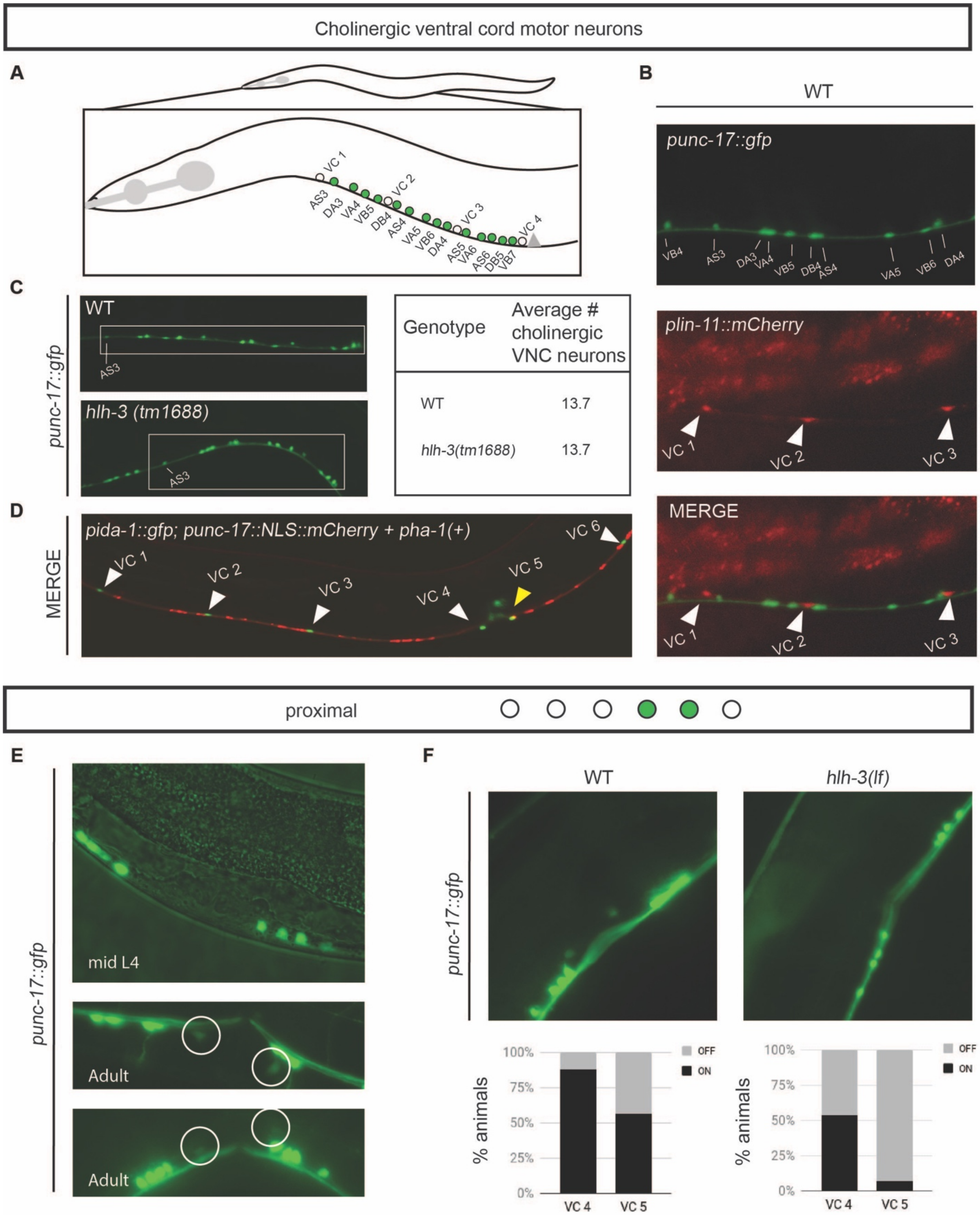


Genotype	Average # cholinergic VNC neurons
WT	13.7
hlh-3(tm1688)	13.7

Figure S1. Cholinergic, sex-shared ventral cord motor neurons differentiate normally in *hlh-3(lf)*.

A: Schematic of the number and position of cholinergic, sex-shared VNC neurons in the anterior body region, and between VC 1 and VC 4 (n = 14).

