Figures and figure supplements

A cellular and regulatory map of the cholinergic nervous system of *C. elegans*

Laura Pereira *et al*

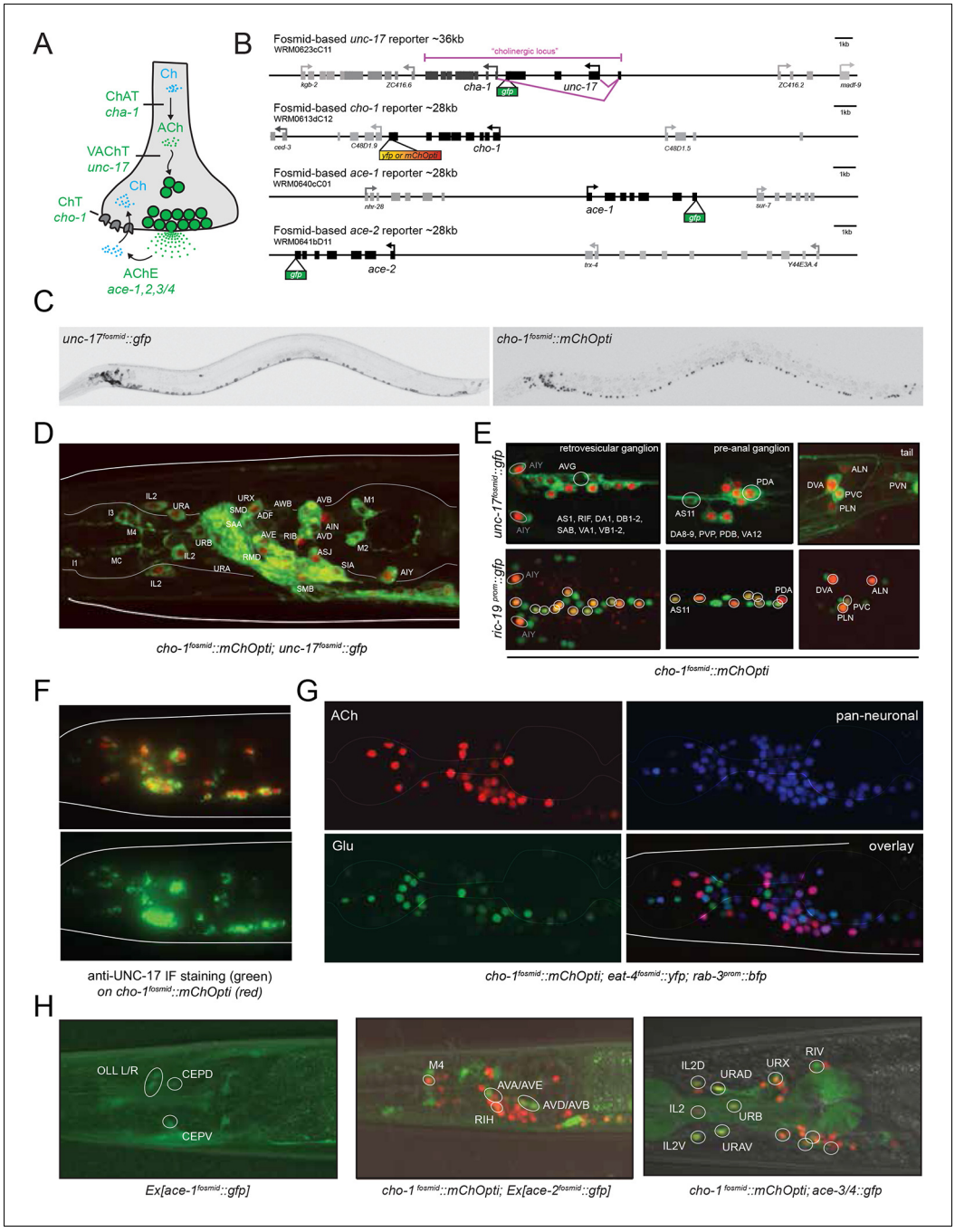

Figure 1. Expression of cholinergic pathway genes in the adult *C. elegans* hermaphrodite. (**A**) Cholinergic pathway genes. Ch = choline; ACh = acetylcholine; ChAT = choline acetyltransferase; VAChT = vesicular ACh transporter, AChE = ACh esterase, ChT = choline transporter. (**B**) Fosmid reporters used in this study. The *unc-17* fosmid reporter was kindly provided by the TransgeneOme project (***Sarov et al., 2012***). It was previously reported that the expression of *unc-17/VAChT* and *cha-1/ChAT* overlap completely (***Mathews et al., 2015***). (**C**) *unc-17* and *cho-1* fosmid reporter expression in an L4 hermaphrodite. The fluorescent reporter inserted into the *cho-1* locus is targeted to the nucleus (see Materials and methods), while the fluorescent reporter inserted into the *unc-17* locus is fused directly to the *unc-17* gene (resulting in cytoplasmic localization). (**D, E**) *unc-17* and *cho-1* fosmid reporter expression in head (**D**), retrovesicular ganglion and tail ganglia (**E**). In (**E**) bottom panels, neurons are labeled with a green pan-neuronal marker, *ric-19*. Transgenes: *otIs576* = *unc-17* fosmid reporter; *otIs544* = *cho-1* fosmid reporter, *otIs380* = *ric-19* reporter (***Stefanakis et al., 2015***). (**F**) Immunofluorescent staining for endogenous UNC-17 protein of *unc-104(e1265)* animals that express the *cho-1* fosmid reporter transgene *otIs544*. (**G**) Co-labeling

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cholinergic (*cho-1/ChT*-positive) and glutamatergic (*eat-4/VGLUT*-positive) neurons illustrate no overlap in neurotransmitter ACh and Glu expression, and co-labeling with pan-neuronal marker *rab-3* illustrates that most neurons now have a neurotransmitter assignment. Transgenes: *otIs544* = *cho-1* fosmid reporter, *otIs388* = *eat-4* fosmid reporter (***Serrano-Saiz et al., 2013***), *otIs355* = *rab-3 reporter*. (**H**) *ace/AChE* genes are expressed in a subset of cholinergic neurons and in non-cholinergic neurons. *ace-1 fosmid* reporter expression in head neurons (left panel). *ace-2* fosmid reporter expression in head neurons together with *cho-1* fosmid reporter (middle panel). *ace-3/4* reporter expression together with *cho-1* fosmid reporter in head neurons (right panel). Transgenes: *otEx4435* = *ace-1* fosmid reporter; *otEx4431* = *ace-2* fosmid reporter; *fpIs1* = *ace-3/4* transcriptional reporter.
DOI: 10.7554/eLife.12432.004

