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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 Galarreta, 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, AVA 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

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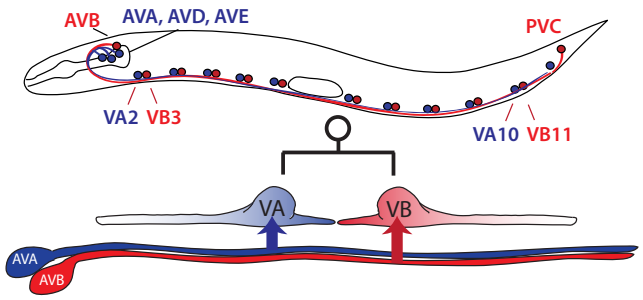

Fig. 1. Diagram of the *C. elegans* motor neuron circuit. Interneurons from the head and tail extend axons into the ventral nerve cord to synapse with specific motor neurons. The forward circuit (red) includes AVB and PVC interneurons and DB (not shown) and VB motor neurons. The backward circuit (blue) includes AVA, 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 VA motor neurons in different ventral cord domains (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::ΔNT-BAR-1 was generated by overlap PCR from plasmid pHCK19 (supplementary material Table S3) (Gleason et al., 2002), microinjected (Fire et al., 1991) with pMH86 (*dpy-20[+]*) to produce NC1847 [*wdEx636[Punc-4::ΔNT-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)* X] 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 *wyEx1845* (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 Hammamatsu 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. Pseudocolors 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).

Pyrvinium assay

Embryos were placed on nematode growth media (NGM) plates streaked with OP50 bacteria, covered with pyrvinium palmoate 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).

Statistical analysis

UNC-7S::GFP puncta, movement assays, and *ceh-12*::GFP expression were quantified using a binary rubric (e.g. GFP was scored as 'present' or 'absent' in a given cell). In all cases, the experimenter was blind to genotype to avoid bias. Fisher's Exact Test was used to calculate a *P*-value for statistical significance (Fisher, 1925). All graphical representations reflect a percentage of total animals scored. Statistical significance was evaluated using a one-way ANOVA ($P<0.05$) for GRASP results.

