscope with a Hamamatsu Orca camera. Images of wdIs85 were obtained using a Leica TCS SP5 confocal microscope. Images of wdIs54 were obtained using an Olympus FV-1000 confocal microscope with a 60×/1.45 Plan-Apochromat lens. Pseudocolours and image overlays were generated using Olympus software and Adobe Photoshop.

Movement assay
A movement assay (‘tapping assay’) detected effects of specific mutants on backward locomotion (Von Stetina et al., 2007b). For each genotype, n=50 L4-young adults were tapped on the head with a platinum wire. Backward movement was scored as either Unc (coiled instantly, no net backward movement) or Suppressed (detectable backward movement of posterior region or entire body in locomotory sinusoidal waves).

Pyruvium assay
Embryos were placed on nematode growth media (NGM) plates streaked with OP50 bacteria, covered with pyruvium palmitoleate dissolved in soybean oil, and allowed to hatch. Tapping assays were performed on young adults (n=50).

Lithium chloride treatment
Lithium chloride was added to NGM media before pouring plates to yield a final concentration of 10 mM LiCl. Synchronized L1 larvae were grown on LiCl plates for 3 days at 20°C and tapping assays performed on young adults (n≥150).
**Results**

**EGL-20 Signaling Promotes ceh-12 Expression in Posterior VA Motor Neurons**

We have previously determined that ceh-12 contributes to the Unc-4 phenotype in posterior VA motor neurons (Von Stetina et al., 2007b). ceh-12 is ectopically expressed in posterior VAs in unc-4 mutants and is specifically required for the mis-wiring of VAs in this region (Von Stetina et al., 2007b). We hypothesized that the biased posterior expression of ceh-12 could result from an inductive signal provided by nearby cells. The diffusible ligand EGL-20 is expressed in this posterior region (Coudreuse et al., 2006; Harterink et al., 2011) and is therefore a candidate for a local cue that could promote ectopic ceh-12 expression.

This idea is substantiated by our finding that ceh-12::GFP expression in posterior VAs is significantly reduced in egl-20; unc-4 double mutants (Fig. 2A,D). We tested the additional Wnt ligands cwn-2, cwn-1 and lin-44 and saw no effect on posterior ceh-12::GFP expression in double mutants with unc-4 (supplementary material Figs S1, S2). mom-2 mutation is lethal (Thorpe et al., 1997) and was not examined. These results are consistent with a model in which EGL-20 is exclusively required for ectopic ceh-12 expression in posterior VA motor neurons. ceh-12::GFP expression in VB motor neurons is not perturbed in the egl-20 mutant and is therefore likely to be activated by additional pathways in these cells.

**Multiple Wnt Receptors Are Required for EGL-20-Dependent Expression of ceh-12**

To test whether the Frizzled receptors MIG-1 and MOM-5 mediate EGL-20-dependent expression of ceh-12, we scored ceh-12::GFP expression in mom-5; unc-4(e120) and mig-1; unc-4(e120) mutants; ectopic ceh-12::GFP expression in posterior VAs was reduced in both of these double mutants (Fig. 2A). We note that mom-5 and mig-1 suppression of ectopic ceh-12::GFP in unc-4 is weaker than that of the egl-20 mutant, and attribute this difference to likely roles for multiple Wnt receptors functioning in parallel.

**Fig. 2. EGL-20 is required for ceh-12 expression in unc-4 mutant VA motor neurons.** (A-D) EGL-20 is secreted from cells in the tail. Boxed area denotes the region depicted in B-D. ceh-12::GFP is expressed in VB motor neurons in L2 stage wild-type (WT) animals (B) and in posterior VAs in unc-4 mutants (A,C). Mutations in egl-20 (D), mig-1 or mom-5 (A) reduce ectopic ceh-12::GFP expression in posterior VAs. *P<0.05, **P<0.01, ***P<0.001 versus unc-4 (A). (E) ceh-12::GFP is detected in anterior VAs when EGL-20 is expressed from the head neuron-specific transgene plim-4::egl-20. †P<0.05, †††P<0.001 versus unc-4. Ectopic ceh-12::GFP in anterior VAs is reduced in mig-1 mutants. *P<0.05 versus plim-4::egl-20; unc-4. (F,G) ceh-12::GFP expression (boxed area in F enlarged in G) depicting anterior VA (arrows) and VB motor neurons. unc-4(e120) was used for all experiments; n=10 for each neuron. Scale bars: 10 μm in B-D, 15 μm in F,G.
(Fig. 2A). Indeed, mutations in the Frizzled homolog cfz-2 and the Ryk family member lin-18 also reduce ectopic ceh-12::GFP expression in a subset of posterior VAs (supplementary material Fig. S1), suggesting that cfz-2 and lin-18 might be partially required for ectopic ceh-12 expression.