B: An annotated image of an adult WT hermaphrodite expressing *vsIs48 [punc-17::gfp]* in non-VC neurons (top panel), *plin-11::mCherry* in VCs (middle panel, filled arrowheads), and a merge of both images (bottom image). Anterior is left, ventral is down.

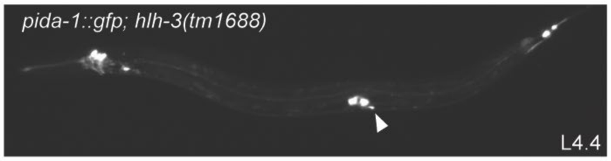
C: Quantification of number of *vsIs48 [punc-17::gfp]* positive nuclei in the anterior region of the vulva in WT (n = 10) and *hlh-3 (lf)* (n = 10) hermaphrodites. Representative images are shown on the left. The average number of positive nuclei is reported on the right for each genotype.

D: An annotated image of an adult WT hermaphrodite expressing *mdEx865 [punc-17::NLS::mCherry + pha-1 (+)]* in the proximal VC 5 (yellow arrowhead), but not other VCs. VC 5 is co-labeled with a VC marker *pida-1::gfp*. All arrowheads point to VCs. Anterior is left, ventral is down.

E: Representative images of L4 and adult WT hermaphrodites harboring the *vsIs48 [punc-17::gfp]* reporter. There is no detectable expression in mid-L4 development (top panel), but the expression is detected in adults (middle and bottom panels).

F: Quantification of *vsIs48 [punc-17::gfp]* reporter expression in proximal VCs of WT (n = 16) and *hlh-3(lf)* (n = 15) in adulthood. On = detectable, Off = undetectable.

A



B

L4 substages	VC 1	VC 2	VC 3	VC 4	VC 5	VC 6
L4.0-L4.3	0%	0%	0%	0%	0%	0%
L4.4-L4.9	5%	2%	7%	40%	67%	10%

Figure S2. *hlh-3* function is required in early L4

A: Image of an early L4 *hlh-3(lf)* hermaphrodite expressing *pida-1::gfp* only in VC 5 (white arrowhead). In WT individuals this reporter is detectable in all VCs (Figure 2) as well as the round-shaped bodies near the vulva, a pair of uv1 cells. Expression in uv1 cells is not affected in *hlh-3(lf)* individuals.

B: Quantification analysis of *pida-1::gfp* detection in each VC of *hlh-3(lf)* individuals during early L4 substages (L4.0-L4.3) (n = 14), or mid-late substages (L4.4-L4.9) (n = 48)

