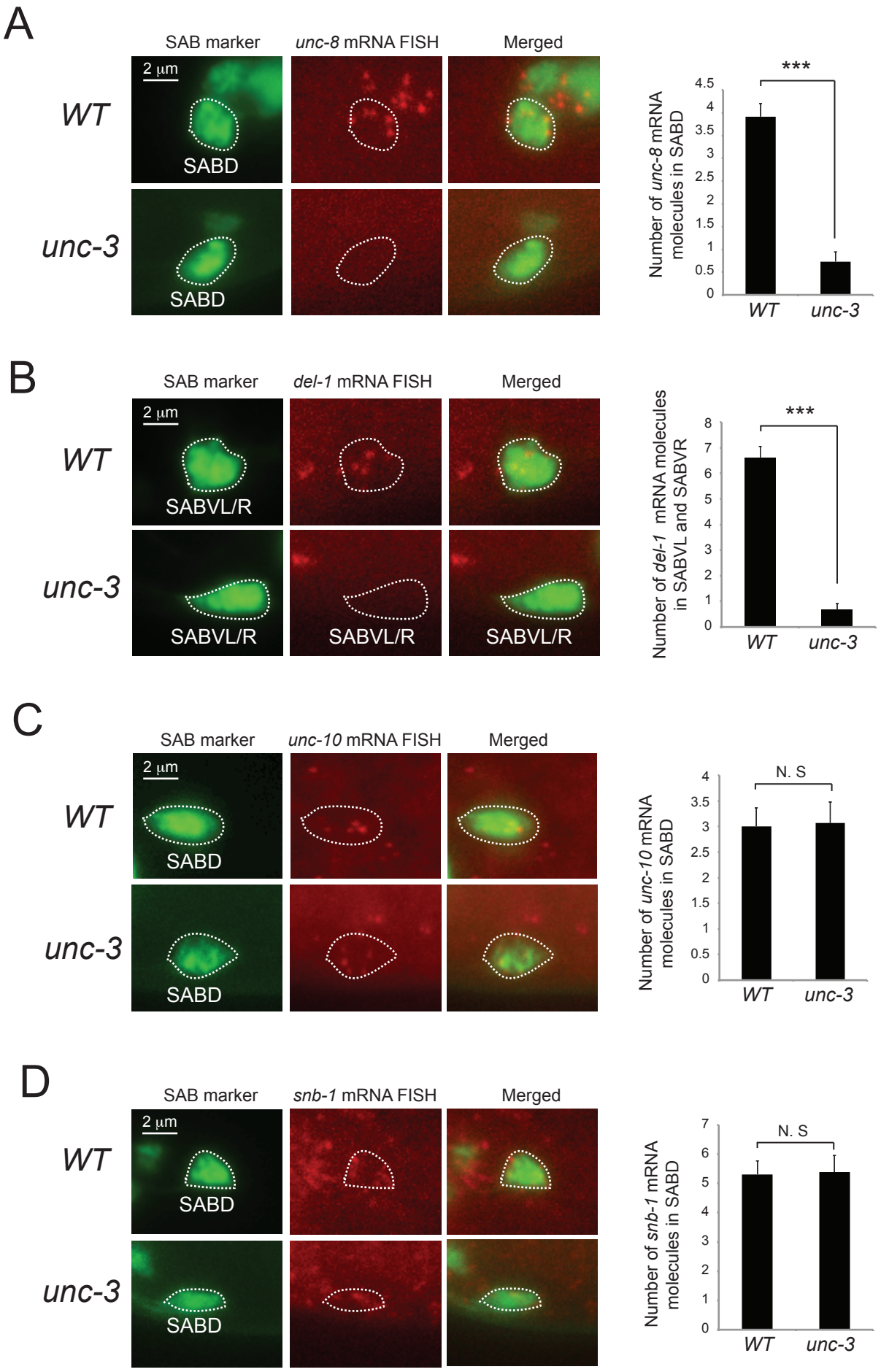

A
SAB marker
unc-8 mRNA FISH
Merged
2 μm
WT
SABD
unc-3
SABD
***
Number of unc-8 mRNA molecules in SABD
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
unc-3
B
SAB marker
del-1 mRNA FISH
Merged
2 μm
WT
SABVL/R
unc-3
SABVL/R
SABVL/R
SABVL/R
***
Number of del-1 mRNA molecules in SABVL and SABVR
8
7
6
5
4
3
2
1
0
WT
unc-3
C
SAB marker
unc-10 mRNA FISH
Merged
2 μm
WT
SABD
unc-3
SABD
N. S
Number of unc-10 mRNA molecules in SABD
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
unc-3
D
SAB marker
snb-1 mRNA FISH
Merged
2 μm
WT
SABD
unc-3
SABD
N. S
Number of snb-1 mRNA molecules in SABD
7
6
5
4
3
2
1
0
WT
unc-3

Figure S1

SABD axons present

	0 axons	1 axon	2 axons
WT (N=31)	3.2 %	6.5 %	90.3 %
unc-3 (N=33)	3 %	30.3 %	66.7 %

SABV axons present

	0 axons	1 axon	2 axons
WT (N=31)	0 %	16.1 %	83.9 %
unc-3 (N=33)	18.2 %	51.5 %	30.3 %