**EGL-20 is sufficient to induce ceh-12::GFP expression**

To determine whether EGL-20 is sufficient to promote ceh-12::GFP expression in VAs, we used a transgene, plim-4::EGL-20, that ectopically expresses EGL-20 in anterior cells (Pan et al., 2006). This treatment enlarged the region of the ectopic ceh-12::GFP signal (Fig. 2E-G) to include anterior VA motor neurons in addition to the posterior VAs that express ceh-12::GFP in an unc-4 mutant. ceh-12::GFP in anterior VAs was not observed in a wild-type background (supplementary material Fig. S3) nor in an unc-4 mutant in the absence of anterior egl-20 (Fig. 2E). Mutation of mig-1 attenuated ectopic ceh-12::GFP expression in anterior as well as posterior VAs, indicating that mig-1 is necessary for EGL-20-dependent ceh-12::GFP expression throughout the ventral nerve cord (Fig. 2E). We attribute residual ceh-12::GFP expression in these anterior cells to the partially redundant functions of mom-3, lin-18 and cfz-2 in this pathway (Fig. 2A; supplementary material Fig. S1). These results confirm that loss of unc-4 is necessary for activation of ceh-12 expression by egl-20 and that egl-20 functions through MIG-1 upstream of ceh-12 (Fig. 2E). This finding also establishes that the unc-4 mutation effectively sensitizes VA motor neurons throughout the length of the VNC to an available EGL-20 cue.

**EGL-20 signaling contributes to the Unc-4 movement defect**

We have previously shown that ectopic expression of ceh-12 contributes to the backward movement defect in unc-4 mutants (Von Stetina et al., 2007b). Because EGL-20 is necessary for ceh-12::GFP expression in posterior VAs, we reasoned that loss-of-function egl-20 mutants should partially suppress the Unc-4 backward movement phenotype (Fig. 3). This trait can be detected in a ‘tapping assay’ in which animals are touched on the head to stimulate backward locomotion (Fig. 3A-C) (Von Stetina et al., 2007b). The missense mutants unc-4(e2323) and unc-4(e2322ts) (Miller et al., 1992) were used to sensitize this assay because ceh-12 mutations afford strong suppression of ‘weak’ or hypomorphic unc-4 alleles (Von Stetina et al., 2007b). Our results show that the egl-20 alleles n585 and m239 and egl-20 RNAi restore backward movement to unc-4(e2323) and unc-4(e2322ts) animals (Fig. 3D,E; supplementary material Fig. S4, Table S5). egl-20(n585) also partially suppresses the backward Unc defect of a hypomorphic allele of the co-repressor unc-37 (Pflugrad et al., 1997; Winnier et al., 1999) (Fig. 3D). The finding that ceh-12 and egl-20 mutations improve backward movement of an unc-4 mutant (Fig. 3D,E) is predicted for a linear pathway in which egl-20 functions upstream to activate ceh-12 expression. To test this model, we used the unc-4(e120) and unc-4(e2320) null alleles, which are weakly suppressed by a mutation in ceh-12. egl-20(n585) does not enhance ceh-12 suppression of unc-4(e120) and RNAi of egl-20 does not improve backward locomotion in ceh-12; unc-4(e2320) (Fig. 3F; supplementary material Fig. S4A). These observations favor a model in which egl-20 and ceh-12 function in a common pathway. We note that the ceh-12 null allele does enhance egl-20 suppression of unc-4 and we attribute this effect to the hypomorphic egl-20(n585) allele used for this experiment (Fig. 3F). Experiments with the null allele egl-20(hu120) confirmed that EGL-20 is necessary for the mis-wiring of posterior VAs with gap junctions from AVB in unc-4 but also suggest that a minimum level of EGL-20 activity might be required to promote the creation of normal backward locomotory inputs to VAs (supplementary material Fig. S4B,C).

To detect potential roles for Wnt receptors in VA input specificity, we tested mutant alleles of mig-1, cfz-2 and lin-18 for suppression of the Unc-4 backward movement defect. Mutations in mig-1 but not cfz-2 or lin-18 result in significant restoration of backward movement in unc-4(e2322ts) (Fig. 3E; supplementary material Fig. S6). Although cfz-2 and lin-18 are partially required for ectopic ceh-12::GFP expression (supplementary material Fig. S1), this effect might be too weak to be detected in the tapping
assay. RNAi of mom-5 results in strong suppression of Unc-4 movement (supplementary material Fig. S6). These results are consistent with a model in which MIG-1 and MOM-5 are the principle receptors in an EGL-20 signaling pathway that contributes to synaptic mis-wiring in unc-4 mutants.