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

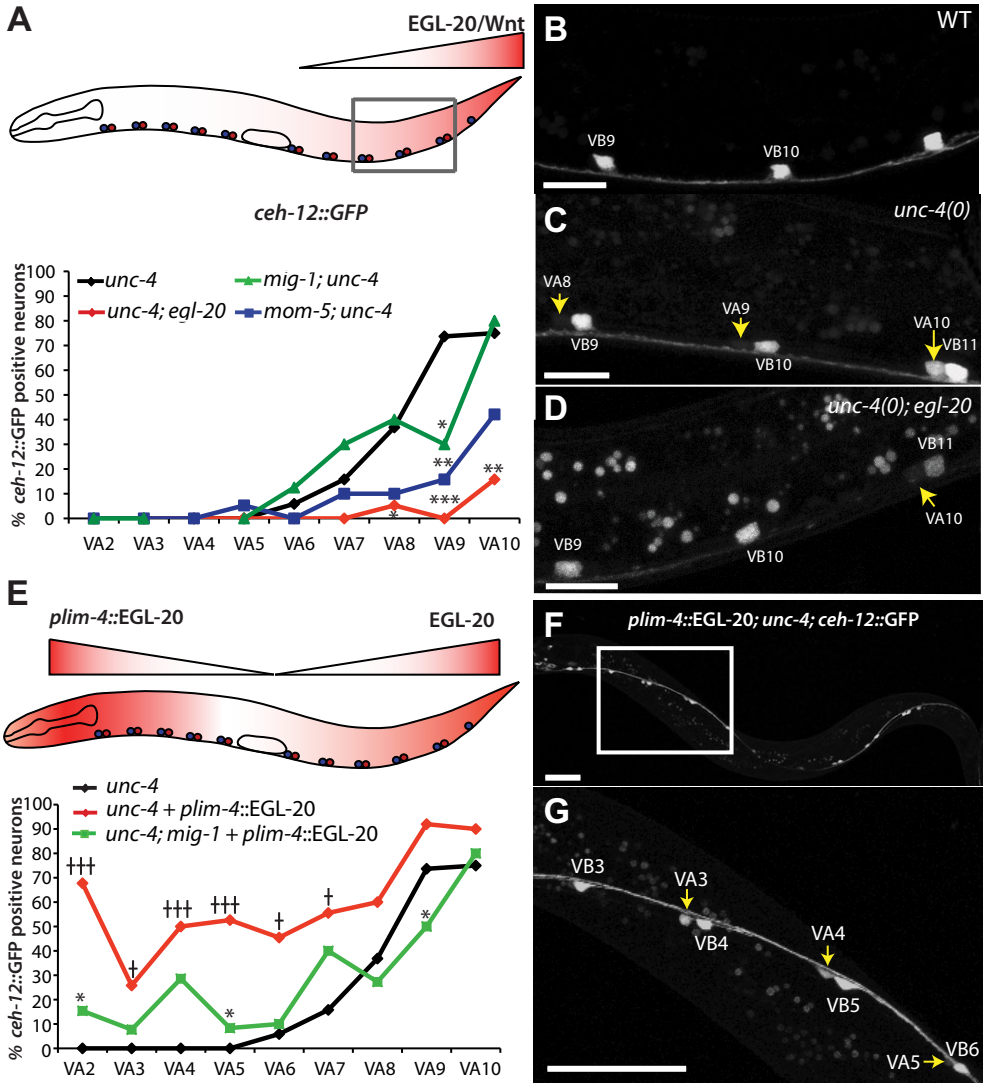

Fig. 2. EGL-20 is required for *ceh-12* expression in *unc-4* mutant VA motor neurons. (**A-D**) EGL-20 is secreted from cells in the tail. Boxed area denotes the region depicted in B-D. *ceh-12*::GFP is expressed in VB motor neurons in L2 stage wild-type (WT) animals (B) and in posterior VAs in *unc-4* 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. $n \geq 10$ for each neuron. Scale bars: 10 μm in B-D; 15 μm in F,G.

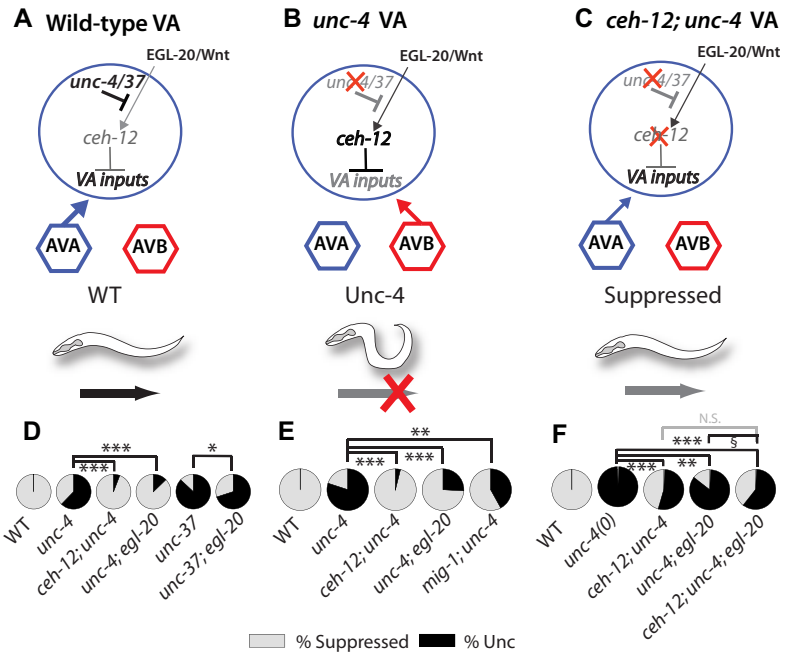

Fig. 3. UNC-4 regulates connectivity in the motor neuron circuit. (**A**) UNC-4 functions with co-repressor UNC-37 to block expression of the VB gene CEH-12 and to preserve VA-type inputs (blue). (**B**) De-repression of CEH-12 in posterior VAs in *unc-4* and *unc-37* mutants results in the mis-wiring of VAs with VB-type inputs (red) and disrupted backward locomotion. EGL-20 promotes CEH-12 expression. (**C**) Mutations in *ceh-12* eliminate ectopic connections with AVB and partially suppress the Unc-4 phenotype. (**D-F**) Locomotion assays. 'Unc' animals cannot crawl backward. 'Suppressed' worms show detectable backward movement. *ceh-12(gk391)* and *egl-20(n585)* mutants strongly suppress the Unc-4 phenotype of hypomorphic alleles *unc-4(e2323)* and *unc-37(e262)* (D) and *unc-4(e2322ts)* (E). A *mig-1(e1787)* mutation partially suppresses Unc-4 movement (E). *ceh-12* and *egl-20* mutants partially suppress the null allele *unc-4(e120)* (F). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$. $^{\S}P<0.05$ versus *unc-4; egl-20*. $n\geq 50$. N.S., not significant.