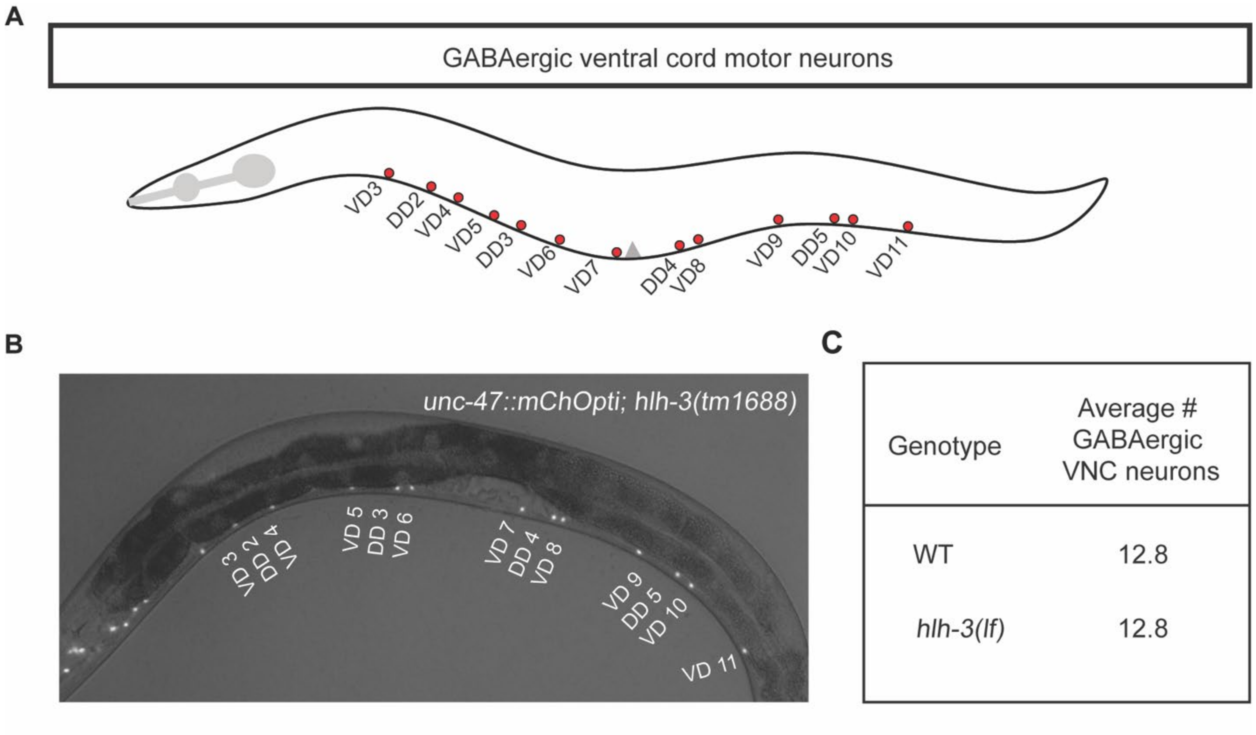


Figure S3. GABAergic, sex-shared, ventral cord motor neurons differentiate normally in *hlh-3(lf)*.

A: Illustration of the positions of the GABAergic VNC motor neurons scored (only VD 3 through VD 11 were scored, n = 13).

B: Representative image of *unc-4*7 reporter expression *otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+)]* in a *hlh-3 (lf)* mutant individual in L4 development. The gene *unc-47* encodes a vesicular GABA transporter; it marks GABAergic neurons in the VNC. Both WT and *hlh-3 (lf)* individuals express the *unc-47* marker (WT not shown).

C: Quantification of VNC neurons expressing *otIs564* reported as averages per genotype in one day old WT (n = 14) and *hlh-3 (lf)* (n = 14) hermaphrodites.