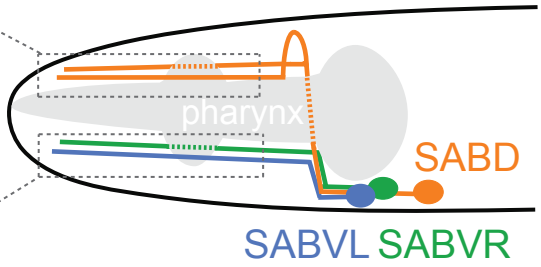


Figure S2

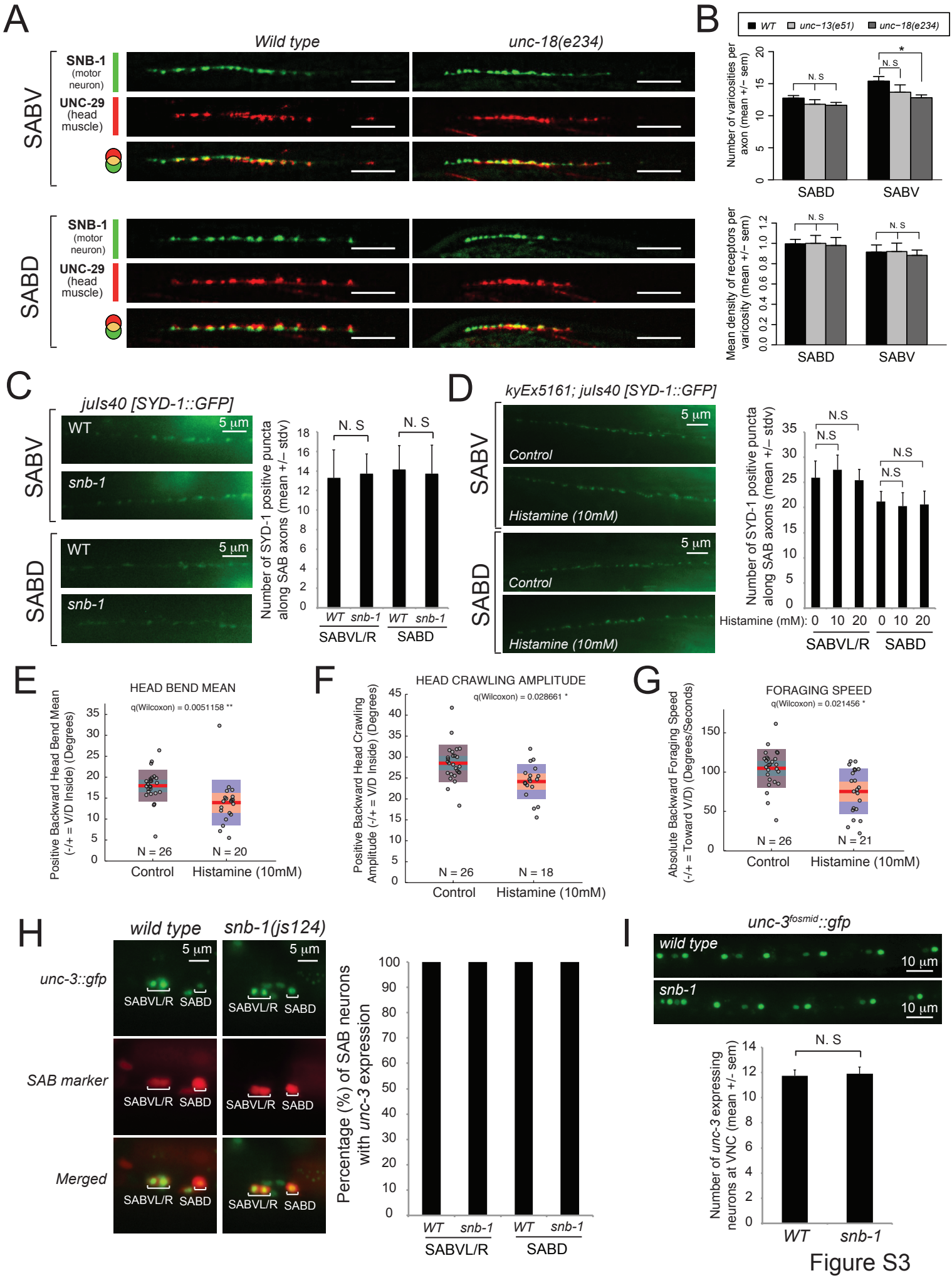

A
Wild type
unc-18(e234)
SABV
SABD
SNB-1 (motor neuron)
UNC-29 (head muscle)
B
WT
unc−13(e51)
unc−18(e234)
Number of varicosities per axon (mean +/− sem)
Mean density of receptors per varicosity (mean +/− sem)
N. S
*
SABD
SABV
C
juIs40 [SYD-1::GFP]
WT
snb-1
5 µm
Number of SYD-1 positive puncta along SAB axons (mean +/− stdv)
WT snb-1 WT snb-1
SABVL/R
SABD
D
kyEx5161; juIs40 [SYD-1::GFP]
Control
Histamine (10mM)
Histamine (mM): 0 10 20 0 10 20
E
HEAD BEND MEAN
q(Wilcoxon) = 0.0051158 **
Positive Backward Head Bend Mean (-/+ = V/D Inside) (Degrees)
N = 26
N = 20
Control
Histamine (10mM)
F
HEAD CRAWLING AMPLITUDE
q(Wilcoxon) = 0.028661 *
Positive Backward Head Crawling Amplitude (-/+ = V/D Inside) (Degrees)
N = 26
N = 18
G
FORAGING SPEED
q(Wilcoxon) = 0.021456 *
Absolute Backward Foraging Speed (-/+ = Toward V/D) (Degrees/Seconds)
N = 26
N = 21
H
wild type
snb-1(js124)
unc-3::gfp
SAB marker
Merged
SABVL/R
SABD
Percentage (%) of SAB neurons with unc-3 expression
I
unc-3fosmid::gfp
wild type
snb-1
10 µm
Number of unc-3 expressing neurons at VNC (mean +/- sem)