**UNC-4 limits expression of mom-5 and mig-1 in VA motor neurons**

Expression of mig-1, mom-5 and cff-2 in VAs is supported by microarray results and by experiments with GFP reporters (Von Stetina et al., 2007a) (supplementary material Fig. S7, Table S4). Microarray data also indicate that mom-5 and mig-1 transcripts are significantly upregulated in VA motor neurons when the unc-4 pathway is disabled (supplementary material Fig. S7, Table S4) (Von Stetina et al., 2007b). Thus, unc-4 might effectively quell the VA response to EGL-20 by preventing MOM-5 and MIG-1 levels from exceeding a critical threshold. To test this idea, we used a quantitative method of in situ hybridization (Harterink et al., 2011) to measure mom-5 and mig-1 transcripts in VAs (Fig. 4A). mig-1 mRNA is significantly elevated in VA9 in unc-37 and unc-4 mutants (Fig. 4B). This result is consistent with the observed requirement for mig-1 function for ectopic ceh-12::GFP expression in VA9 in unc-4 mutants (Fig. 2A). mom-5 mRNA is increased in VA10 in unc-4 and unc-37 mutants but not in the adjacent VA9 motor neuron where MOM-5 promotes ectopic expression of ceh-12::GFP (Fig. 2A, Fig. 4C). This disparity could indicate that unc-4 also antagonizes mom-5 activity by an additional mechanism in VA9 that does not involve direct transcriptional repression (see Discussion). We observed statistically significant elevation of mom-5 (VA6) and mig-1 (VA2, VA6) in selected anterior VA motor neurons in unc-4 and unc-37 mutants, which might account for the sensitivity of these VA motor neurons to an anterior source of EGL-20 (Fig. 2B, Fig. 4B,C).

If UNC-4 antagonizes MIG-1 and MOM-5 to preserve VA-type inputs, then overexpression of these Frizzled receptors should be sufficient to induce an Unc-4-like phenotype. To test this model, we used the unc-4 promoter to drive expression of MIG-1 in A-class motor neurons in the unc-4(e2322ts) mutant (Fig. 4D). At permissive temperature (16°C), unc-4(e2322ts) animals display wild-type backward locomotion (Miller et al., 1992). However, expression of Punc-4::MIG-1 in unc-4(e2322ts) resulted in strong Unc-4-like movement (Fig. 4D). Suppression of this effect by ceh-12(0) is predicted by the hypothesis that the Unc-4-like phenotype induced by Punc-4::MIG-1 depends on ectopic ceh-12 expression and also rules out a model in which VA function is non-specifically disrupted by overexpression of MIG-1 protein. These results are consistent with the proposal that UNC-4 limits the sensitivity of VA motor neurons to an available Wnt signal by inhibiting expression or function of the Frizzled receptors mig-1 and mom-5.

**A separate Wnt signaling pathway opposes ceh-12 expression in VA motor neurons**

We noted that a lin-17 mutant enhanced the Unc-4 phenotype (Fig. 5A) in contrast to mutations in mig-1 and mom-5, which suppress the Unc-4 backward movement defect (Fig. 3E; supplementary material Fig. S6). unc-4(e2322ts) animals normally display wild-type backward movement at 16°C (Miller et al., 1992). Backward locomotion is significantly impaired, however, in the lin-17; unc-4(e2322ts) double mutant compared with either unc-4(ts) or lin-17 single mutants. In addition, mutation of lin-17 alone shows a weak backward Unc defect. Mutations in lin-44 and cwn-1 also display a similar Unc-4 enhancer effect (Fig. 5A).