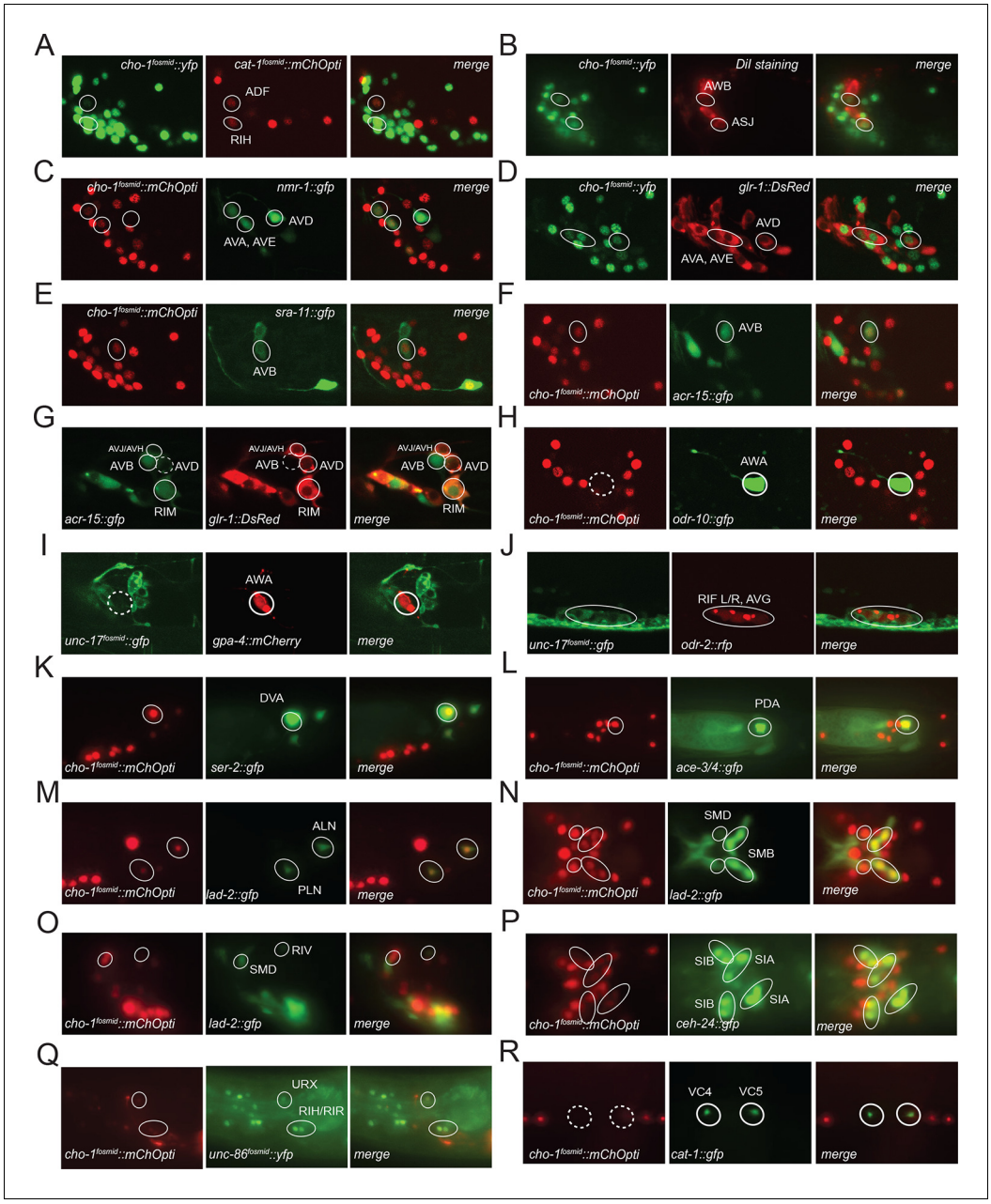

Figure 1—figure supplement 1. Neuronal cell identification. Neuronal identity was confirmed by crossing *cho-1* fosmid reporter and/or *unc-17* fosmid reporter with specific markers. (**A**) ADF and RIH were labeled by *cho-1 (otIs354)* and *cat-1 (otIs625)*. (**B**) ASJ and AWB were labeled by *cho-1 (otIs354)* and DiI staining. (**C**) AVA, AVE and AVD were labeled by *cho-1 (otIs544)* and *nmr-1 (akIs3)*. (**D**) AVA, AVE and AVD were also labeled by *cho-1 (otIs544)* and *glr-1 (hdIs30)*. (**E**) AVB was labeled by *cho-1 (otIs544)* and *acr-15 (wdEx290)*. (**F**) AVB was also labeled by *cho-1 (otIs544)* and *sra-11 (otIs123)*. (**G**) AVB was not labeled by *glr-1(hdIs30)* as had been previously published (***Brockie et al., 2001***). (**H**) AWA was labeled by *odr-10 (kyIs37)* but did not show *cho-1 (otIs544)* expression. (**I**) AWA was labeled by *gpa-4 (otEx6381)* but did not show *unc-17 (otIs576)* expression. (**J**) AVG and RIF were labeled by *cho-1 (otIs544)* and *odr-2 (otEx4452)*. (**K**) DVA was labeled by *ser-2 (otIs358)* and *cho-1 (otIs544)*. (**L**) PDA was labeled by *cho-1 (otIs544)* and *ace-3/4 (fpIs1)*. (**M**) ALN and PLN were labeled by *cho-1 (otIs544)* and *lad-2 (otIs439)*. (**N**) SMB and SMD were labeled by *cho-1 (otIs544)* and *lad-2 (otIs439)*. (**O**) SMD and RIV were labeled by *cho-1 (otIs544)* and *lad-2 (otIs439)*. (**P**) SIA and SIB were labeled by *cho-1 (otIs544)* and *ceh-24 (ccIs4595)*. (**Q**) URX, RIR and RIH were labeled by *cho-1 (otIs544)* and *unc-86 (otIs337)*. (**R**) VC4 and VC5 were labeled by *cat-1 (otIs221)* but not by *cho-1 (otIs544)*.

DOI: 10.7554/eLife.12432.005

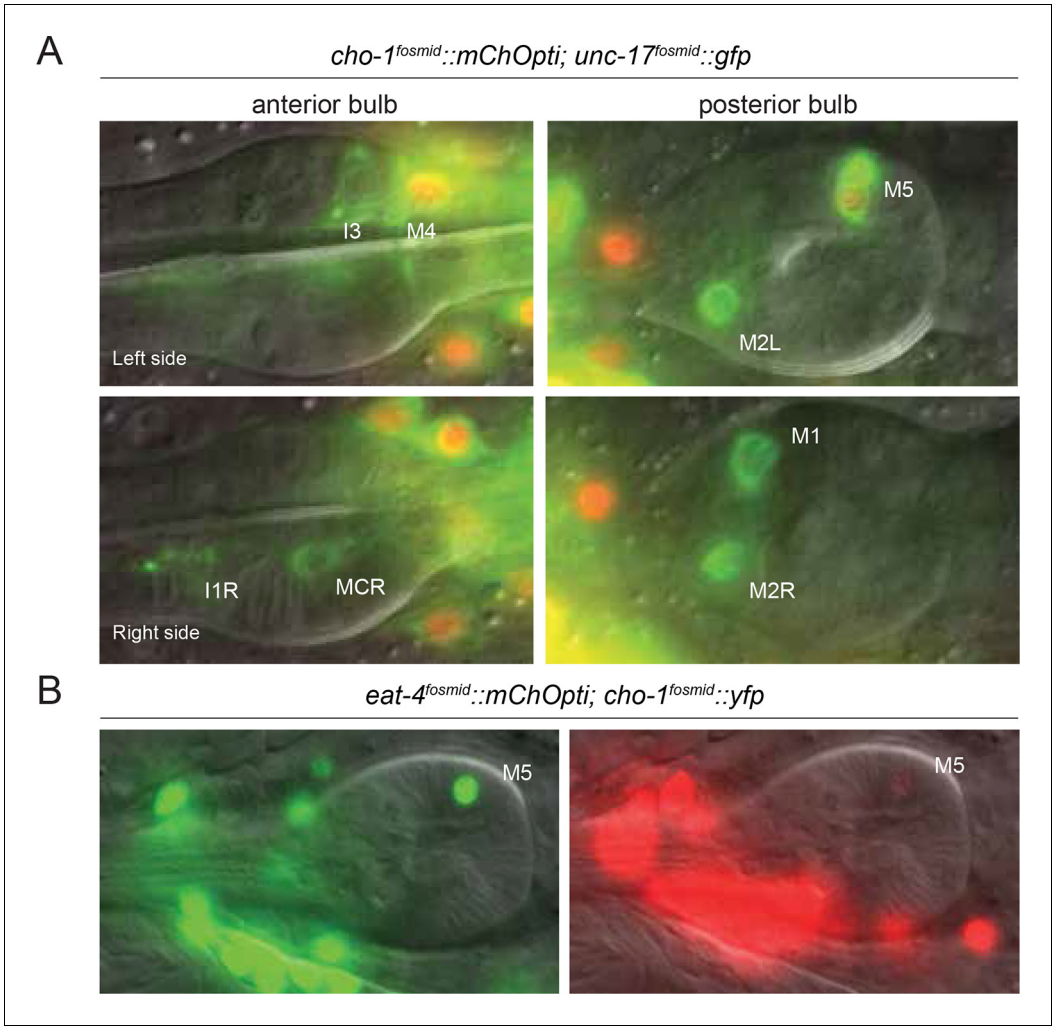

Figure 1—figure supplement 2. Neurotransmitter identity of pharyngeal neurons. (**A**) The different panels show the expression of *unc-17 (otIs576)* and *cho-1 (otIs544)* fosmids in the pharyngeal neurons in the anterior and posterior bulbs. Only M4 and M5 express both fosmids. Schematic for the cholinergic pharyngeal neurons is shown. (**B**) Expression of *eat-4 (otIs518)* and *cho-1 (otIs344)* fosmids in the pharyngeal neuron M5.
DOI: 10.7554/eLife.12432.006

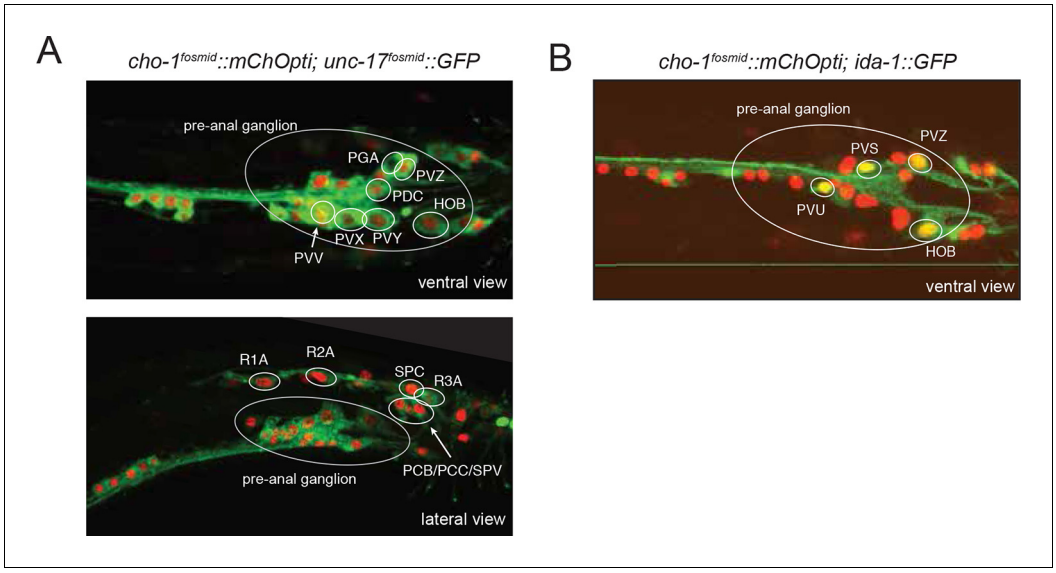

Figure 1—figure supplement 3. Expression of *unc-17* and *cho-1* fosmid reporters in the male tail. (**A**) The top panel shows the male pre-anal ganglion on a ventral view and the bottom panel shows the pre-anal ganglion and tail neurons in a lateral view. (**B**) Male tail ventral view where PVS, PVU and the male-specific neurons PVZ and HOB were labeled by *cho-1* fosmid and *ida-1::gfp* reporter. Transgenes: *otIs576* = *unc-17* fosmid reporter; *otIs544* = *cho-1* fosmid reporter; *inIs179* = *ida-1* reporter.