Figure S3

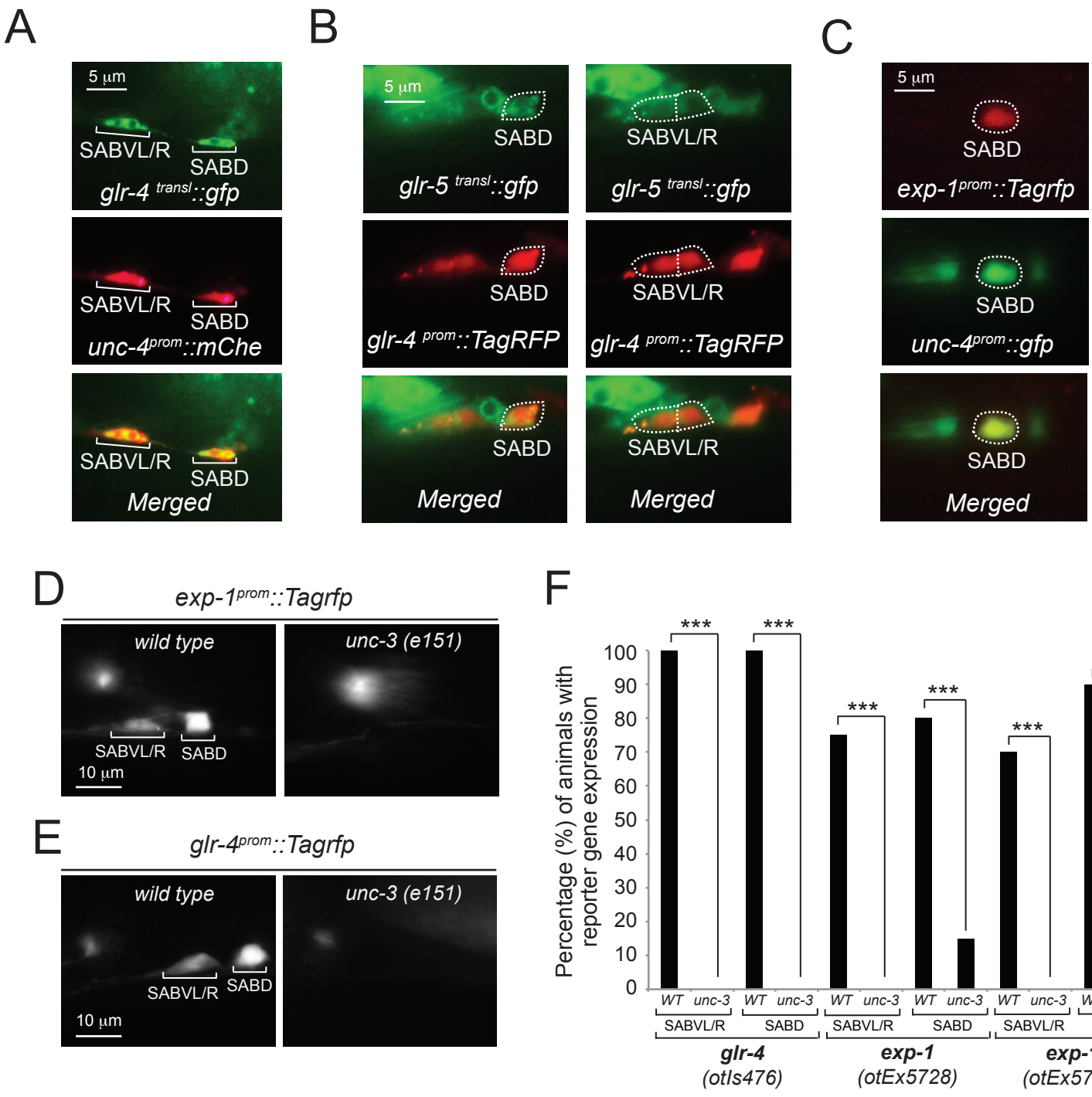

A
B
C
5 µm
SABVL/R
SABD
glr-4 transl::gfp
unc-4prom::mChe
Merged
glr-5 transl::gfp
glr-4 prom::TagRFP
exp-1prom::Tagrfp
unc-4prom::gfp
D
exp-1prom::Tagrfp
wild type
unc-3 (e151)
10 µm
E
glr-4prom::Tagrfp
F
Percentage (%) of animals with reporter gene expression
***
WT
unc-3
glr-4 (otIs476)
exp-1 (otEx5728)
exp-1 (otEx5729)