![Fig. 4. mon-5 and mig-1 are negatively regulated by unc-4 and unc-37 in VA motor neurons.](image)
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One explanation for these results is that LIN-44, CWN-1 and LIN-17 normally function to prevent expression of ceh-12 in VA motor neurons. This model is consistent with experiments showing that overexpression of CEH-12 protein in VAs induces an Unc-4-like movement defect (Von Stetina et al., 2007b). We note that ectopic expression of ceh-12::GFP is enhanced in anterior VA motor neurons (VA2-6) in lin-44; unc-4(0) and cwn-1 unc-4(0) mutants, whereas expression of ceh-12::GFP in posterior VAs (VA7-10) was unaffected (supplementary material Fig. S2, Table S6). ceh-12::GFP expression is detected in lin-17; unc-4(0) in anterior VAs, although it is not statistically significantly different from wild type (Fig. 5B; supplementary material Table S6). A linear pathway involving LIN-44, CWN-1 and LIN-17 that limits ectopic CEH-12 expression could explain these results. Our finding that lin-44 fails to enhance ectopic ceh-12::GFP expression in lin-17 mutants is consistent with this model (Fig. 5B). However, other genetic interactions are also suggestive of a more complex mechanism. For example, the lin-44; cwn-1 double mutant does not result in further elevation of ectopic ceh-12::GFP expression in comparison with each single mutant as would be expected if LIN-44 and CWN-1 exercise similar roles upstream of LIN-17 (Fig. 5B). This effect may be attributed to a partially redundant function for CWN-2 (supplementary material Fig. S1). In addition, loss of egl-20 function fails to suppress ectopic ceh-12::GFP in anterior VA motor neurons in a lin-44; unc-4 mutant (Fig. 5B). This result suggests that ceh-12 expression is not regulated by EGL-20 in most anterior VAs. By contrast, the lin-44 mutation partially restores ceh-12::GFP expression to posterior VAs in an egl-20; unc-4 background (supplementary material Fig. S9). This result is consistent with the proposal that LIN-44 antagonizes EGL-20-dependent expression of ceh-12 in posterior VAs and probably reflects the posterior origins of LIN-44 signals (Fig. 5B) (Harterink et al., 2011). This observation also reveals a ‘ground’ state in which ceh-12::GFP expression is activated by an additional pathway when lin-44 and egl-20 function are eliminated (Green et al., 2008).

Opposing Wnt signaling pathways regulate the specificity of interneuron gap junctions with VA motor neurons

The AVB interneuron normally forms gap junctions with DB and VB motor neurons (White et al., 1986), which can be detected with a GFP-tagged gap junction protein (innexin), UNC-7S::GFP, expressed in AVB. UNC-7S::GFP puncta are localized to motor neuron cell soma and therefore allow identification of AVB motor neuron partners in animals counterstained with a DNA dye (DAPI) (Starich et al., 2009) (Fig. 6). We used this assay to confirm that unc-4 mutant VAs display ectopic AVB to VA gap junctions (White et al., 1992) (Fig. 6B) and that ceh-12 is required for the creation of AVB gap junctions with posterior VAs (Von Stetina et al., 2007b). Therefore, if EGL-20 is necessary for ectopic ceh-12 in posterior VAs (Fig. 2A, Fig. 5D), then these aberrant AVB gap junctions should be reduced in egl-20 mutants. The frequency of UNC-7S::GFP puncta associated with posterior VAs is substantially reduced in unc-4; egl-20(n855) and in unc-4; egl-20(n120) mutants (Fig. 6C; supplementary material Fig. S4C). MIG-1 is also required for ectopic AVB gap junctions with posterior VAs (Fig. 6B,D). A synthetic lethal phenotype prevented us from performing this test with mom-5 (data not shown). These results are consistent with the proposal that EGL-20 and MIG-1 function together to promote ceh-12 expression, thereby leading to the creation of ectopic gap junctions between AVB and VA motor neurons. We note that egl-20, mig-1 and ceh-12 also suppress the Unc-4 AVB gap junction defect for VA2 in the anterior ventral nerve cord (Von Stetina et al., 2007b). This finding suggests
Ectopic AVB to VA gap junctions

Rescue unc-4 induced ectopic AVB to VA gap junctions

EGL-20/Wnt

Fig. 6. Opposing Wnt pathways regulate the specificity of gap junction inputs to VA motor neurons. (A) AVB gap junctions (green) with the cell soma of VB and DB motor neurons (red). Mutants of lin-17 (A) and unc-4 (B) show ectopic AVB to VA gap junctions (yellow arrows). (B) AVB gap junctions with posterior VAs depend on EGL-20. Mutations in ceh-12 and mig-1 suppress ectopic AVB gap junctions with VA10 in unc-4(e120) mutants. (C) lin-17 mutants display ectopic gap junctions near known sources of LIN-44 in the anchor cell (AC) (VA6) and tail region (VA10). (D) ceh-12, mig-1 and egl-20(n585) mutants suppress ectopic AVB gap junctions with posterior VAs in unc-4(e120) mutants and with VA2. *P<0.05 versus WT (C) or unc-4 (D). n=10 for each neuron.

that ceh-12 expression is regulated by the EGL-20 pathway in this anterior VA motor neuron, although we were unable to detect this effect with the ceh-12::GFP reporter as noted above.