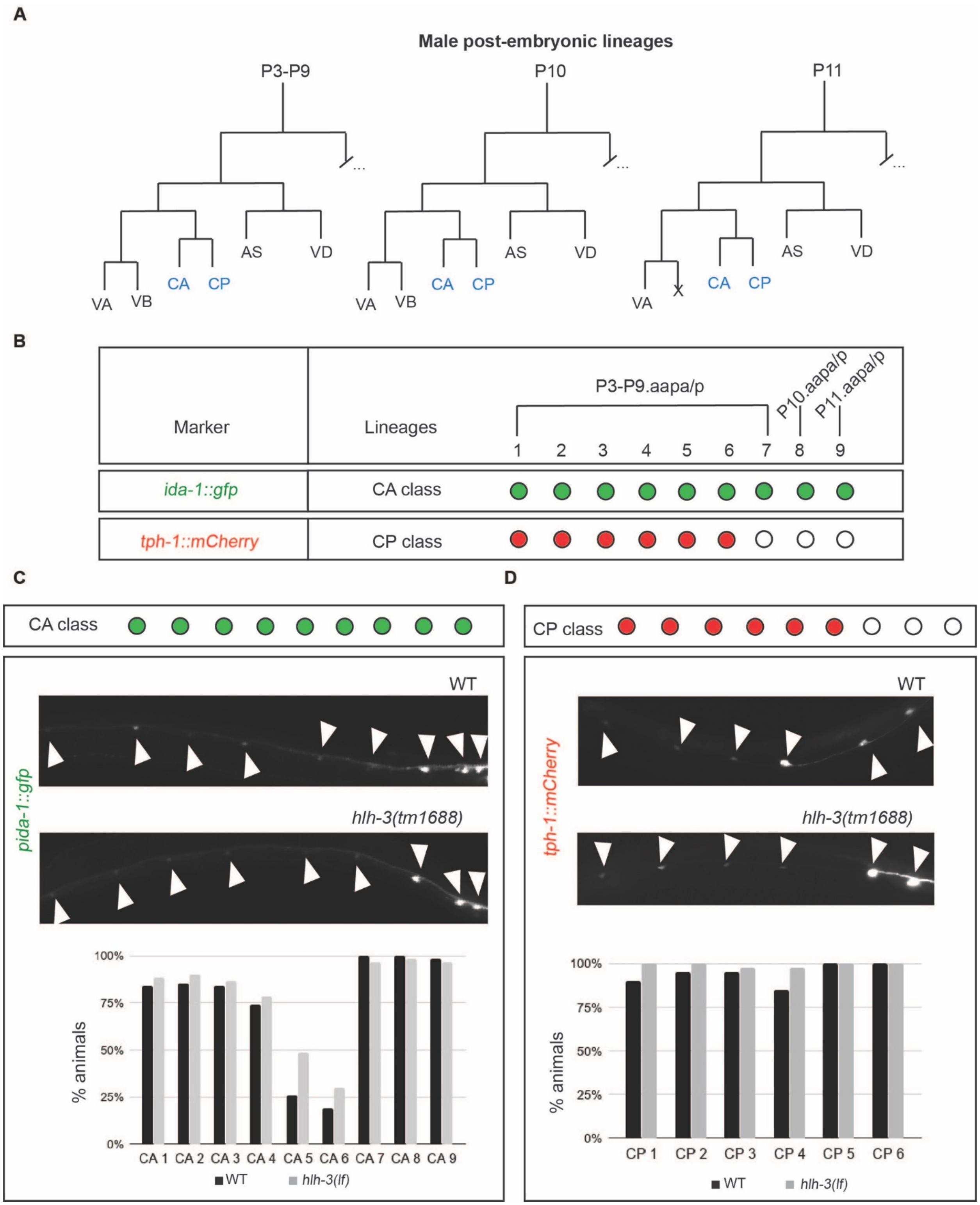

A
Male post-embryonic lineages
P3-P9
P10
P11
VA
VB
CA
CP
AS
VD
X
B
Marker
Lineages
P3-P9.aapa/p
P10.aapa/p
P11.aapa/p
1 2 3 4 5 6 7 8 9
ida-1::gfp
CA class
tph-1::mCherry
CP class
C
CA class
WT
hlh-3(tm1688)
pida-1::gfp
% animals
100%
75%
50%
25%
0%
CA 1 CA 2 CA 3 CA 4 CA 5 CA 6 CA 7 CA 8 CA 9
WT
hlh-3(lf)
D
CP class
WT
hlh-3(tm1688)
tph-1::mCherry
% animals
100%
75%
50%
25%
0%
CP 1 CP 2 CP 3 CP 4 CP 5 CP 6
WT
hlh-3(lf)

Figure S4. The differentiation of the male-specific ventral cord motor neurons derived from P cells is not affected by the absence of *hlh-3* function

A: Diagram of post-embryonic lineages in the ventral nerve cord that gives rise to CA and CP male-specific neurons. Notably, P2.a divisions give rise to CP0 but are not shown (adapted from Sulston *et al.* 1980).

B: Summary of the expression pattern of *ida-1::gfp* and *tph-1::mCherry* in CAs and CPs, respectively (based on data from Kalis *et al.* 2014; Loer and Kenyon 1993).

C: Quantification of expression of *pida-1::gfp* in the adult male ventral cord of wild type and mutant individuals as one day old adults, synchronized as L4s the day before. Arrowheads point to CAs. Representative fluorescent images for each genotype (top). Graph reports the percent of animals with detectable expression in each cell of WT (n = 71) and *hlh-3 (lf)* (n = 61) males.

D: Quantification of expression of *ptph-1::mCherry* expression in the adult male ventral cord of wild type and mutant individuals as one day old adults, synchronized as L4s the day before. Arrowheads point to CPs. Representative fluorescent images for each genotype (top). Graph reports the percent of animals with detectable expression in each cell of WT (n = 20) and *hlh-3 (lf)* (n = 41) males.