(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-5*, *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(2322ts)* (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 *mu39* 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 *cfz-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).

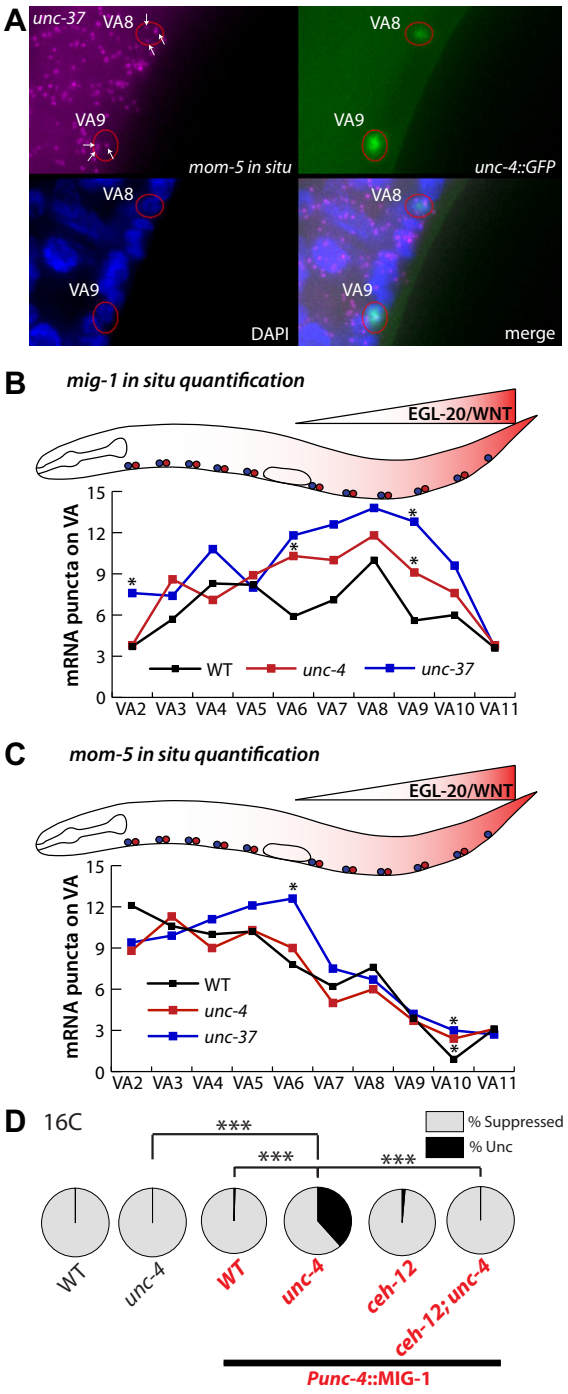

Fig. 4. *mom-5* and *mig-1* are negatively regulated by *unc-4* and *unc-37* in VA motor neurons. (**A**) Fluorescence in situ hybridization detects *mom-5* transcripts as fluorescent puncta (purple, arrows) in VA motor neurons marked with *unc-4*::GFP (green) and DAPI (blue). Puncta overlapping each VA (red circles) were counted for wild type (WT), *unc-4(e120)* and *unc-37(e262)*. (**B**) *mig-1* mRNA is elevated in VA2 and VA9 in *unc-37* mutants and in VA6 and VA9 in *unc-4* mutants. *P<0.05. n≥5 for each neuron. (**C**) *mom-5* mRNA is upregulated in VA10 in *unc-4* mutants and in VA6 and VA10 in *unc-37* mutants. *P<0.05. (**D**) Overexpression of MIG-1 in VAs enhances Unc-4 movement. WT and *unc-4(e2322ts)* show normal backward movement at 16°C. MIG-1 expression in A-class motor neurons with *Punc-4*::MIG-1 (red text) enhances the backward movement defect of *unc-4(e2322ts)* but not WT. *ceh-12(gk391)* suppresses the MIG-1-induced backward movement phenotype. ***P<0.001 versus *unc-4*. n≥50.