To determine whether lin-17 normally antagonizes the formation of AVB to VA gap junctions, we investigated UNC-7S::GFP localization in a lin-17 mutant; this test detected ectopic UNC-7S::GFP puncta for VA6 and VA10 (Fig. 6A,C). Although aberrant AVB gap junctions are limited to a subset of VAs in this experiment, these effects are statistically significant and are consistent with the mild backward movement defect of the lin-17 mutant (Fig. 6A; supplementary material Fig. S8). Moreover, the strong lin-17 dependent enhancement of Unc-4 movement for the hypomorphic unc-4(e120) allele (Fig. 5A) suggests that LIN-17 might also act in additional VAs to oppose the creation of AVB to VA gap junctions. Similarly, a cwn-1 mutation results in ectopic AVB gap junctions with anterior VAs (supplementary material Fig. S8). Owing to synthetic lethality, lin-44 could not be tested (data not shown). Taken together, these data support the hypothesis that LIN-17 and CWN-1 prevent the imposition of ectopic AVB inputs to VA motor neurons by opposing the activity of an EGL-20 signaling pathway that functions principally through mom-5 and mig-1 (Fig. 5C,D).

EGL-20 opposes the formation of AVA to VA chemical synaptic connections

Mutations in unc-4 alter the connectivity of VA motor neurons by replacing gap junctions and chemical synapses from AVA interneurons with gap junctions from AVB (White et al., 1992; Von Stetina et al., 2007b). We have established that a posterior EGL-20 signal, functioning via ceh-12, directs the formation of ectopic gap junctions between posterior VAs and AVB in unc-4 mutants (Fig. 6B,D) (Von Stetina et al., 2007a). To determine whether EGL-20 signaling is also necessary for eliminating normal VA inputs from AVA in unc-4 animals, we utilized a GRASP (GFP reconstitution across synaptic partners) assay (Fig. 7A). GRASP uses split-GFP to label chemical synapses between specific pairs of neurons (Fig. 7A-C). These split-GFP puncta include AVA in unc-4 mutants with both DA and VA motor neurons, which cannot be readily distinguished except within a region between VA10 and DA7 (Fig. 7B). We confirmed that the AVA to VA10 GRASP signal is significantly weaker in unc-4 mutants (Feinberg et al., 2008) (Fig. 7C,D) and show that mutations in ceh-12 or egl-20 restore wild-type levels of split-GFP signal to AVA synapses with VA10 (Fig. 7C,D). These results indicate that EGL-20 signaling, acting through ceh-12, is responsible for eliminating AVA synapses with posterior VAs in addition to its role in establishing ectopic gap junctions with AVB.

UNC-4 antagonizes a canonical Wnt signaling pathway

In C. elegans, canonical Wnt signaling involves the β-catenin protein BAR-1, whereas non-canonical or atypical Wnt pathways utilize other members of the β-catenin family (Korswagen et al., 2000). In the canonical pathway, Wnt interaction with a Frizzled receptor stabilizes BAR-1 by inhibiting the activity of a ‘destruction complex’ that includes the proteins Axin, GSK3β and
Casein Kinase Iα. In the absence of Wnt, the destruction complex phosphorylates cytosolic BAR-1 leading to its degradation (Gleason et al., 2002). Thus, if BAR-1/β-catenin functions in a canonical Wnt pathway to promote VA mis-wiring, then a loss-of-function bar-1 mutation should suppress this defect. We observe weak suppression of Unc-37 backward movement by the bar-1(mu63) allele but not unc-4 (supplementary material Fig. S10). One explanation for this ambiguous result is that bar-1 mutant animals are Unc possibly owing to a required function in another motor neuron class (Vashlishan et al., 2008). The stronger effect of the bar-1 mutation on the Unc-37 phenotype could also result from dual roles of UNC-37 as a co-repressor with UNC-4 and with the TCF-LEF family member POP-1 (Calvo et al., 2001), both of which probably function to block ceh-12 expression.

To confirm that canonical Wnt signaling is involved upstream of ceh-12, we tested for potential roles of the Wnt pathway destruction complex in the Unc-4 phenotype. Genetic ablation of the Axin-like pry-1 effectively activates canonical Wnt signaling by preventing degradation of BAR-1 (Gleason et al., 2002). It follows that if PRY-1 negatively regulates EGL-20-dependent signaling in VA motor neurons, then a mutation in pry-1 should constitutively activate this pathway. Backward locomotion is strongly impaired in the pry-1; unc-4(e2322ts) double mutant compared with the unc-4(e2322ts) and pry-1 single mutants (Fig. 8A). This synthetic unc-4 phenotype is consistent with a model in which both unc-4 and pry-1 normally function as negative regulators of a Wnt pathway that leads to VA mis-wiring (Fig. 8E).