DOI: 10.7554/eLife.12432.007

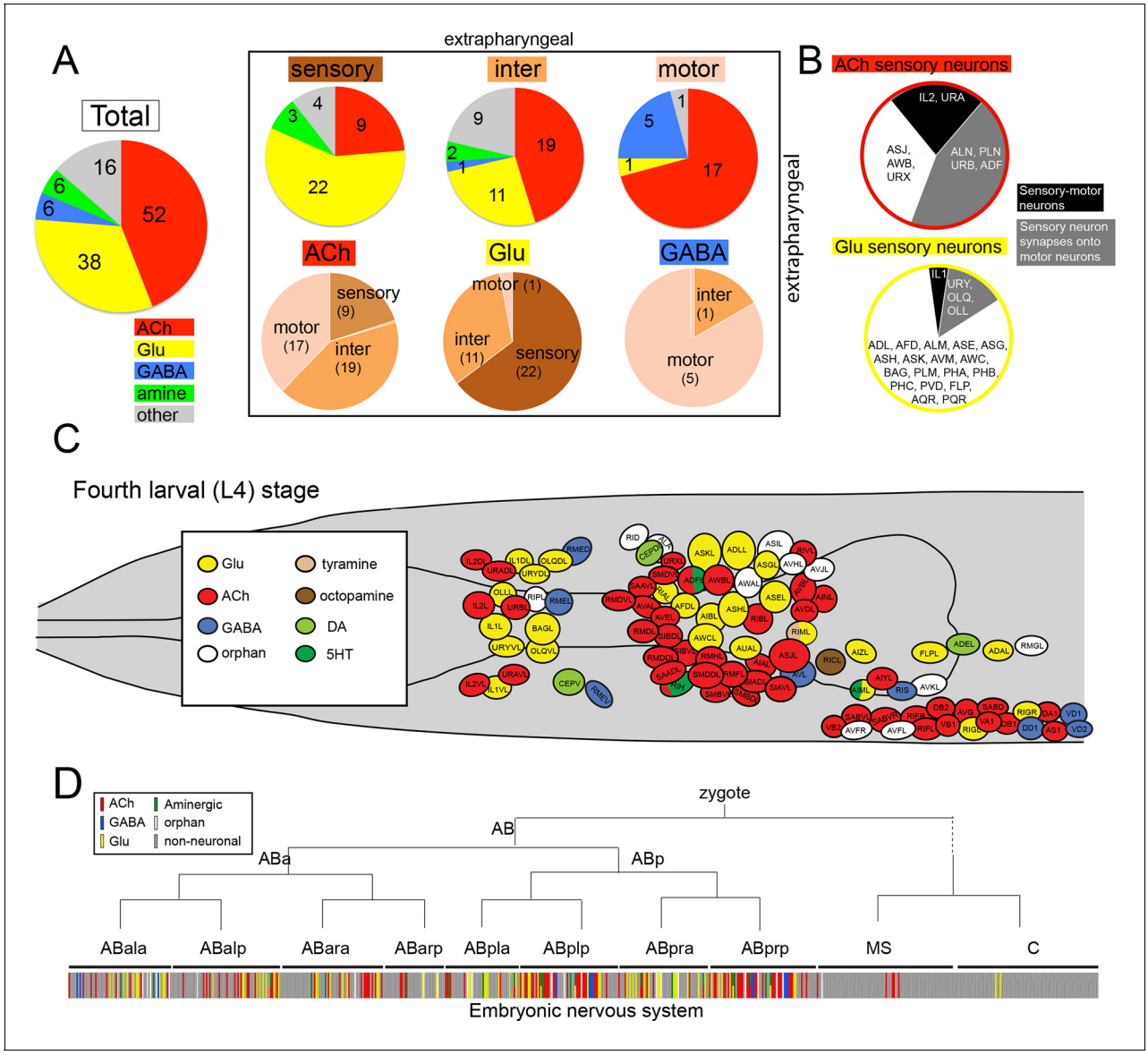

Figure 2. Distribution of neurotransmitters throughout the nervous system of the hermaphrodite. (**A**) Pie chart with numbers/distributions of cholinergic (this study), glutamatergic (***Serrano-Saiz et al., 2013***), GABAergic (***McIntire et al., 1993***) and aminergic (***Chase and Koelle, 2007***) neurons (including pharyngeal neurons). Inset: Pie charts of extrapharyngeal sensory, motor- and interneurons. Neurons that contain a classic fast transmitter plus an aminergic transmitter (e.g. RIH) are counted in the fast transmitter category. Classification of *C. elegans* neurons into sensory, inter- and motor neurons is complicated by the fact that a subset of sensory neurons are also motor neurons, i.e. synapse directly onto muscle (we count those neurons here only as sensory neurons). Conversely, a large number of motor neurons also extensively synapse onto other motor neurons or interneurons and hence classify as 'interneuron' as well; these neurons are shown exclusively in the motor neuron category. A number of neurons that were originally assigned as 'interneurons' by John White and colleagues are now considered motor neurons (because of the more recent identification of NMJs; e.g. SIA, SIB, SAB neurons), or are considered sensory neurons (because of their position in connectivity diagrams or expression of molecular markers; e.g. URA, URB, URXY, URY). See ***Table 2*** for a complete list of neurons and their neurotransmitter assignment. Lastly, we note that unpublished results from our lab demonstrate that at least two additional interneurons, not shown here, utilize GABA (M. Gendrel and O.H., unpubl. data). (**B**) Distance of sensory neurons to motor output (processing depth) of cholinergic and glutamatergic sensory neurons. (**C**) Location of neurons with different neurotransmitter identities in the head ganglia. (**D**) Neurotransmitter identity does not track with lineage history. Neurotransmitter identity is superimposed on the embryonic lineage diagram (***Sulston et al., 1983***), with each color line indicating one neuron type with a defined identity. White lines indicate no known neurotransmitter identity, gray lines indicate non-neuronal cells. Lines with two colors illustrate co-transmitter identities.

DOI: 10.7554/eLife.12432.008

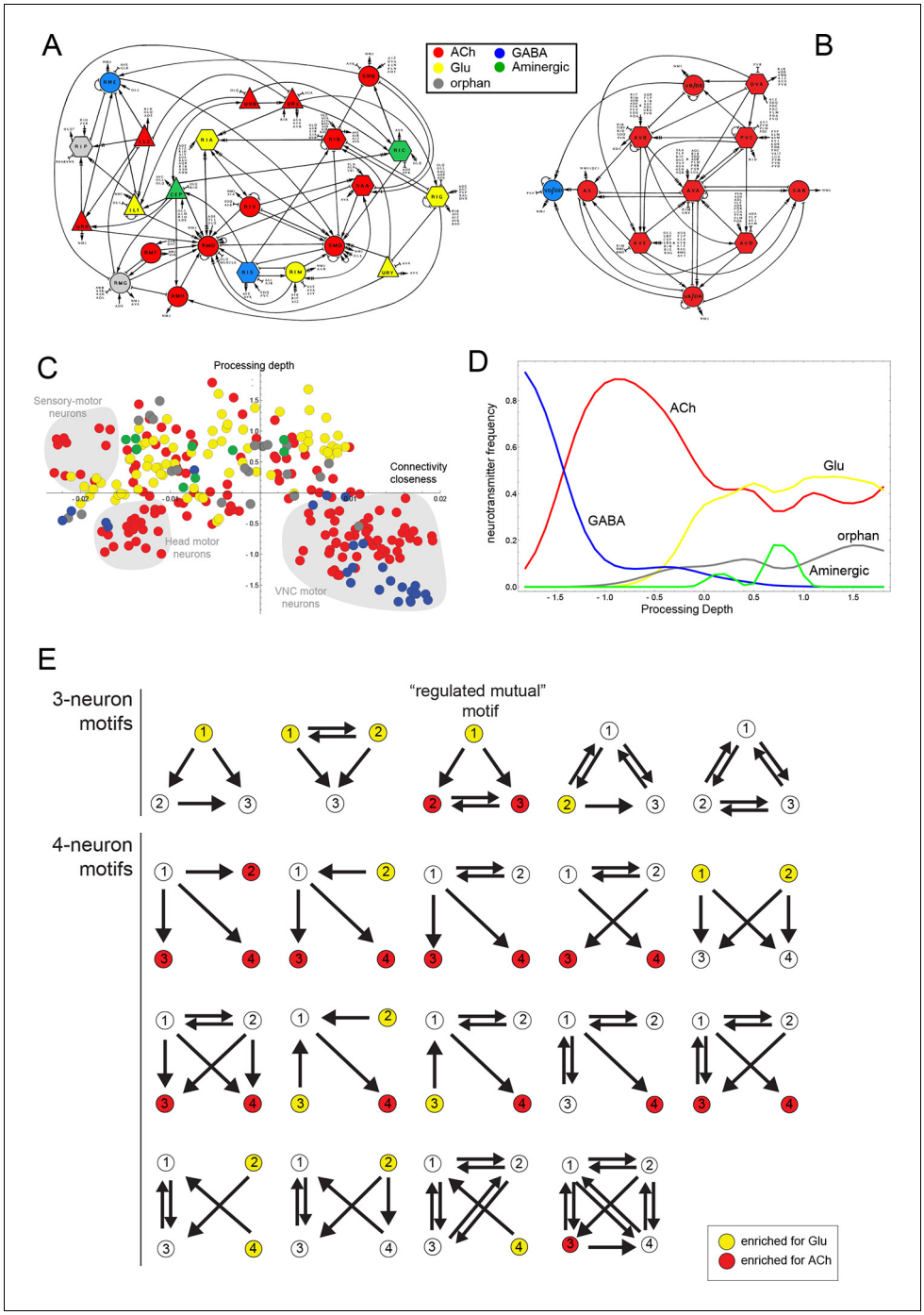

Figure 3. Neurotransmitter distribution in nervous system-wide circuit diagrams. (**A, B**) Circuit diagrams, taken from ***White et al. (1986)***, with neurotransmitter identities added in colors, as indicated. Panel A shows what White et al. called the "Circuitry associated with motoneurons in the nerve ring" and panel B shows the "Circuitry associated with the motoneurons of the ventral cord". (**C**) A visualization of the *C. elegans* connectome that reflects signal flow through the network as well as the closeness of neurons in the network, as previously proposed and described (***Varshney et al., 2011***). Coordinates from the diagram were kindly provided by Lav Varshney. The vertical axis represents the signal flow depth of the network, i.e. the number of synapses from sensory to motor neurons. The horizontal axis represents connectivity closeness. We superimpose here neurotransmitter identity onto this network diagram, illustrating some network cluster enriched for ACh usage (shaded gray). (**D**) A graphic representation that focuses on processing depth, illustrating whether a neurotransmitter is used more frequently in upper (sensory) or lower (motor) layers of the network. (**E**) Network motifs enriched in the *C. elegans* connectome

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and their neurotransmitter usage. Colors indicate if the neurons in this position are enriched for the usage of Glu or ACh.