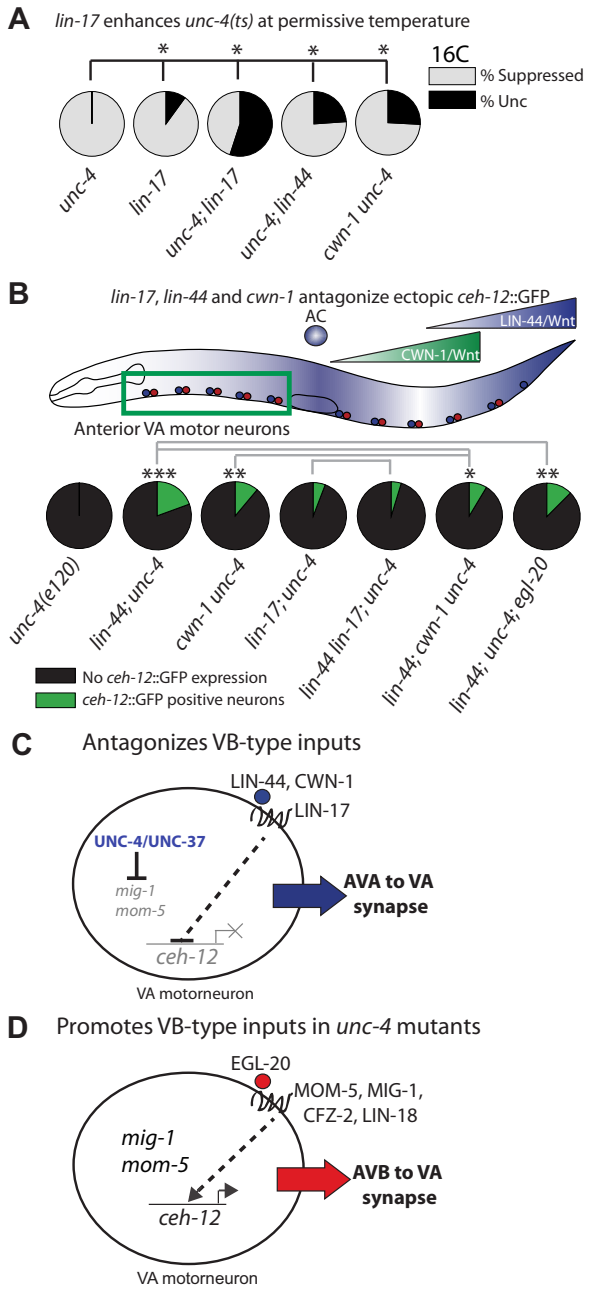

Fig. 5. *lin-17* promotes VA-type inputs in opposition to *egl-20* signaling. (**A**) *unc-4(e2322ts)* shows wild-type backward locomotion at 16°C. Mutations in *lin-17*, *lin-44* and *cwn-1* enhance Unc-4 backward movement. *lin-44* and *cwn-1* single mutants show wild-type movement (data not shown). (**B**) LIN-44 is expressed in the tail and in the anchor cell (AC); CWN-1 is expressed in posterior cells. Mutations in *cwn-1* and *lin-44* enhance ectopic *ceh-12*::GFP expression in anterior VAs in *unc-4(e120)*. ***P<0.001, **P<0.01, *P<0.05 versus *unc-4*. $n\geq16$ for each neuron. Green box denotes group of anterior VA neurons scored in B. Gray brackets denote no significant difference (P>0.05, Fisher's Exact Test) between compared strains. (**C**) LIN-44, CWN-1 and LIN-17 inhibit *ceh-12* expression to preserve AVA inputs to anterior VAs. UNC-4 and UNC-37 antagonize *mig-1* and *mom-5* activity by transcriptional repression or by an indirect mechanism involving an intermediate target gene. (**D**) EGL-20 signaling promotes the creation of AVB inputs to VAs. Elevated expression or function of MOM-5 and MIG-1 in *unc-4* and *unc-37* mutants confers sensitivity to a local EGL-20 cue that activates *ceh-12* expression and the creation of VB-type inputs. CFZ-2 and LIN-18 might also function in this pathway (Fig. 2).