Figure S4

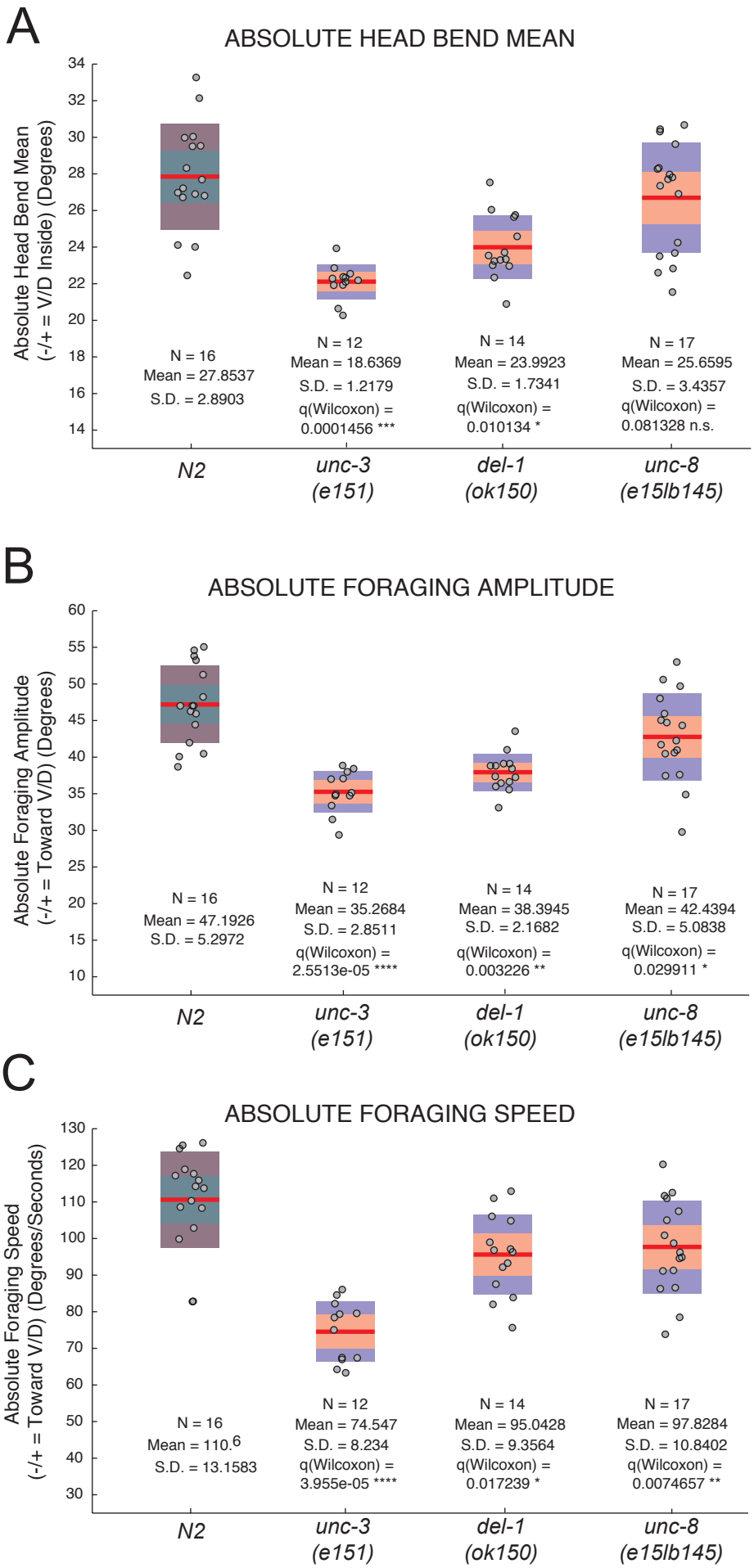
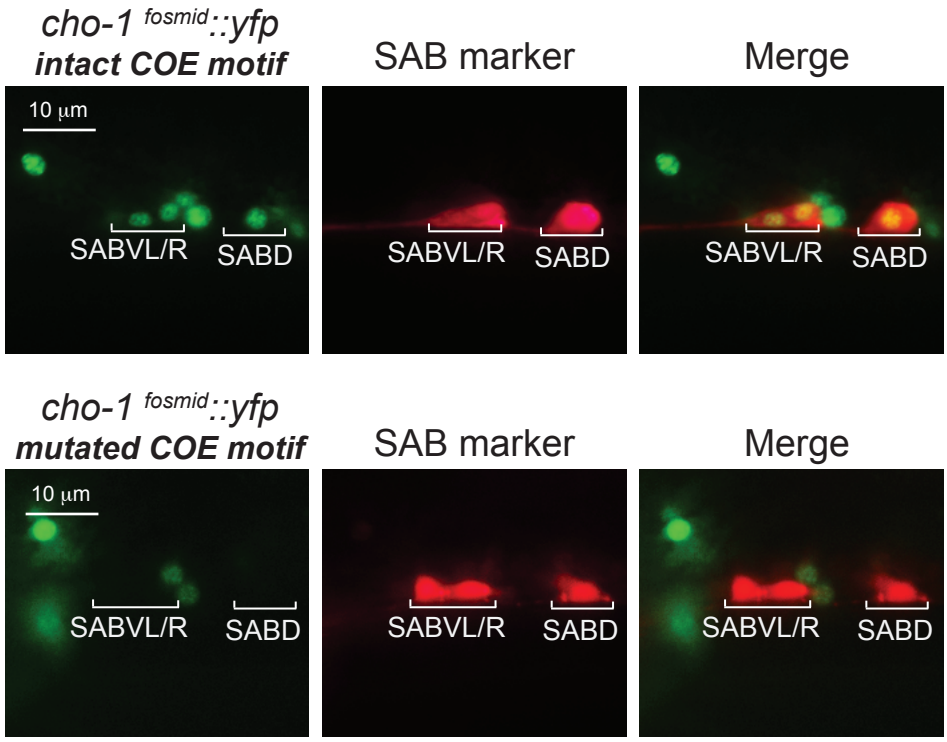