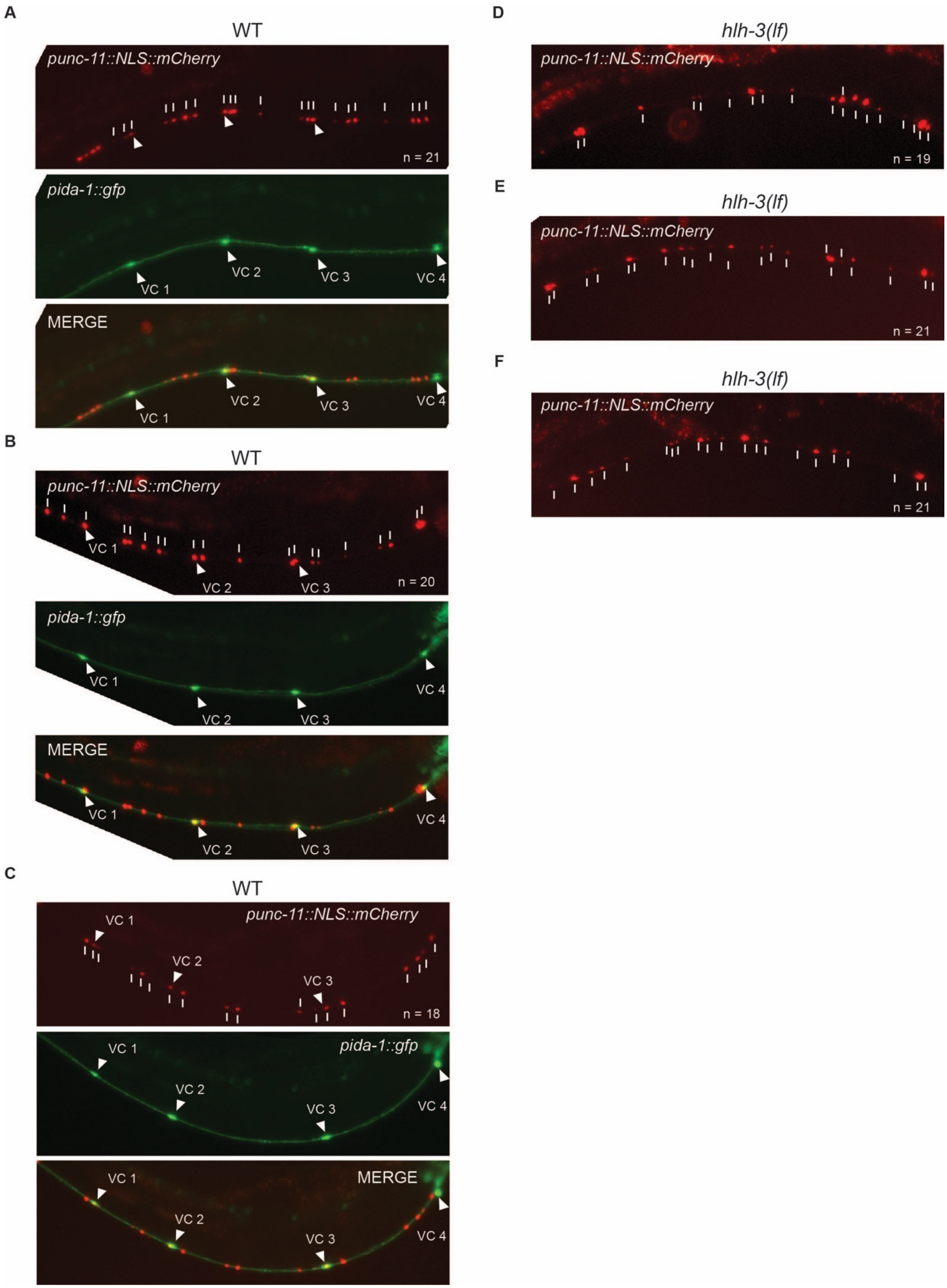

A
WT
punc-11::NLS::mCherry
n = 21
pida-1::gfp
VC 1
VC 2
VC 3
VC 4
MERGE
VC 1
VC 2
VC 3
VC 4
B
WT
punc-11::NLS::mCherry
VC 1
VC 2
VC 3
n = 20
pida-1::gfp
VC 1
VC 2
VC 3
VC 4
MERGE
VC 1
VC 2
VC 3
VC 4
C
WT
punc-11::NLS::mCherry
VC 1
VC 2
VC 3
n = 18
pida-1::gfp
VC 1
VC 2
VC 3
VC 4
MERGE
VC 1
VC 2
VC 3
VC 4
D
hlh-3(lf)
punc-11::NLS::mCherry
n = 19
E
hlh-3(lf)
punc-11::NLS::mCherry
n = 21
F
hlh-3(lf)
punc-11::NLS::mCherry
n = 21

Figure S5. Pan-neuronal expression in the anterior ventral cord with and without *hlh-3* function

A-C: One-day-old WT individuals (n = 3) express both a pan-neuronal marker *unc-11^{promoter8}::NLS::mCherry* and *pida-1::gfp*. Only the anterior half of the body is shown. The total number of ventral cord motor neurons expressing the pan-neuronal marker was quantified from the region containing VC 1 through VC 4 and is denoted at the bottom right of the first panel in each series.