DOI: 10.7554/eLife.12432.012

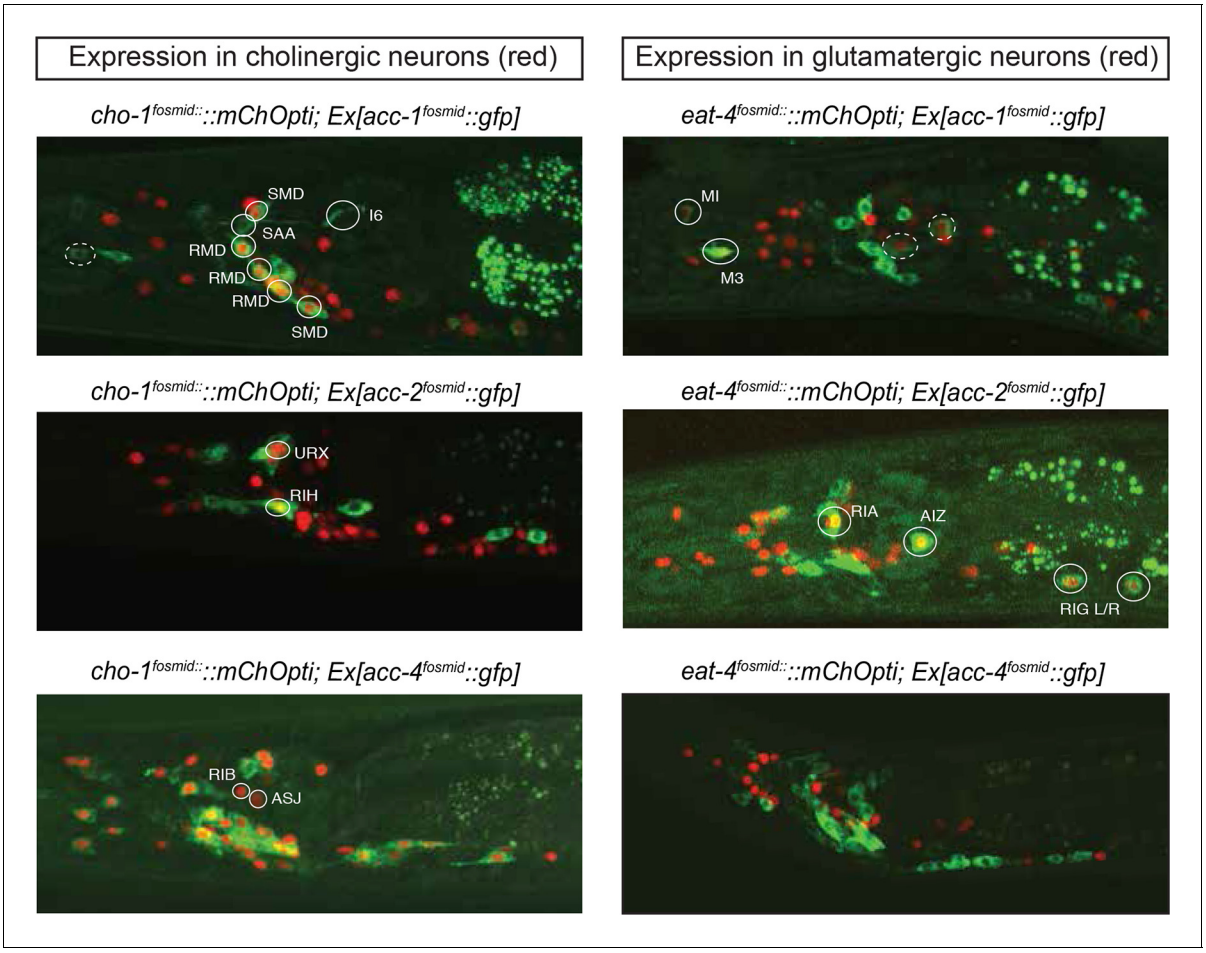

Figure 4. Expression pattern of ACh-gated chloride channels. Expression pattern of *acc* fosmid reporters in L4 stage animals are shown. Transgenes: *otEx6374* = *acc-1* fosmid reporter; *otEx6375* = *acc-2* fosmid reporter; *otEx6376* = *acc-4* fosmid reporter; *otIs545* = *cho-1* fosmid reporter; *otIs518* = *eat-4* fosmid reporter. Besides the neurons shown here, *acc-1* and *acc-2* are expressed in a small number of additional neurons (not shown).
DOI: 10.7554/eLife.12432.014