Next, we treated animals with LiCl to inhibit GSK3β activity (McColl et al., 2008) and, thus, hyper-activate canonical Wnt signaling. Exposure of wild-type animals to 10 mM LiCl induces a strong backward movement defect (Fig. 8B). Although lithium-dependent inhibition of other targets in C. elegans (Tanizawa et al., 2006) could potentially impede locomotion, this LiCl-induced Unc-4 phenotype is attenuated in a ceh-12 null mutant (Fig. 8B). This result is consistent with the model that VA mis-wiring depends on activation of ceh-12 expression by canonical Wnt signaling (Fig. 8E).

If inhibition of the destruction complex by either the pry-1 mutation or by LiCl prevents degradation of BAR-1, then a constitutively active BAR-1 protein should produce a similar phenotypic effect. We tested this prediction with ΔNT-BAR-1, a truncated β-catenin protein lacking N-terminal phosphorylation sites that trigger Axin/GSK3-mediated degradation (Gleason et al., 2002). Expression of Punc-4:ΔNT-BAR-1 in A-class motor neurons results in Unc-4-like movement resembling that produced by CEH-12 overexpression (Fig. 8C) (Von Stetina et al., 2007b). Moreover, the Punc-4:ΔNT-BAR-1 transgene induced ectopic ceh-12::GFP expression in VA motor neurons in which wild-type unc-4 function is intact (Fig. 8D), thereby supporting the idea that canonical Wnt signaling is sufficient to activate ceh-12 expression in VA motor neurons.

In a final experiment, we utilized the canonical Wnt-pathway inhibitor pyrvinium which interacts with casein kinase Iα to activate the destruction complex and downregulate β-catenin. Experiments in C. elegans have confirmed that pyrvinium disrupts canonical Wnt signaling (Thorne et al., 2010). Thus, if the Unc-4 movement defect arises from ectopic activation of canonical Wnt signaling then this effect should be ameliorated by treatment with pyrvinium. This prediction is substantiated by the finding that pyrvinium suppresses the Unc-4 movement phenotype of unc-4(e2322ts) (supplementary material Fig. S11). Taken together, our results support the hypothesis that UNC-4 preserves VA-type synaptic inputs by antagonizing an EGL-20-dependent canonical signaling pathway.

DISCUSSION

Neural networks are defined by the creation of synapses between specific neurons. In C. elegans, the UNC-4 homeodomain protein functions in VA motor neurons to prevent the formation of inputs...
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No ceh-12::GFP expression  ceh-12::GFP positive VAs

% Suppressed  % Unc

A B C

unc-4  ppf-1  WT

WT  ceh-12  WT  L4

WT  ceh-12  WT  L4

D

WT  wdx636(punc-4::WNT-BAR-1)

E

WT  Promotes VB-type inputs

WNT/EGL-20

Frizzled/MIG-1, MOM-5

β-Catenin/BAR-1

CEH-12/HB9

AXIN/PRY-1

GSK3

Fig. 8. Canonical Wnt signaling functions upstream of ceh-12 in unc-4 mutant VA motor neurons. (A) pry-1(mu38) enhances the Unc-4 backward movement phenotype of unc-4(e2322ts) at 16°C. *p<0.05; n=50. (B) The GSK-3β inhibitor LiCl induces a backward movement defect in WT animals that is partially rescued by ceh-12(gk391). *p<0.05; n=150. (C) Constitutive activation of BAR-1 in WT A-class motor neurons with Punc-4::AXIN/PRY-1 results in Unc-4-like movement. *p<0.05; n=50. (D) ceh-12::GFP is ectopically expressed in VAs when BAR-1 is constitutively activated in a wild-type background. ***p<0.001 versus WT. n=20 for each neuron. (E) UNC-4 antagonizes a canonical Wnt signaling pathway to preserve VA-type inputs.

normally reserved for VB motor neurons (Miller et al., 1992; White et al., 1992; Miller and Niemeyer, 1995). We have shown that this synaptic choice depends on UNC-4 inhibition of a canonical Wnt signaling pathway that promotes VA-type inputs. UNC-4 is required in posterior VAs to block the effects of an EGL-20 cue secreted from adjacent epithelial cells in the tail. The outcome of EGL-20 signaling is also opposed by a separate pathway involving LIN-44, CWN-1 and LIN-17. We propose that this mechanism could be generally employed by neuron-specific transcription factors to diversify local circuits during neural development in which graded Wnt signals are widely utilized to specify neurogenic fates.