A
ABSOLUTE HEAD BEND MEAN
Absolute Head Bend Mean
(-/+ = V/D Inside) (Degrees)
34
32
30
28
26
24
22
20
18
16
14
N = 16
Mean = 27.8537
S.D. = 2.8903
N = 12
Mean = 18.6369
S.D. = 1.2179
q(Wilcoxon) =
0.0001456 ***
N = 14
Mean = 23.9923
S.D. = 1.7341
q(Wilcoxon) =
0.010134 *
N = 17
Mean = 25.6595
S.D. = 3.4357
q(Wilcoxon) =
0.081328 n.s.
N2
unc-3
(e151)
del-1
(ok150)
unc-8
(e15lb145)
B
ABSOLUTE FORAGING AMPLITUDE
Absolute Foraging Amplitude
(-/+ = Toward V/D) (Degrees)
60
55
50
45
40
35
30
25
20
15
10
N = 16
Mean = 47.1926
S.D. = 5.2972
N = 12
Mean = 35.2684
S.D. = 2.8511
q(Wilcoxon) =
2.5513e-05 ****
N = 14
Mean = 38.3945
S.D. = 2.1682
q(Wilcoxon) =
0.003226 **
N = 17
Mean = 42.4394
S.D. = 5.0838
q(Wilcoxon) =
0.029911 *
N2
unc-3
(e151)
del-1
(ok150)
unc-8
(e15lb145)
C
ABSOLUTE FORAGING SPEED
Absolute Foraging Speed
(-/+ = Toward V/D) (Degrees/Seconds)
130
120
110
100
90
80
70
60
50
40
30
N = 16
Mean = 110.6
S.D. = 13.1583
N = 12
Mean = 74.547
S.D. = 8.234
q(Wilcoxon) =
3.955e-05 ****
N = 14
Mean = 95.0428
S.D. = 9.3564
q(Wilcoxon) =
0.017239 *
N = 17
Mean = 97.8284
S.D. = 10.8402
q(Wilcoxon) =
0.0074657 **
N2
unc-3
(e151)
del-1
(ok150)
unc-8
(e15lb145)

Figure S5

A


cho-1 fosmid::yfp
intact COE motif
SAB marker
Merge
10 μm
SABVL/R
SABD
cho-1 fosmid::yfp
mutated COE motif
SAB marker
Merge
10 μm
SABVL/R
SABD

B

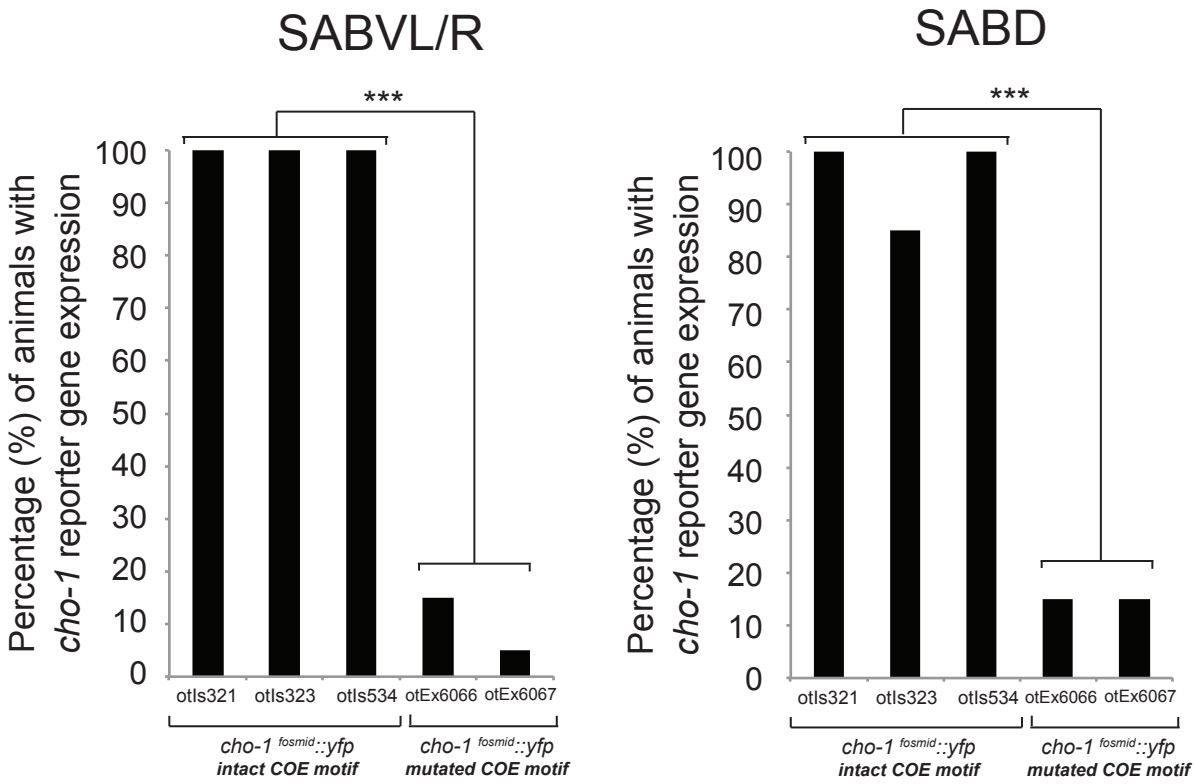

SABVL/R
SABD
***
***
Percentage (%) of animals with cho-1 reporter gene expression
100
90
80
70
60
50
40
30
20
10
0
otIs321
otIs323
otIs534
otEx6066
otEx6067
cho-1 fosmid::yfp
intact COE motif
cho-1 fosmid::yfp
mutated COE motif