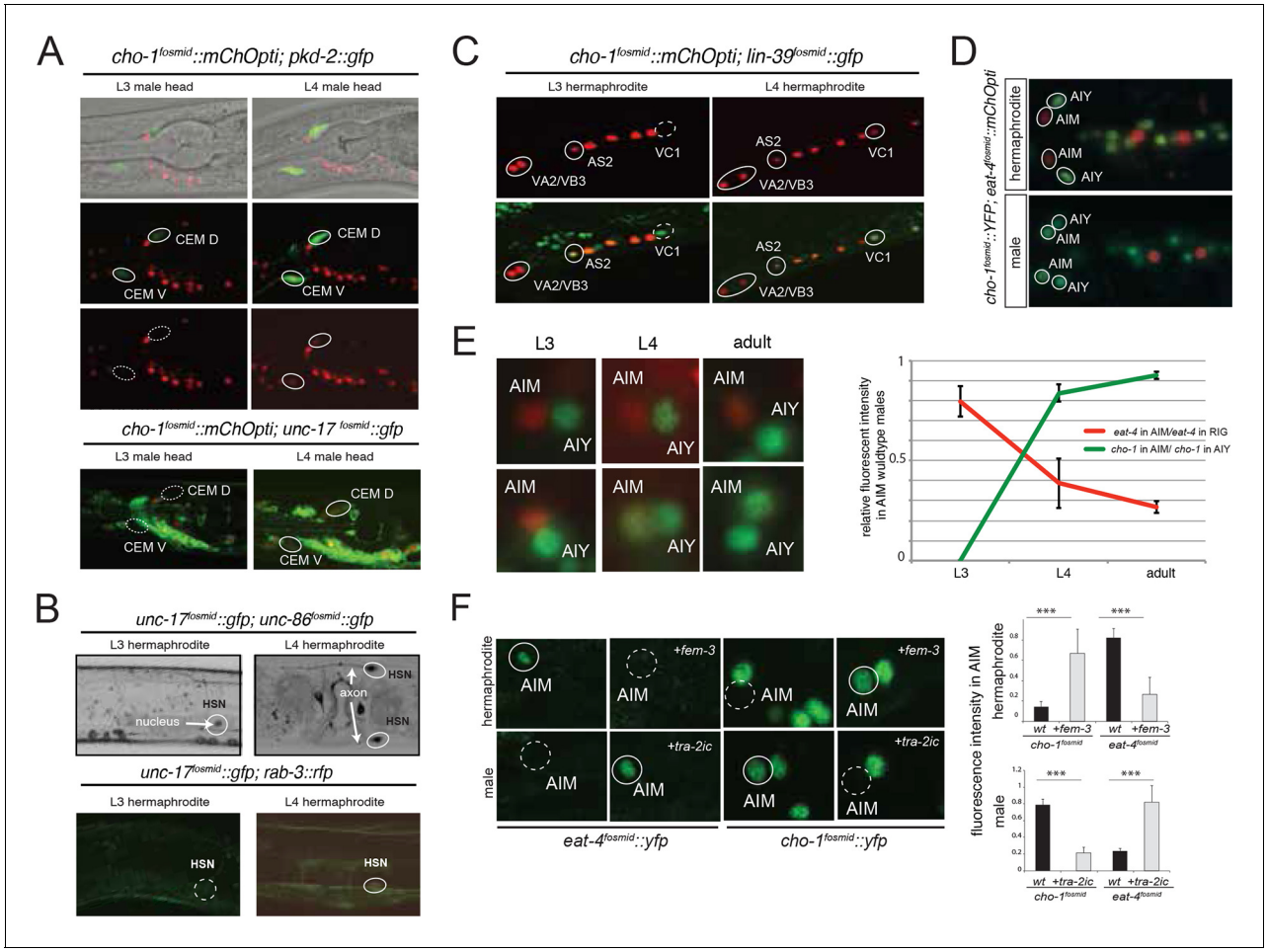

Figure 5. Sexual and temporal dynamics of cholinergic identity. (**A**) Male-specific CEM neurons are cholinergic, but turn on *cho-1* (*otIs544*) and *unc-17* (*otIs576*) only in late L4. In the top panels CEM neurons are labeled by the *pkd-2* reporter (*bxIs14*). See ***Figure 1—figure supplement 3*** and ***Table 5*** for a list of all male-specific cholinergic neurons. (**B**) Hermaphrodite-specific HSN neurons turn on the cholinergic marker *unc-17* and pan-neuronal *rab-3* also in late L4. HSN neurons are labeled by a nuclear localized *unc-86* fosmid reporter (*otIs337*). At L4 and later stages, *unc-17* fosmid expression (*otIs576*) becomes apparent in both soma and axon (top panels). The expression of the pan-neuronal marker *rab-3* (*otIs355*) is also first observed in late L4 (bottom panels). (**C**) Hermaphrodite-specific VC neurons turn on *unc-17* and *cho-1* only in late L4 (note that *cho-1* is NOT in VC4/5); this is later than the onset of the same genes in VA and VB neurons (VA, VB and VC neurons are labeled with the HOX gene *lin-39*). Transgenes: *wgIs18 = lin-39 fosmid reporter; otIs544 = cho-1 fosmid reporter*. (**D**) Sexually dimorphic neurotransmitter identity of a sex-shared neuron class. The AIM neuron expresses *cho-1* (and *unc-17*; not shown) in adult males, but expresses *eat-4/VGLUT* instead in hermaphrodites Transgenes: *otIs354 = cho-1* fosmid reporter; *otIs518 = eat-4* fosmid reporter. (**E**) Sexually dimorphic neurotransmitter switch. Until the L3 stage, both male and hermaphrodite AIM neurons are glutamatergic (express *eat-4/VGLUT*). While hermaphrodites continue to express *eat-4*, males downregulate *eat-4* and turn on *cho-1* (and *unc-17*; not shown). (**F**) The neurotransmitter switch is cell-autonomously controlled by the sex-determination pathway. In the upper panels, the masculinizing *fem-3* gene is force-expressed in the AIM neurons (with the *eat-4prom11* driver) in otherwise hermaphroditic animals; in the lower panels, the masculinizing *tra-2* intracellular domain ('*tra-2ic*') is expressed in AIM neurons of the male. Quantification is provided on the right.

DOI: 10.7554/eLife.12432.015

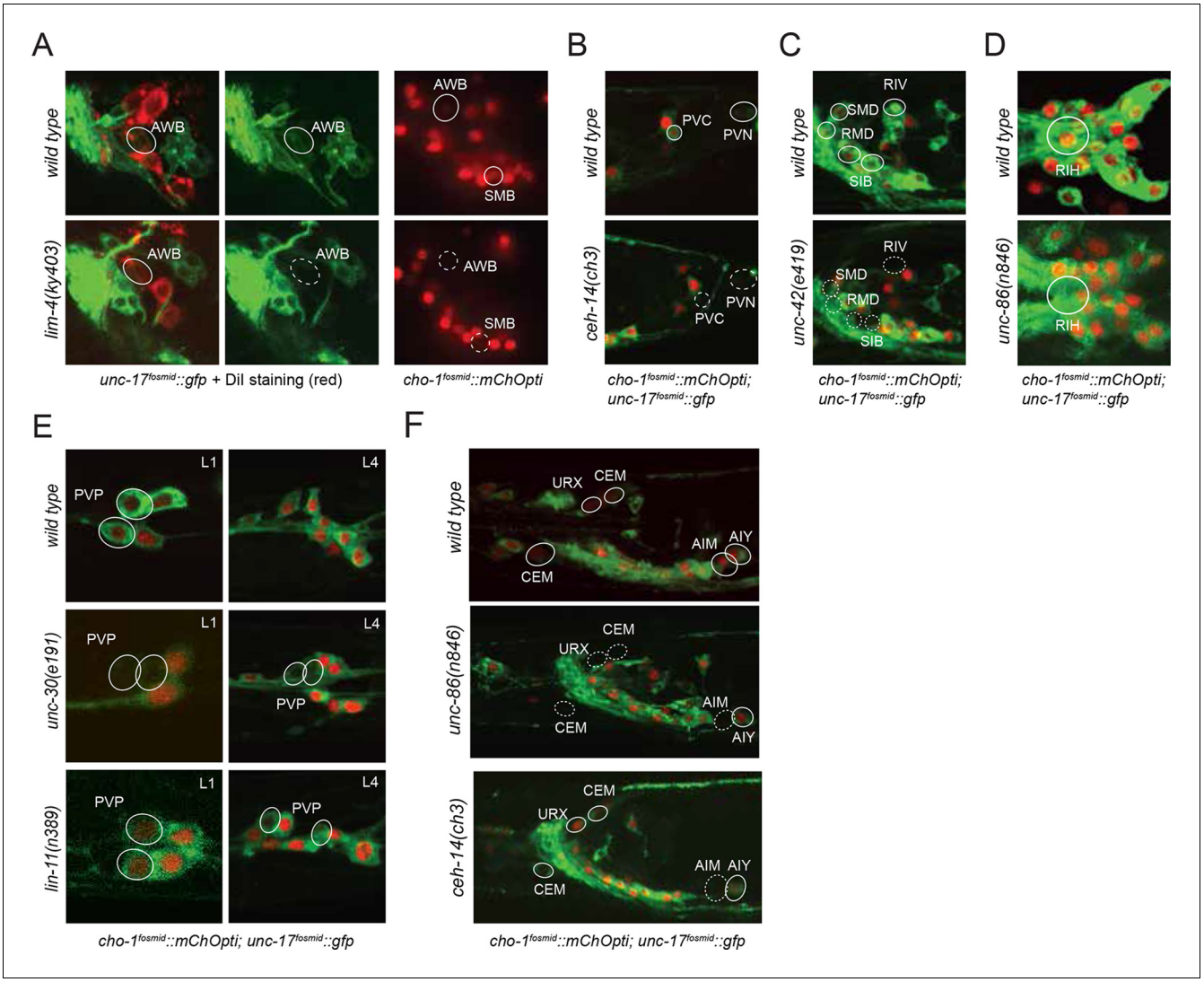

Figure 6. Regulatory factors affecting cholinergic identity. We examined 20 animals for each genotype and for every mutant strain the described phenotype was observed in >80% of animals. (**A**) The LIM homeobox transcription factor *lim-4* is required for *unc-17* fosmid reporter expression (left panel) and *cho-1* fosmid reporter expression (right panel) in AWB and SMB neurons . AWB neurons were visualized by DiI staining in the *unc-17* fosmid reporter expressing strain. AWB and SMB show no fosmid reporter expression in the *lim-4* mutant. (**B**) The Otx-type homeobox transcription factor *ceh-14* is required for *unc-17* and *cho-1* fosmid reporter expression in PVC and *unc-17* fosmid reporter expression in PVN. PVC neurons show a decrease in *unc-17* and *cho-1* fosmid reporter expression in the *ceh-14* mutant compared to *wild type*. PVN neurons show no *unc-17* fosmid reporter expression in the *ceh-14* mutant. Note that PVN does not express *cho-1* fosmid reporter in wild type animals. (**C**) The homeobox transcription factors *unc-30* and *lin-11* are required for normal expression of the *unc-17* and *cho-1* fosmid reporters. Cholinergic identity genes are downregulated in PVP neurons starting at L1 (top panels) and continuing until the L4/adult stage (bottom panels) in *unc-30* and *lin-11* mutant strains compared to *wild type*. (**D**) The homeobox transcription factor *unc-42* is required for *unc-17* and *cho-1* fosmid reporter expression in RIV, SMD, RMD and SIB. (**E**) The POU homeobox transcription factor *unc-86* is required for *unc-17* and *cho-1* fosmid reporter expression in RIH. (**F**) A *wild type* male is shown in the top panel for reference. *unc-86* (middle panel) is also required for *unc-17* and *cho-1* fosmid reporter expression in URX and in the CEM male-specific neurons. In the absence of *unc-86* the AIM neurons did not show expression of *unc-17* and *cho-1* fosmid reporters in the L4/adult male. The LIM homeobox transcription factor *ceh-14* is required for the AIM neurons to express *unc-17* and *cho-1* fosmid reporters in the L4/adult male (bottom panel). Transgenes: *otIs576* = *unc-17* fosmid reporter; *otIs544* = *cho-1* fosmid reporter.