**Wnt signaling regulates synaptic choice**

Wnt signaling can either promote or inhibit synaptic assembly. Wnt exercises a positive role at central synapses and at motor neuron inputs to muscle (Budnik and Salinas, 2011). For example, in the cerebellum, Wnt7a is provided by granule cell target neurons to activate presynaptic assembly in migrating mossy fiber axons (Ahmad-Annuar et al., 2006). Wnt7a also promotes postsynaptic development of excitatory neurons in the hippocampus (Ciani et al., 2011). At the mammalian neuromuscular junction, Wnt activates postsynaptic clustering of acetylcholine receptors and can be provided by either the innervating motor neuron (Henriquez et al., 2008) or by nearby non-neuronal tissues (Jing et al., 2009). In these instances, the Wnt signal is transduced by non-canonical pathways that act locally and apparently do not require transcription (Cerpa et al., 2008; Miech et al., 2008; Purro et al., 2008; Sáhores et al., 2010; Budnik and Salinas, 2011). Wnt signals may also act as negative cues at neuromuscular synapses. In *C. elegans*, LIN-44 functions through LIN-17 to exclude synapses from a nearby motor neuron axonal compartment (Klassen and Shen, 2007). In *Drosophila*, Wnt4 is selectively expressed in a specific embryonic muscle to prevent inappropriate motor neuron inputs (Inaki et al., 2007). Together, these results are indicative of multiple alternative pathways for Wnt signaling that either stimulate or limit synaptogenesis. However, with the exception of the negative role of Wnt4 in blocking input from specific motor axons in the fly embryonic musculature, Wnt signaling has not been previously shown to drive the selection of particular pairs of synaptic partners. We describe a mechanism in which EGL-20 favors the creation of synapses between one set of neurons versus another. EGL-20 activates a canonical pathway that promotes expression of the homeodomain transcription factor CEH-12. In turn, CEH-12 exercises the dual function of favoring the creation of VB-type inputs (e.g. gap junction from AVB; Fig. 6) with VA motor neurons while simultaneously blocking the formation of endogenous VA-type connections (e.g. synapses from AVA; Fig. 7) (Von Stetina et al., 2007b). The identification of CEH-12-regulated genes should help to elucidate the mechanism of these effects. The central roles of HB9 homologs of CEH-12 in *Drosophila* and vertebrate motor neuron differentiation (Arber et al., 1999; Thaler et al., 1999; Broihier and Skeath, 2002) suggest that these targets could also function in more complex motor circuits (Von Stetina et al., 2007b). The function of UNC-4 in VA-input specificity must involve additional downstream genes, however, as the Unc-4 miswiring defect in VA motor neurons in the anterior ventral nerve cord does not depend on ceh-12 (Von Stetina et al., 2007b). The recent isolation of Unc-4 suppressor mutations that function in parallel to ceh-12 is consistent with this model (J.S., R.L.S., D. Ruley, Z. Xu, I. Boothby and D.M.M., unpublished). The existence of alternative pathways for driving the creation of VB-type inputs could explain why VB motor neurons, which normally express CEH-12, are not mis-wired in either ceh-12 or egl-20 mutants (Fig. 6) (Von Stetina et al., 2007b).

**Wnt signaling regulates the specificity of electrical synapses in the motor circuit**

In the *C. elegans* motor circuit, gap junctions are assembled between specific neuron pairs and in specific neuronal compartments. For example, the gap junction component UNC-7 is expressed in AVB interneurons where it localizes adjacent to the cell soma of target B-class (DB and VB) motor neurons (White et al., 1986; Von Stetina et al., 2007b; Starich et al., 2009). Our results show that the UNC-4 pathway controls both the location and specificity of UNC-7 gap junctions. UNC-4 normally prevents the formation of AVB gap junctions with VA motor neurons, and ectopic CEH-12 expression in posterior unc-4 mutant VAs is required for this effect (Von Stetina et al., 2007b). We have shown that EGL-20 signaling is also necessary for the creation of AVB to
VA gap junctions, presumably through the activation of ectopic CEH-12 expression in unc-4 mutant VAs. In these cases, gap junctions with AVB are placed on the VA cell soma whereas the usual wild-type gap junctions with AVA are located on VA neuron processes (White et al., 1986; Von Stetina et al., 2007b) (Fig. 6). Thus, UNC-4 is likely to antagonize a cellular biological mechanism that places AVB gap junctions on the VA cell soma, whereas EGL-20 signaling acting through CEH-12 favors this choice. Gap junctions are widespread in the developing nervous system where they mediate synchronous activity that presages mature circuits of chemical synaptic connections (Bennett and Zukin, 2004). Gap junctions are more limited in the mature vertebrate brain and spinal cord (Rash et al., 2000) but recent results indicate that these electrical synapses are important for function (Van Der Giessen et al., 2008; Li et al., 2009). The mechanisms that define the specificity and localization of gap junction components are largely unknown (Hestrin and Galarreta, 2005). Thus, future studies of the downstream genes that are regulated by the antagonistic activities of UNC-4 versus EGL-20 and CEH-12 could provide a foundation for understanding the cell biology of neuron-selective gap junction assembly.