Figure S6

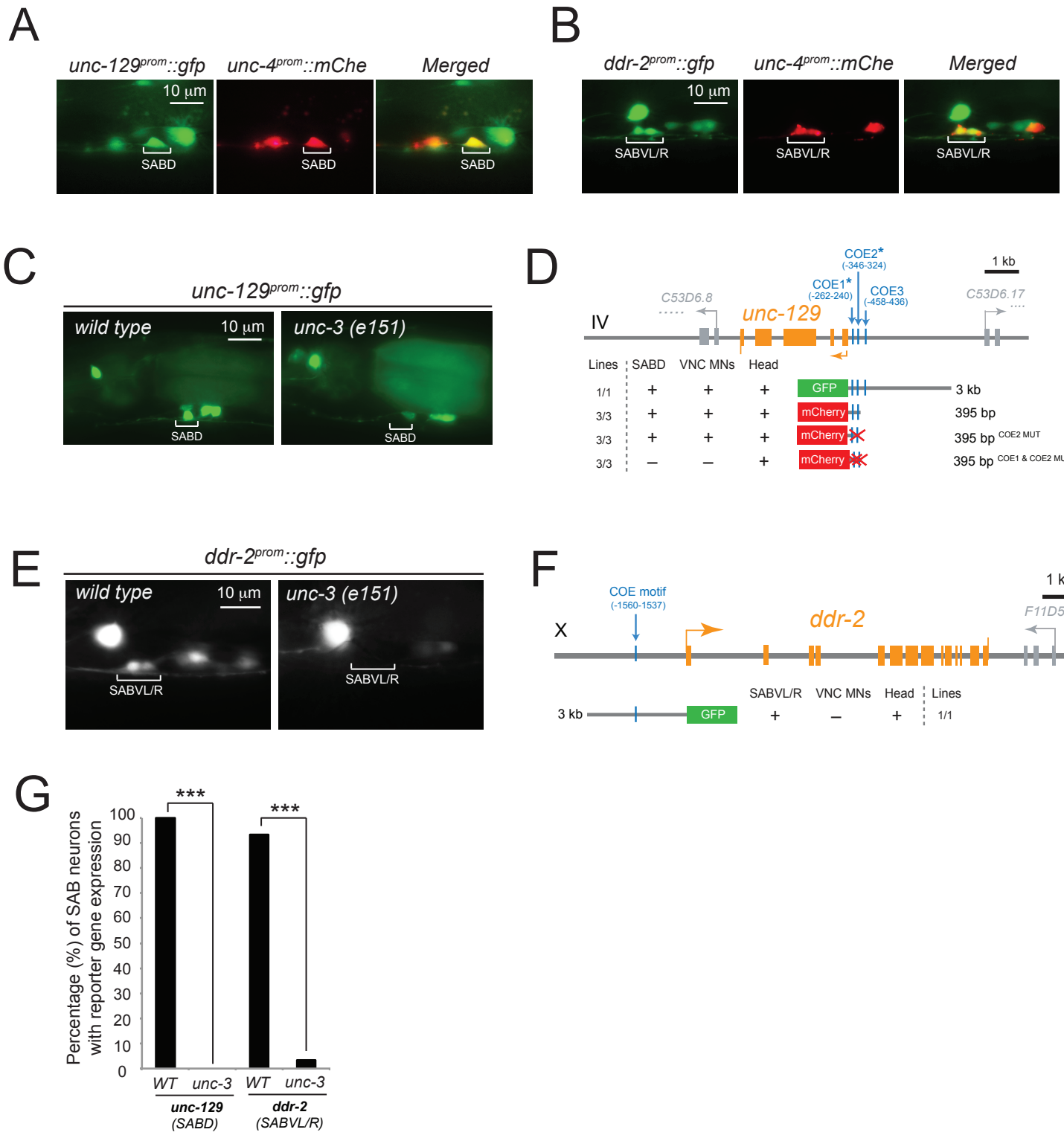

A
unc-129prom::gfp
unc-4prom::mChe
Merged
10 µm
SABD
B
ddr-2prom::gfp
unc-4prom::mChe
Merged
SABVL/R
C
unc-129prom::gfp
wild type
unc-3 (e151)
D
COE2*
(-346-324)
COE1*
(-262-240)
COE3
(-458-436)
1 kb
C53D6.8
unc-129
C53D6.17
IV
Lines
SABD
VNC MNs
Head
1/1
3/3
3/3
3/3
GFP
mCherry
3 kb
395 bp
395 bp COE2 MUT
395 bp COE1 & COE2 MUT
E
ddr-2prom::gfp
F
COE motif
(-1560-1537)
ddr-2
F11D5.6
X
SABVL/R
VNC MNs
Head
Lines
G
Percentage (%) of SAB neurons with reporter gene expression
***
WT
unc-3
unc-129
(SABD)
ddr-2
(SABVL/R)

Figure S7

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 – Related to Figure 4F-G: **Single mRNA molecule fluorescent *in situ* hybridization (FISH) analysis in *unc-3 (e151)* mutants. A-B:** A decrease in the number of *unc-8* and *del-1* mRNA molecules is observed in SABD and SABV neurons of *unc-3* mutants, respectively. Individual *unc-8* and *del-1* mRNA molecules appear as red dots as the *unc-8* and *del-1* probes are coupled with Quasar 670 dye. The SABD and SABV cell bodies are labeled with green fluorescence using *wdIs5[unc-4^{prom}::gfp]* as marker. Quantification of the number of individual *unc-8* mRNA molecules (mean ± standard error of the mean) found in SABD is provided on the right. Quantification of the number of individual *del-1* mRNA molecules (mean ± standard error of the mean) in SABVL and SABVR is also provided. Due to the close proximity of the SABV cell bodies, the number of individual *del-1* mRNA molecules reflects the mean of *del-1* mRNA molecules found in both SABVL and SABVR neurons. N = 10-15 animals per genotype. *** = P < 0.001. **C-D:** The number of *unc-10* and *snb-1* mRNA molecules in the SABD neuron of *unc-3* mutants appears unaltered. Individual *unc-10* and *snb-1* mRNA molecules appear as red dots. The SABD cell body is labeled with green fluorescence using *wdIs5 [unc-4^{prom}::gfp]* as marker as the *unc-10* probe was coupled with Cy5 and the *snb-1* probe with Quasar 670 dye. Quantification (mean ± standard error of the mean) is provided on the right. N = 10-15 animals per genotype. Scale bar = 2 μm.