DOI: 10.7554/eLife.12432.017

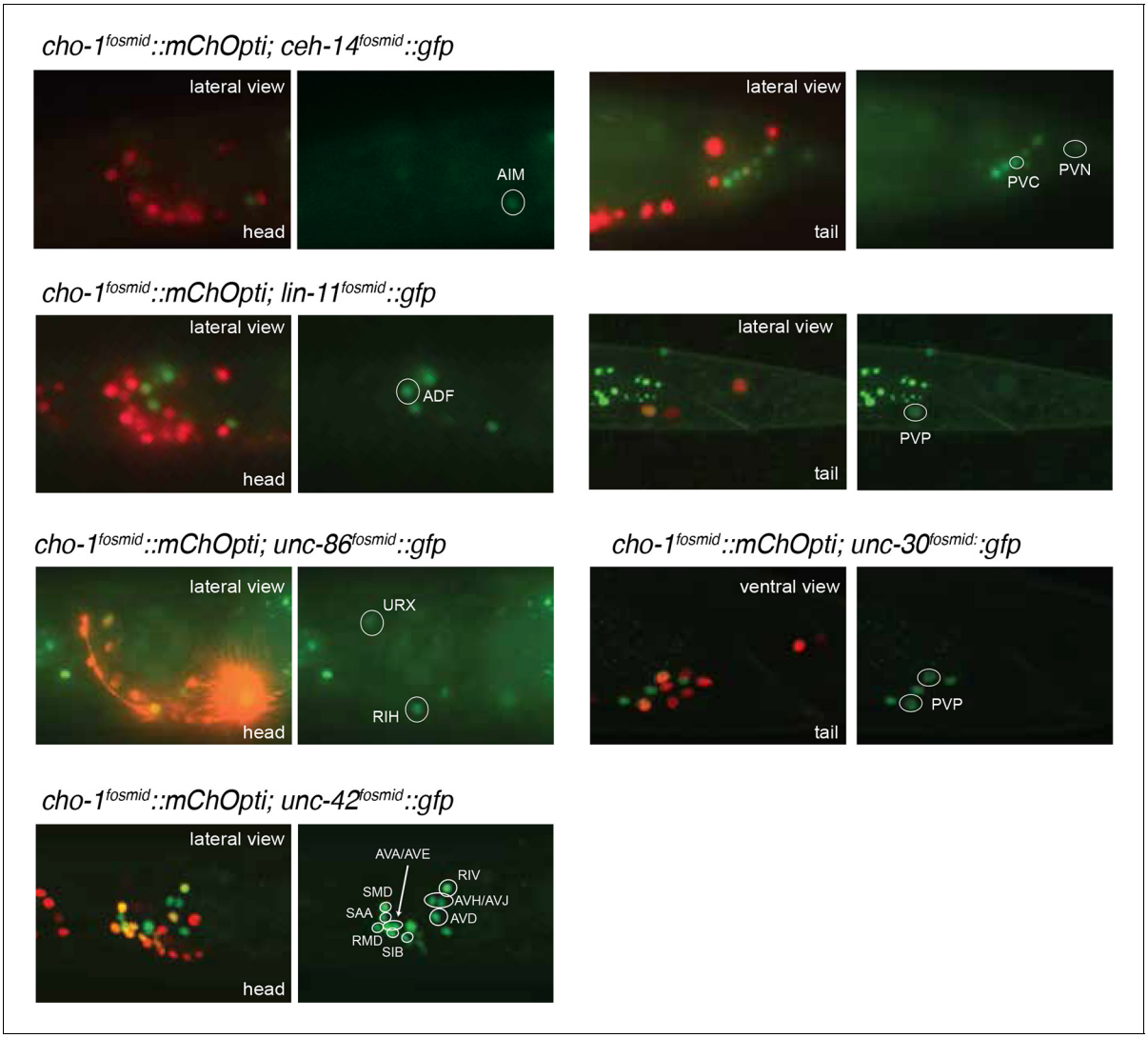

Figure 6—figure supplement 1. Continuous expression of transcription factors fosmid reporters in cholinergic neurons. AIM and PVC were labeled by *cho-1* and *ceh-14* fosmid reporters. PVN was labeled by *ceh-14* fosmid reporter but it did not express *cho-1* (see ***Table 1***). ADF and PVP were labeled by *cho-1* and *lin-11* fosmid reporters. URX, RIR and RIH were labeled by by *cho-1* and *unc-86* fosmid reporters. PVP was labeled by *cho-1* and *unc-30* fosmid reporters. AVA, AVE, AVEs, RIV, RMD, SAA, SIB and SMD were labeled by *cho-1* and *unc-42* fosmid reporters.

DOI: 10.7554/eLife.12432.018

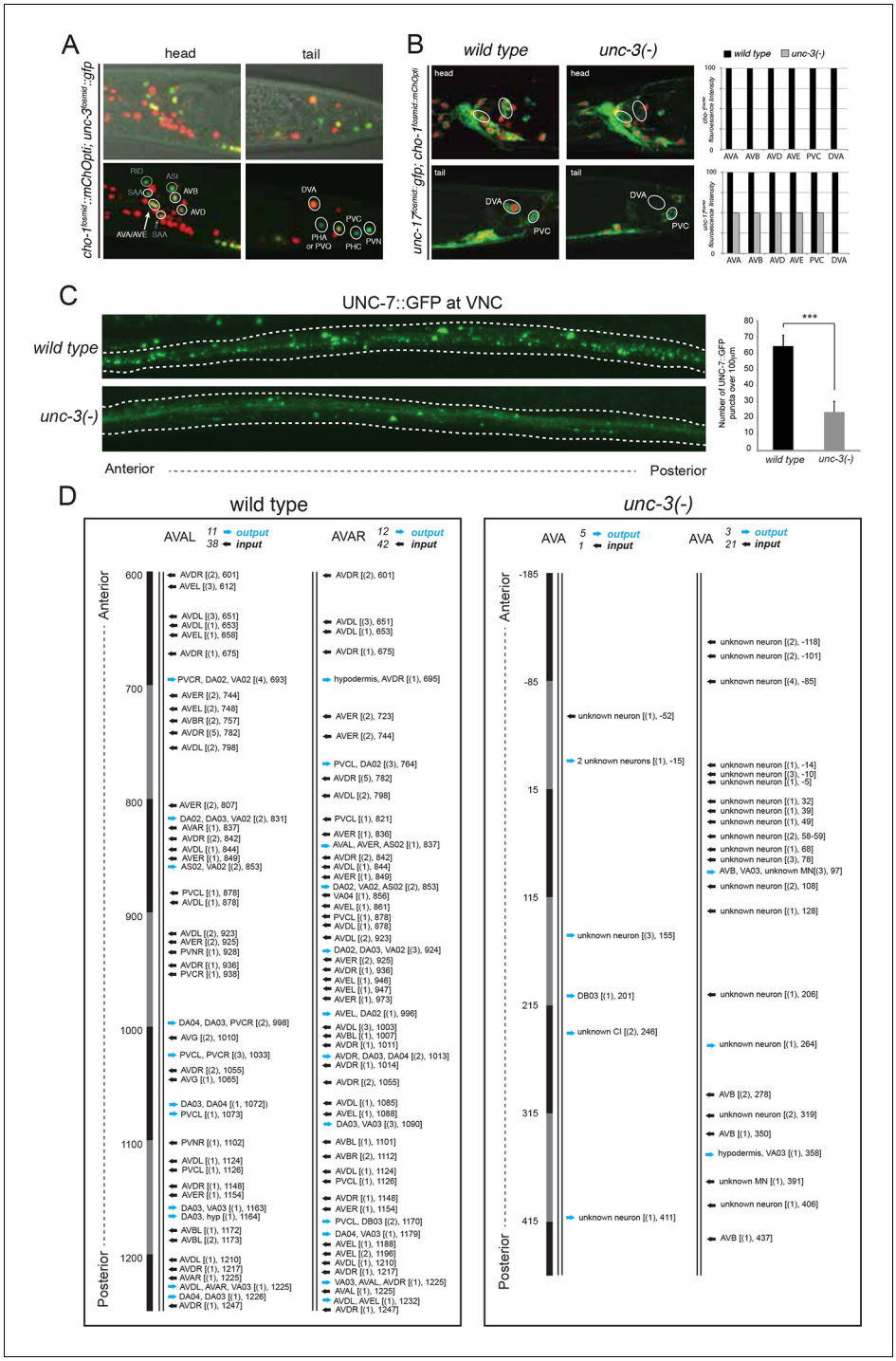

Figure 7. *unc-3* is a circuit-associated transcription factor. (**A**) Expression pattern of an *unc-3* fosmid-based reporter (*otIs591*). Overlap with a *cho-1* fosmid-based reporter (*otIs544*) is shown in all panels. The upper panels are the same as the lower, but a Nomarski image has been added for orientation purposes. *unc-3* expression was also detected in PDA, PDB and PVP in the pre-anal ganglion (data not shown). (**B**) The expression of the *unc-17* and *cho-1* fosmid reporters is downregulated in command interneurons (AVA, AVB, AVD, AVE, PVC) and the tail neuron DVA in *unc-3* mutant animals (identical results were obtained using two *unc-3* alleles, *e151* generates a premature STOP and *n3435* is a deletion allele). Quantification is shown on the right. Twenty animals were analyzed at the fourth larval stage (L4) per genotype. Note that the effect of *unc-3* on *unc-17* expression in the command interneurons (this figure) is not as fully penetrant as it is in VNC motor neurons (***Kratsios et al., 2011***). (**C**) Gap junctions that command interneurons make are visualized with *gfp* tagging the innexin protein UNC-7, as

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previously described (***Starich et al., 2009***) (transgene: *iwIs47*). Dotted white lines delineate the location of the VNC. A significant decrease in the number of the UNC-7::GFP puncta was observed in the VNC of *unc-3(n3435)* mutant animals (quantification shown on the right with average values and standard deviation). A student's t test was performed. ***p value <0.0001. (**D**) Reconstruction of the chemical synapse connectivity of the AVA command interneurons in a wild type and an *unc-3(e151/MnH205)* mutant animal. Less synaptic input onto AVA neurons and output from the AVA neurons was observed in the *unc-3* mutant animal. This is not merely an effect of axonal process misplacement since in *unc-3* mutants, AVA processes still run adjacent to the processes of the neurons it normally makes synaptic contacts to. More than 600 electron micrographs were reconstructed per genotype. In square brackets, the location (number of electron micrograph) for each chemical synapse is shown, and the number of consecutive micrographs in which a synapse was detected is also shown in parenthesis.
DOI: 10.7554/eLife.12432.020