**Multiple Wnt cues and receptors regulate synaptic specificity**

The *C. elegans* genome encodes five Wnt ligands (CWN-1, CWN-2, EGL-20, LIN-44 and MOM-2) and six Wnt receptors (LIN-18, CAM-1 and the Frizzled homologs CFZ-2, LIN-17, MIG-1, MOM-5) (Eisenmann, 2005; Green et al., 2007). Studies in *C. elegans* have revealed roles for these components in cell migration, axon guidance, synaptogenesis and cell fate determination (Zinovyeva and Forrester, 2005; Goldstein et al., 2006; Hilliard and Bargmann, 2006; Pan et al., 2006; Klassen and Shen, 2007; Green et al., 2008; Moro et al., 2009; Song et al., 2010; Harterink et al., 2011). Our results parallel the finding that multiple Wnts and receptors might contribute to each of these features (Zinovyeva et al., 2008). For example, we show that EGL-20 and the Frz receptors MIG-1 and MOM-5 activate expression of CEH-12 in VA motor neurons to promote the creation of VB-type inputs (i.e. gap junctions with AVB) (Fig. 6) and to oppose VA-type inputs (i.e. synapses from AVA) (Fig. 7). This observation is consistent with earlier studies that detected overlapping functions for MOM-5 and MIG-1 in other EGL-20-dependent signaling pathways (Pan et al., 2006; Zinovyeva et al., 2008). Conversely, a LIN-17-dependent function opposes the outcome of the EGL-20 signal in a pathway that probably responds to LIN-44 and CWN-1. Opposing roles for MIG-1 versus LIN-17-dependent signaling have been previously noted but the downstream mechanisms that account for this effect are not known (Pan et al., 2006). Our evidence indicates that the EGL-20 interaction with MIG-1 and MOM-5 functions through a canonical signaling pathway involving BAR-1 to activate expression of CEH-12. The independent identification of *unc-4*-interacting (i.e. suppressor) mutations in the canonical signaling protein POP-1 (J.S., R.L.S. and D.M.M., unpublished) is consistent with this model. We did not detect evidence of roles for these components in LIN-17-dependent regulation of VA input specificity, which therefore probably utilizes an atypical or non-canonical pathway. A recent report of opposing roles for canonical versus noncanonical Wnt signaling in hippocampal neuron synaptogenesis (Davis et al., 2008) suggests that related antagonistic mechanisms for modulating Wnt signaling output might also be utilized during vertebrate neural development.

**Transcriptional mechanisms preserve motor circuit fidelity by preventing selected neurons from responding to available Wnt cues**

Our results are consistent with the idea that UNC-4 functions in VA motor neurons via a transcriptional mechanism that limits expression of MOM-5 and MIG-1 and thereby effectively quells the sensitivity of VA motor neurons to an available EGL-20 cue. The effect of UNC-4 on *mig-1* and *mom-5* transcript levels is modest, however. Thus, our results do not rule out the possibility that UNC-4-dependent inhibition of the EGL-20 signaling pathway could also require transcriptional repression of additional UNC-4 target genes. In any case, the function of UNC-4 reported here is important because it preserves VA inputs that are required for normal locomotion (White et al., 1992). The extensive occurrence of graded Wnt signals in vertebrate nervous systems (Ciani and Salinas, 2005; Fradkin et al., 2005) suggests that comparable mechanisms could be utilized to differentiate the Wnt sensitivity of local groups of neurons in order to diversify functional circuits. For example, a Wnt signal (Wnt4, Wnt5a, Wnt5b) originates from the floorplate on the ventral side of the spinal cord to promote differentiation of motor neurons in the median motor column (MMC). These MMC neurons, which sit on the proximal edge of the developing spinal column, eventually innervate axial muscles that control body movement. However, progenitors in the P3 ventral domain that directly abut the floorplate, and are therefore likely to experience a higher concentration of Wnt4/5 than more dorsal regions, do not adopt the MMC fate. The insensitivity of P3 precursors to Wnt4/5 is maintained by the transcription factor Nkx2.2 but the mechanism of this effect is unknown (Agalliu et al., 2009). We note a striking parallel to the role of UNC-4 in the *C. elegans* ventral nerve cord, which prevents VA motor neurons from responding to an EGL-20 gradient originating from adjacent epithelial cells.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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