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(n585)* and in *unc-4; egl-20(hu120)* 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

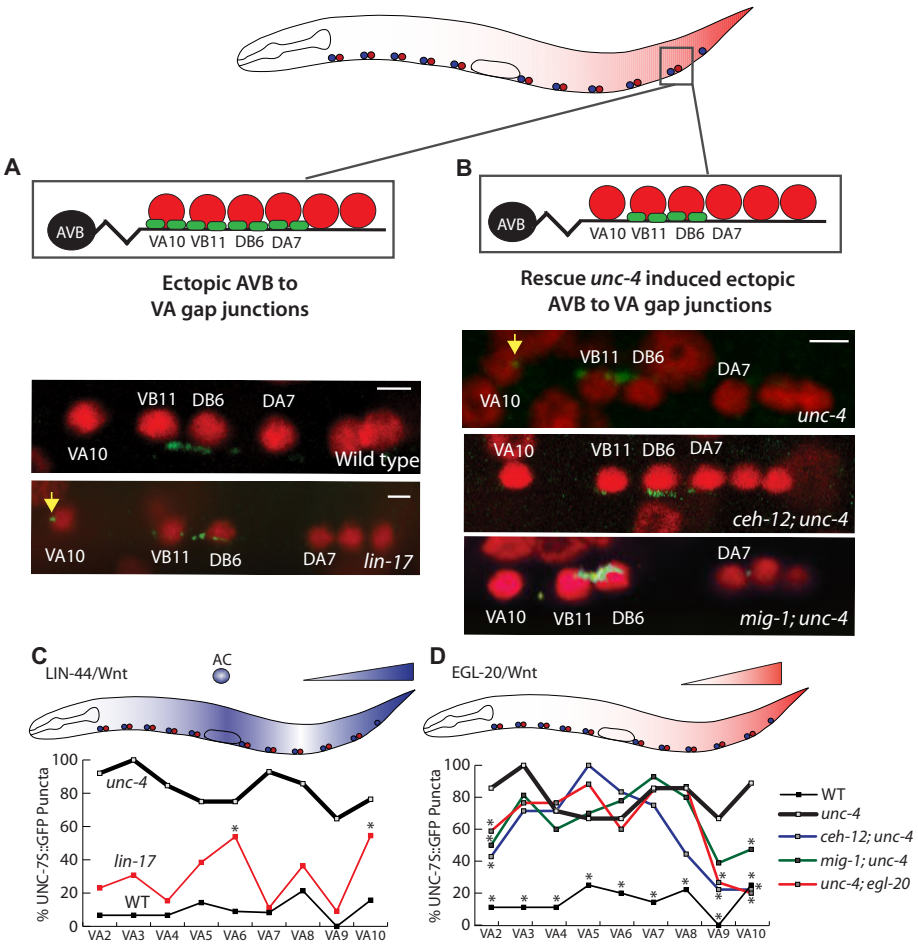

Fig. 6. Opposing Wnt pathways regulate the specificity of gap junction inputs to VA motor neurons. (**A**) AVB gap junctions (green) with the cell soma of VB and DB motor neurons (red). Mutants of *lin-17* (A) and *unc-4* (B) show ectopic AVB to VA gap junctions (yellow arrows). (**B**) AVB gap junctions 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). $n \geq 10$ 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(e2322ts)* 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 synapses 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