Figure S2 – Related to Figure 2: Quantification of SAB axonal defects in *unc-3 (e151)* mutants. Axon branching defects are observed in the SABD of *unc-3(e151)* mutants. Only one SABD axon was detected in 30.3% of *unc-3(e151)* mutants, suggesting a branching defect. While 66.7% of *unc-3(e151)* mutants display two SABD axons, the majority of wild-type (WT) animals display two SABD axons (90.3%). Axon outgrowth defects are observed in the SABVs of *unc-3(e151)* mutants. Zero SABV axons are observed in 18.2 % of *unc-3(e151)* mutants, one SABV axon is observed in 51.5% of *unc-3(e151)* mutants, and 2 SABV axons are observed in 30.3% of *unc-3(e151)* mutants. In comparison, the majority of wild-type (WT) animals (83.9%) display two SABV axons on the left and right side. SAB axons were visualized using the *otEx5422 (snb-1* translational fosmid-based *yfp* reporter*)* transgene. Due to mosaicism of the *otEx5422* transgene, we were able to detect only one of the two SABV axons in 16.1% of wild-type animals and zero SABD axons in 3.2% of wild-type animals. N = number of animals analyzed.

Figure S3 – Related to Figure 4B-C: Inhibition of SAB neuronal activity does not affect synapse formation.

A: Representative images of SABV and SABD innervation zones in *wild type* and *unc-18 (e234)* mutant animals. The SAB synaptic boutons (green) are labeled with the transgene *jsIs42 [unc-4^{prom}::snb-1::GFP]* and L-AChR are visualized (red) using the knock-in *kr208* allele (*unc-29::tagRFP*). Images (cropped with dimensions 580_80px, scale 0:11 _m=px) are shown following max-projection and background subtraction. Scale bar: 10 μm.

B: The average number of synaptic boutons between the SAB MNs and head muscle was counted in young adult animals. In SABD, the number of presynaptic varicosities is not significantly reduced in *unc-13 (e51)* and *unc-18 (e234)* general transmission mutants (ANOVA, p=0.23). In SABV, the number of varicosities is reduced in transmission mutants (ANOVA, p=0.01; Tukey's HSD p=0.0075 between WT and *unc-18*, p**>**0.1 between *unc-13* and WT or *unc-18*). However, this SABV defect is small compared to *unc-3* mutants (See Figure 2H). Mean number of varicosities for WT: 15.4, *unc-13 (e51):* 13.7, *unc-18 (e234):* 12.8). Since 50% of *unc-13 (e51)* mutants display SAB sprouting defects accompanied by the formation of ectopic synaptic varicosities [S16], we excluded from our counts the additional synapses (due to sprouting) and focused only on the synapses along the “main” SAB innervation zones to avoid any bias resulting from the longer SAB sprouting axons of *unc-13 (e51)* animals. For each synaptic bouton, the mean density of associated L-AChR receptors was measured. In SABD and SABV innervation zones, the mean density of receptors associated with each varicosity is not significantly different between WT and *unc-13 (e51)* or *unc-18 (e234)* general transmission mutants (Kruskal-Wallis, p=0.88 and p=0.75). For SABD, the number of axons measured is 20 for WT, 30 for *unc-18*, and 18 for *unc-13.* For SABV, the number of axons measured is 15 for WT, 37 for *unc-18*, and 6 for *unc-13.* **C:** The number of SYD-1 positive puncta (mean +/– standard deviation) at the SAB innervation zone is not altered in *snb-1(js124)* animals. Animals were analyzed at the first larval stage (L1). Transgenic *juIs40 [syd-1^{prom}::SYD-1::GFP]* animals were used to visualize the SAB presynapse. Wild type, N = 12; *snb-1(js124),* N = 12. **D:** Silencing of SAB neuronal activity using the histamine chloride channel 1(HisCl1) does not affect SAB presynapse assembly. Animals carrying the *kyEx5161 [unc-4^{prom}::HisCl1::SL2::mCherry]* were allowed to grow on 10mM or 20mM histamine-containing NGM plates till they reached the fourth larval stage

(L4). The number of SYD-1 positive puncta in the SAB innervation zone was calculated using *juIs40 [syd-1^{prom}::SYD-1::GFP]* as a presynaptic marker. Quantification is provided as mean +/– standard deviation. As a control, *kyEx5161* animals were allowed to grow on NGM plates without histamine till the L4 stage. N > 20 animals per condition. **E-G:** Silencing of SAB neuronal activity using the HisCl1 system (See also Supplemental Experimental Procedures) results in head bending defects. In the presence of 10mM histamine, *kyEx5161 [unc-4^{prom}::HisCl1::SL2::mCherry, elt-2^{prom}::mCherry])* animals show reduced backward head bend mean (**E**), reduced backward head crawling amplitude, and (**F**) reduced backward foraging speed (**G**). **H-I:** The expression of *unc-3* in SAB and VNC motor neurons is not affected in *snb-1(js124)* mutants. Animals were photographed at the L1 stage. Transgenic animals carrying a fosmid-based reporter for *unc-3 (otIs591 [unc-3^{fosmid}::gfp])* were used. SAB neurons were identified using the SAB specific-marker *otIs476 [glr-4^{prom}::TagRFP].* The percentage of animals with *unc-3* expression in SAB neurons, as well as the number of neurons expressing *unc-3* in the VNC was quantified. N = 15 animals per genotype.