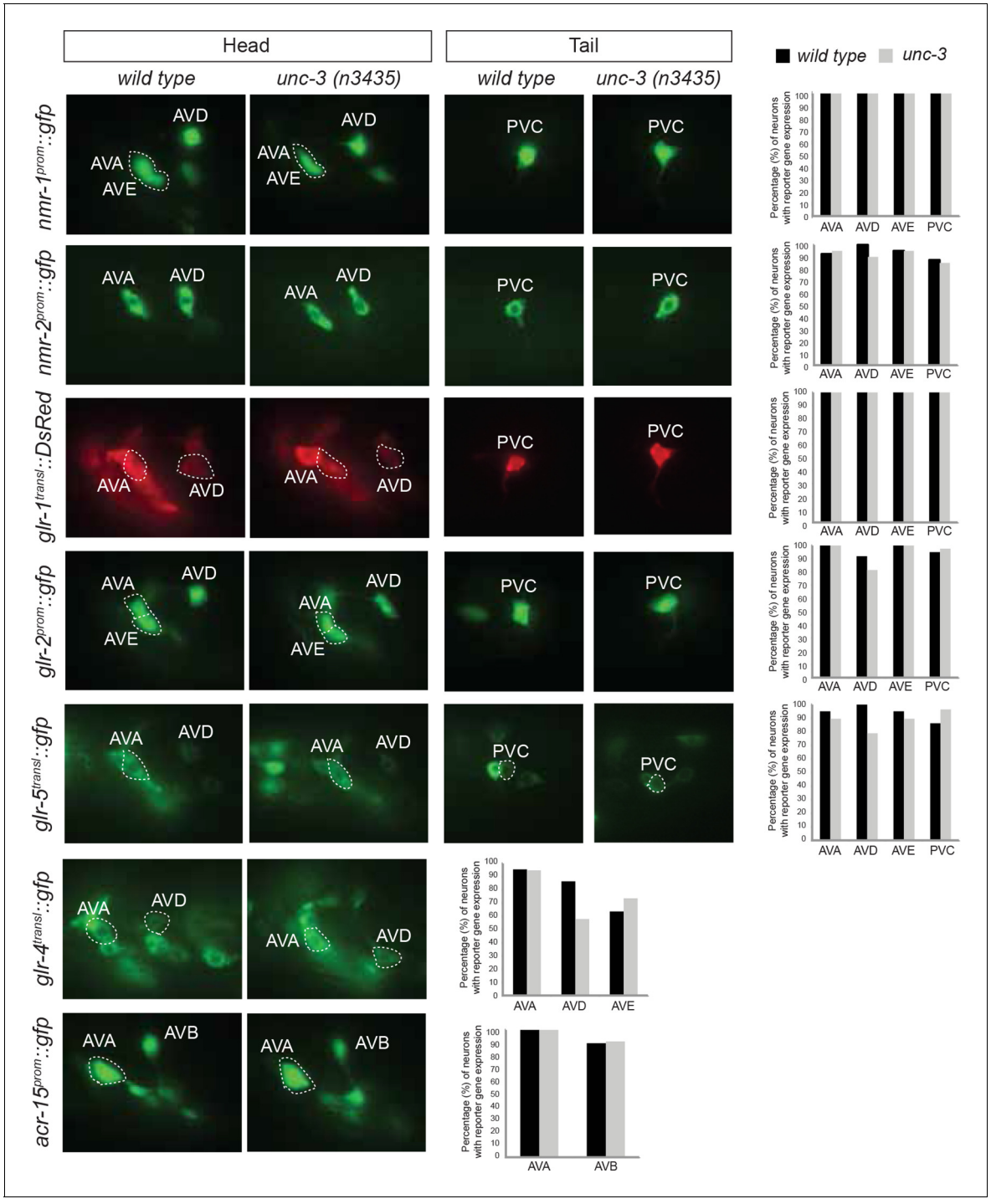

Figure 7—figure supplement 1. UNC-3 has no effect on glutamate receptor expression in command interneurons. (**A**) The expression of PDA identity markers *exp-1, ace-3/4,* and *cog-1* is affected in *unc-3* mutant animals. Quantification is provided on the right. For *cog-1*^{prom}*::gfp*, n = 25 for wild type and *unc-3(e151).* For *ace-3/-4*^{prom}*::gfp and exp-1*^{prom}*::gfp*, n = 20 for wild type and *unc-3(e151).* Fisher's exact test was performed. **p value <0.01; ***p value < 0.0001. (**B**) The expression of multiple glutamate receptor genes (*nmr-1, nmr-2, glr-1, glr-2, glr-4, glr-5*) is unaffected in command interneurons (AVA, AVB, AVD, AVE, PVC) of *unc-3* null animals. Similarly, the expression of the ACh receptor subunit encoding gene *acr-15* is not affected in the AVA and AVB neurons of *unc-3* mutants. Quantification is provided on the right. Number of animals examined = 20 animals per reporter gene per genotype. Moreover, the expression of *flp-18* and *rig-3* (AVA markers), as well as *opt-3* (AVE marker) is not affected in *unc-3* mutants (N = 20, data not shown). In addition, the expression of several identity genes (*glr-5,* glutamate receptor; *twk-16,* potassium channel; *nlp-12,* neuropeptide; *zig-5,* immunoglobulin superfamily gene; *ser-2,* serotonin receptor) for the DVA interneuron is unaffected in *unc-3* mutants (data not shown).

DOI: 10.7554/eLife.12432.021

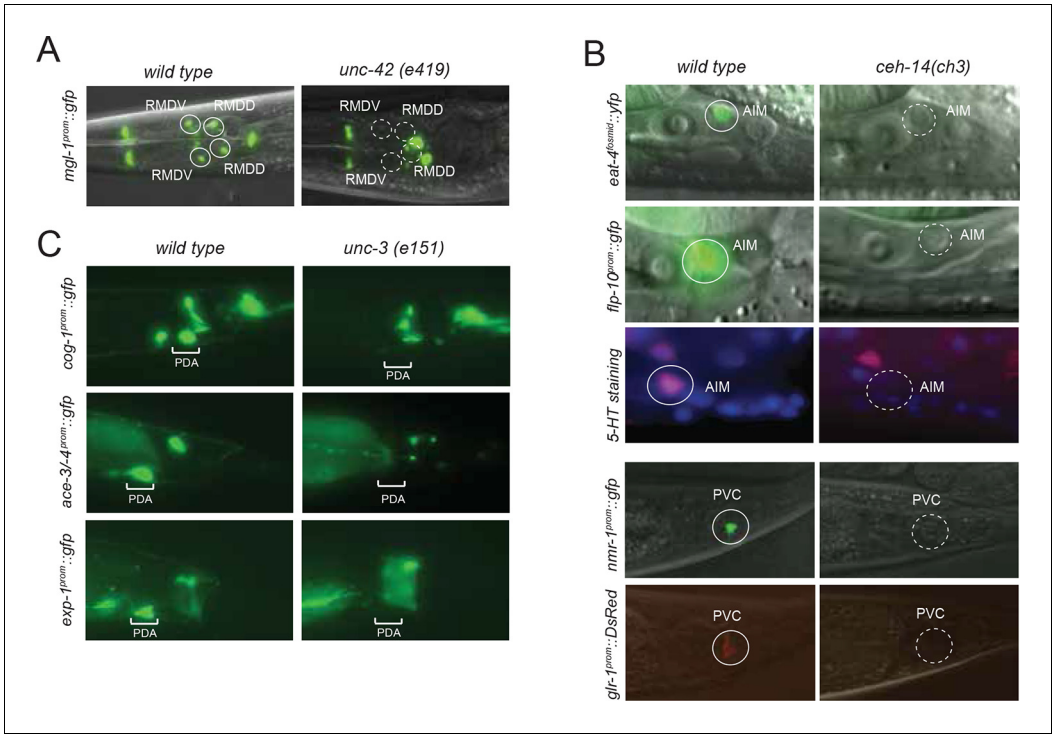

Figure 8. Coupling of cholinergic identity with other identity features. (**A**) An *mgl-1* reporter transgene does not show expression in RMD neurons in the absence of *unc-42*. (**B**) In the absence of *ceh-14* the AIM neurons do not show *eat-4* fosmid reporter and *flp-10* reporter expression. 5-HT staining is not detectable in AIM neurons in the *ceh-14* mutant. In the absence of *ceh-14* the PVC neurons do not show *nmr-1* or *glr-1* reporter expression. Number of animals examined = 20 animals per reporter gene per genotype. (**C**) The expression of PDA identity markers *exp-1*, *ace-3/4*, and *cog-1* is lost in *unc-3* mutant animals. For *cog-1*^{prom}*::gfp*, 25 of 25 wild-type and 1 of 25 *unc-3(e151)* animals showed *cog-1*^{prom}*::gfp* expression in PDA. For *ace-3/-4*^{prom}*::gfp*, 20 of 20 wild-type and 0 of 20 *unc-3(e151)* animals showed *ace-3/-4*^{prom}*::gfp* expression in PDA. For *exp-1*^{prom}*::gfp*, 20 of 20 wild-type and 11 of 20 *unc-3(e151)* animals showed *exp-1*^{prom}*::gfp* expression in PDA.