D-F: One-day old *hlh-3(lf)* individuals (n = 3) express the pan-neuronal marker *unc-11^{promoter8}::NLS::mCherry*. Only the anterior half of the body is shown. The total number of ventral cord motor neurons expressing the pan-neuronal marker was quantified from the region containing VC 1 through VC 4, denoted at the bottom right of the first panel. Since *hlh-3(lf)* animals rarely express the VC reporter *pida-1::gfp* in distal VCs, we did not include the *pida-1::gfp* VC reporter in this strain.

Strain	Genotype
AL166	*inIs179 [pida-1prom::gfp] II ; him-8(e1489) IV ; hlh-3(tm1688) II*
AL184	*vsIs48 [punc-17::gfp; him-5(e1490) V*
AL195	*vsIs48 [unc-17::gfp; him-5(e1490) V; hlh-3(tm1688) II*
AL262	*cccIs1 [tph-1::mCherry]; wgIs18 [lin-39::TY1::EGFP::3xFLAG + unc-119(+)]; hlh-3(tm1688)II*
AL270	*icIs270 [pglr-5::gfp + lin-15(+)]*
AL273	*hlh-3(tm1688) II ; icIs270 [pglr-5::gfp + lin-15(+)]*
AL281	*uIs45 [punc-4::MDM2::GFP + rol-4(+)]; hlh-3(tm1688) II*
AL284	*icIs270 [pglr-5::gfp]; ced-3(n717), unc-26(e205) IV; hlh-3(tm1688) II*
AL287	*icIs270 [pglr-5::gfp]; ced-3(n717), unc-26(e205) IV*
AL303	*otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+); him-5(e1490); him-5(e1490) V hlh-3(tm1688) II*
AL325	*hlh-3(tm1688) II; mjIs27 [mir-124p::gfp + lin-15(+)]*
AL331	*hlh-3(ic271[hlh-3::gfp]) II*
AL338	*hlh-3(tm1688) II; otIs456 [plin-11::mCherry; pmyo-2::GFP]*
AL341	*otIs456 [plin-11::mCherry; pmyo-2::GFP]*
AL346	*hlh-3(tm1688) II; otIs456 [plin-11::mCherry; pmyo-2::GFP]; icEx274 [VC::hlh-3cDNA::GFP]*
AL347	*inIs179 [pida-1prom::gfp] II; otIs619 [punc-11*^{promoter8}*::NLS::mCherry]*
AL348	*hlh-3(ic271[hlh-3::gfp]) II; otIs456 [plin-11::mCherry; pmyo-2::GFP]*
AL349	*hlh-3(tm1688) II; otIs619 [punc-11*^{promoter8}*::NLS::mCherry]*
AL360	*inIs179 [pida-1::gfp] II; mdEx865 [unc-17p::NLS::mCherry + pha-1(+)]*
BL5717	*inIs179 [pida-1::gfp] II; him-8(e1489) IV*
OH11954	*otIs456 [plin-11::mCherry; pmyo-2::GFP]*
OH13105	*otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+)]; him-5(e1490) V*
SX621	*lin-15B&lin-15A(n765) X; mjIs27 [mir-124p::gfp + lin-15(+)]*
Tu3067	*uIs45 [punc-4::MDM2::GFP + rol-4(+)]*
JRW29	*cccIs1 [tph-1::mCherry]; wgIs18 [lin-39::TY1::EGFP::3xFLAG + unc-119(+)]*

Supplemental Table 1. List of strains.

LITERATURE CITED

Kalis, A. K., D. U. Kissiov, E. S. Kolenbrander, Z. Palchick, S. Raghavan *et al.*, 2014 Patterning of sexually dimorphic neurogenesis in the *Caenorhabditis elegans* ventral cord by Hox and TALE homeodomain transcription factors. Developmental Dynamics 243: 159–171.

Loer, C. M., and C. J. Kenyon, 1993 Serotonin-Deficient Mutants and Male Mating Behavior in the Nematode *Caenorhabditis elegans*. The Journal of Neuroscience 13: 5407–5417.

Sulston, J. E., D. G. Albertson, and J. N. Thomson, 1980 The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. Developmental Biology 78: 542–576.