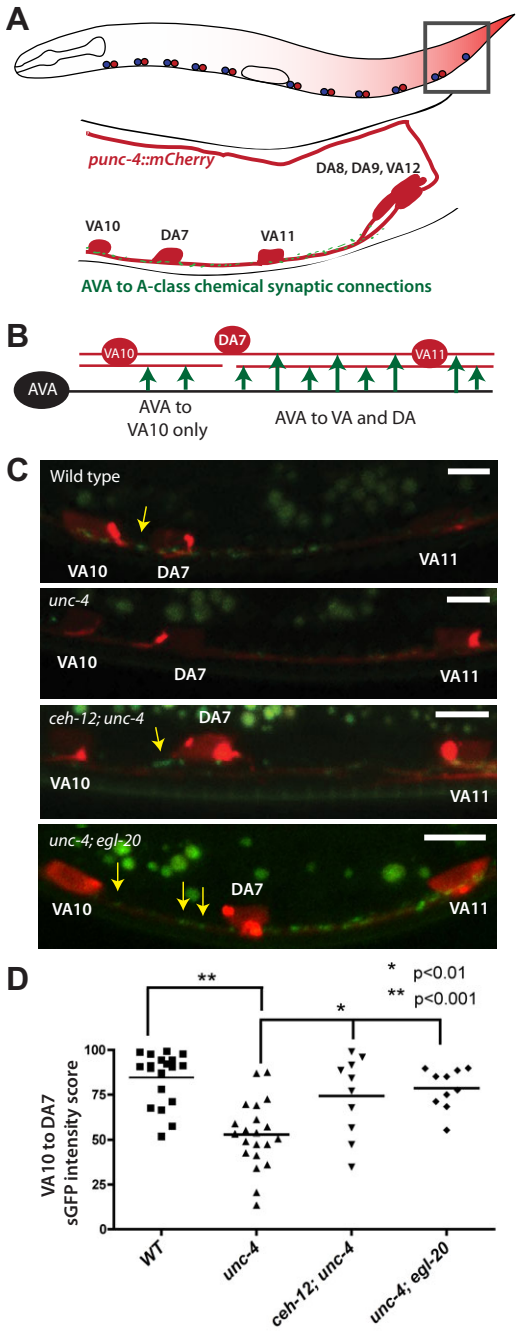

Fig. 7. GRASP markers detect chemical synapses between AVA and A-motor neuron partners. (**A**,**B**) AVA synapses with DA and VA motor neurons in wild type. Boxed area shows the posterior region between VA10 and DA7 where AVA synapses specifically with VA10 (B). (**C**) GRASP-dependent GFP puncta (yellow arrows) with VA10 (red) are reduced in *unc-4(e120)* and restored by *ceh-12(gk391)* or *egl-20(n585)*. (**D**) Intensity values of GFP puncta were quantified from line scans of this region (see Materials and methods). **P*<0.01, ***P*<0.001, analysis of variance (ANOVA). $n \geq 10$ for each neuron.

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 1α 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

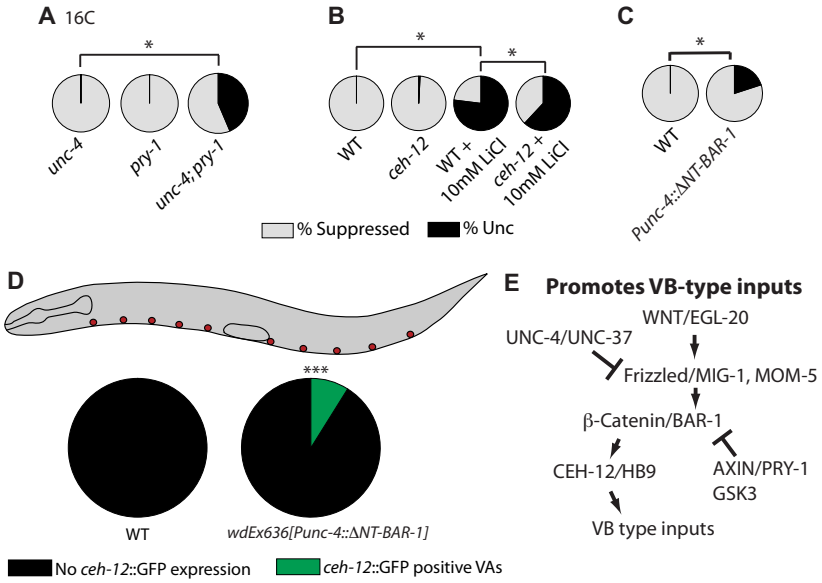

Fig. 8. Canonical Wnt signaling functions upstream of *ceh-12* in *unc-4* mutant VA motor neurons. (**A**) *pry-1(mu38)* enhances the Unc-4 backward movement phenotype of *unc-4(e2322ts)* at 16°C. **P*<0.05. *n*≥50. (**B**) The GSK-3β inhibitor LiCl induces a backward movement defect in WT animals that 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::ΔNT-BAR-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 VB-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; Sahores 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 mis-wiring 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 cell 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; Maro 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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