DOI: 10.7554/eLife.12432.022

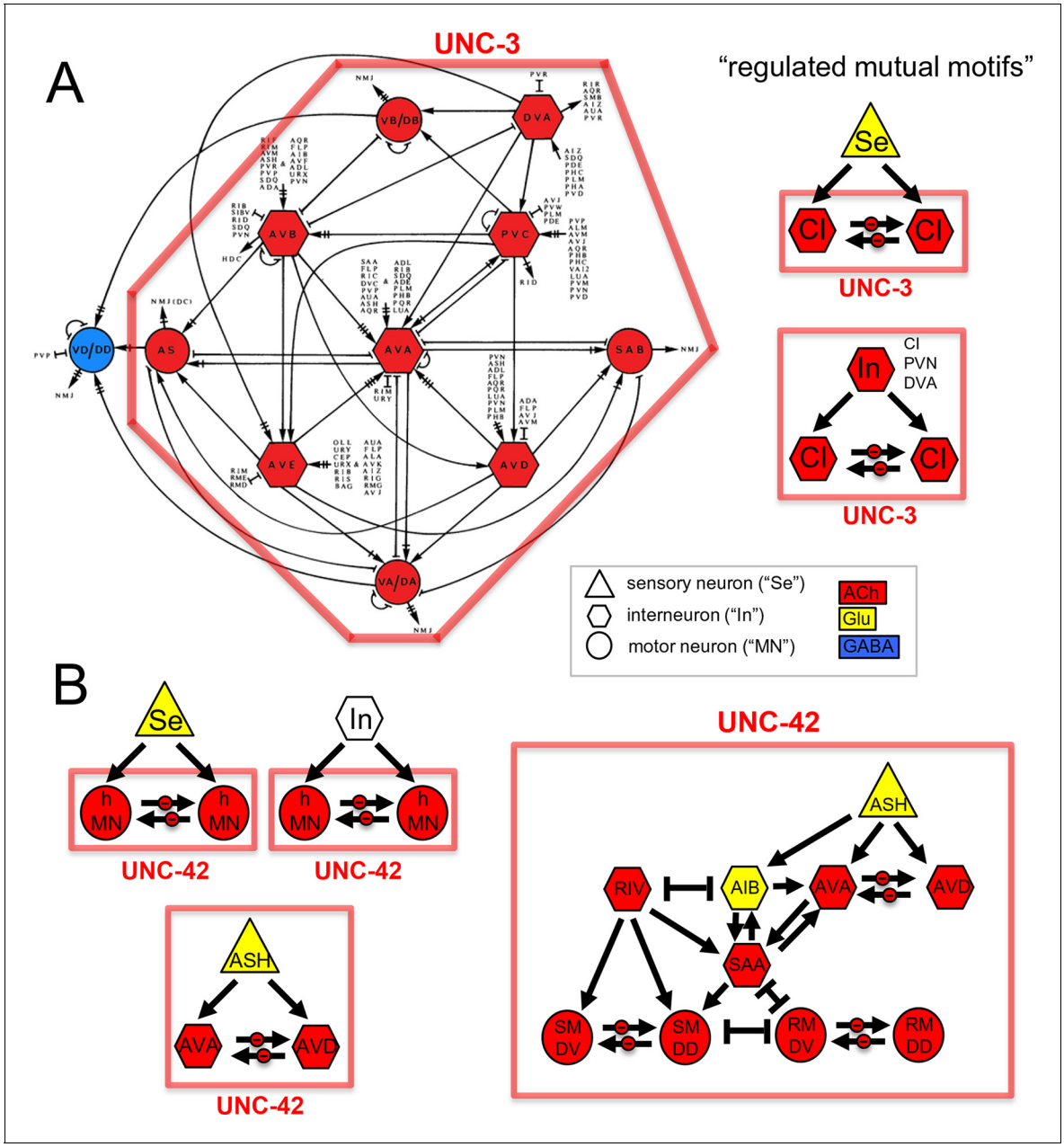

Figure 9. Circuit-associated transcription factors. (A) Ventral cord motor circuit as shown in ***White et al. (1986)***, but now superimposed with neurotransmitter identity and expression pattern of the *unc-3* transcription factor. *unc-3* controls the cholinergic identity of every single neuron in this circuit. Next to the circuit diagram, a number of different regulated mutual 3-neuron networks motifs are shown. These motifs are either embedded in the circuit and provide a connection to neurons located outside the circuit (e.g. glutamatergic sensory neurons). In all cases *unc-3* controls cholinergic identity of the mutually connected command interneurons ('CI') and in those cases where the mutually connected neurons receive cholinergic interneuron input, *unc-3* controls the identity of the entire microcircuit. (B) *unc-42* controls the cholinergic identity of interconnected head motor neurons, and glutamatergic signaling between ASH sensory neurons (whose glutamatergic identity is controlled by *unc-42* (***Serrano-Saiz et al., 2013***) and cross-connected command interneurons in which *unc-42* controls glutamate receptor expression (***Brockie et al., 2001***) (shown in ***Table 4***). Red boxes indicate the neurons affected by the indicated transcription factor.

DOI: 10.7554/eLife.12432.025

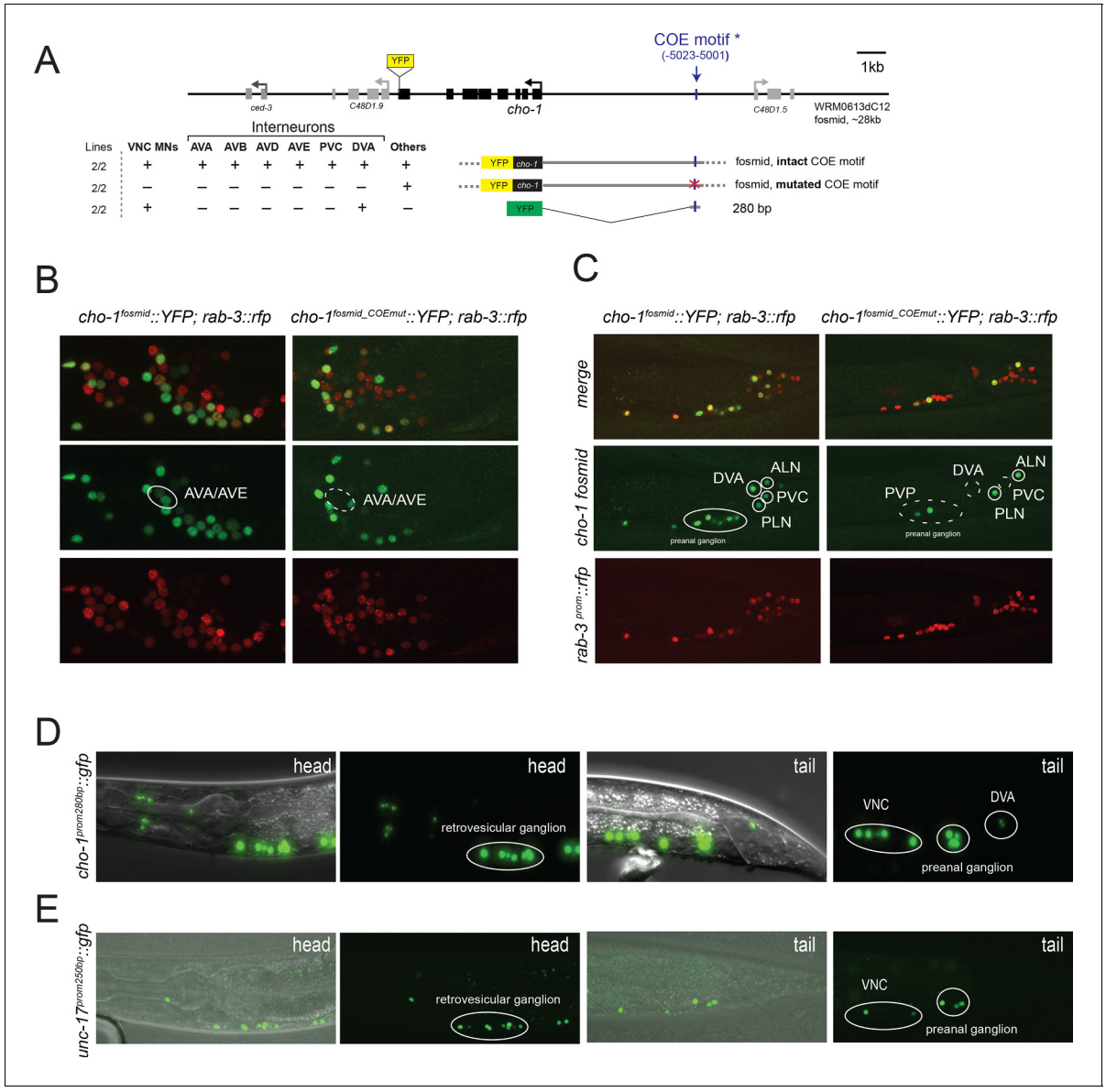

Figure 10. A single UNC-3 binding site is required for *cho-1* expression in all distinct *unc-3*-dependent cholinergic neuron types. (**A**) Schematic showing of the *cho-1* locus and the location of the UNC-3 binding site (COE motif) relative to ATG for the fosmid reporters and 280bp promoter fusion. (**B, C**) A *cho-1* fosmid reporter (~28 kb) that contains an intact COE motif shows expression in all cholinergic neurons including the ventral nerve cord (VNC) motor neurons (MNs), the command interneurons (AVA, AVB, AVD, AVE, PVC), and the interneuron DVA. Mutation of the COE motif in the context of this *cho-1* fosmid-based reporter results in selective loss of reporter gene expression in VNC MNs residing at the retrovesicular ganglion and all command interneurons (only AVA and AVE head interneurons are shown). (**B**) *cho-1* fosmid reporter versus *cho-1_COEmut* fosmid reporter in an adult head. (**C**) *cho-1* fosmid reporter versus *cho-1_COEmut* fosmid reporter in an adult tail. Reporter gene expression is also lost in tail neurons DVA and PVC. The transgenic line *rab-3*^{prom}*::rfp* drives reporter gene expression in the entire nervous system and was used in the background to facilitate neuronal identification. (**D**) A short fragment (280 bp) of the *cho-1 cis*-regulatory region containing the COE motif is sufficient to drive reporter gene expression only in VNC MNs. This fragment does not show expression in command interneurons located at the head and tail of the animal. (**E**) A short fragment (250 bp) of the *unc-17 cis*-regulatory region containing the COE motif is sufficient to drive reporter gene expression only in VNC MNs.
DOI: 10.7554/eLife.12432.026