Figure S4 – Related to Figure 4F-G: **The expression of the neurotransmitter receptor-encoding genes *glr-4, glr-5* and *exp-1* is affected in the SAB neurons of *unc-3(e151)* mutants. A:** A translational reporter for *glr-4 (glr-4^{transl}::GFP)* shows expression in all three SAB neurons. The SAB identity was confirmed by crossing transgenic animals carrying the *akEx32 [glr-4^{transl}::GFP]* array with animals carrying the SAB specific-marker *otEx6068 [unc-4promC::Cherry].* **B:** A translational reporter for *glr-5 (glr-5^{transl}::GFP)* shows expression in all three SAB neurons. The SAB identity was confirmed by crossing transgenic animals carrying the *akEx31 [glr-5^{transl}::GFP]* array with animals carrying the SAB specific-marker *otIs476 [glr-4^{prom}::TagRFP].* The SABD and SABVL/R images were acquired from different focal planes of the same *akEx31;otIs476* transgenic animal. **C-D**: A transcriptional reporter for *exp-1 (exp-1^{prom}::TagRFP)* shows expression in SAB neurons. The SABD identity was confirmed by crossing transgenic animals carrying the *otEx5728 [exp-1^{prom}::TagRFP]* array with animals carrying the SAB specific-marker *wdIs5[unc-4^{prom}::gfp*]. In *unc-3(e151)* mutants, the expression of *exp-1* is severely affected in SABV and SABD neurons. Quantification is provided in **F** for two *exp-1* transgenic reporter lines *otEx5728 [exp-1^{prom}::TagRFP]* and *otEx5729 [exp-1^{prom}::TagRFP].* **E**: The expression of a *glr-4* transcriptional reporter (*otIs476*

[glr-4^{prom}::Tagrfp]) is abrogated in the SAB neurons of *unc-3* mutants. Quantification provided in **F**. N = 20 animals per genotype.

Figure S5 – Related to Figure 4: **Automated worm-tracking analysis of *unc-3, del-1* and *unc-8* mutant animals. A**: A significant decrease in the absolute head bend mean was observed in *unc-3(e151)* and *del-1(ok150)* mutants. **B-C**: Foraging speed and amplitude are significantly decreased in *unc-3(e151), del-1(ok150),* and *unc-8(e15lb145)* null mutants when compared to wild-type animals. Similar results were reported for *unc-3(e151), del-1(ok150),* and *unc-8(e15lb145)* mutants in [S10]. N = number of animals per genotype. q values are shown in each graph. Mean values are represented with a horizontal red line.

Figure S6 – Related to Figure 6B: **Mutation of the COE motif in the context of a *cho-1* fosmid-based reporter abrogates expression in SAB neurons**. **A:** A 30kb fosmid-based reporter for *cho-1* shows expression in the entire cholinergic nervous system including the SAB neurons (See also Figure 6B). Mutation of the COE motif in the context of this 30kb reporter selectively affects reporter gene expression in the SAB neurons (and VNC MNs that express *unc-3*), suggesting that *unc-3* directly regulates *cho-1* expression through a single binding site (COE motif). SAB identity was confirmed using the SAB-specific markers *otIs476 [glr-4^{prom}::Tagrfp]* and *otEx6068 [unc-4^{promC}::Cherry].* **B:** Quantification is provided for multiple transgenic lines that carry the "intact COE motif" and the "mutated COE motif" in the context of the *cho-1* fosmid-based reporter. N = 20 animals per transgenic line. ***, P < 0.001. Similar results were found in the VNC cholinergic MNs (See Figure 6B).

Figure S7 – Related to Figure 4A: The expression of two novel SAB-specific genes is *unc-3*-dependent. A: A reporter for the TGFbeta-like molecule *unc-129* shows expression only in SABD (not SABV) neurons. Transgenic *evIs82B[unc-129^{prom}::gfp]* animals were crossed with animals carrying the SAB specific-marker *otEx6068 [unc-4promC::Cherry].* **B:** A reporter for the receptor tyrosine kinase encoding-gene *ddr-2* shows expression only in SABV (not SABD) neurons. **C:** The expression of an *unc-129^{prom}::gfp* reporter is severely affected in SABD neurons of *unc-3* mutants (Quantification in **G**, N = 25). **D:** Evidence for direct regulation of *unc-129* by *unc-3*. Three COE motifs are present in the *cis*-regulatory region of *unc-129*. Combined mutation of COE1 and COE2 motifs results in loss of reporter gene

expression in SABD and VNC MNs. Multiple transgenic lines were analyzed for each construct. Lines indicate genomic region fused to *gfp* (green). (**+**) indicates robust expression in the SAB neurons, or ventral nerve cord MNs, or head (at least 80% of the animals) in at least 2 independent transgenic lines. (**–**) indicates complete loss of reporter gene expression in the SAB neurons and very faint residual reporter gene expression in a fraction of ventral nerve cord MNs in at least 90% of the animals (in at least 2 independent transgenic lines) when compared to transgenic animals carrying longer genomic fragments of the *cis*-regulatory region. (*) indicates that the COE motif (vertical light blue line) is conserved in at least 3 other nematode species. MUT indicates that COE motif has been mutated by substituting the same 2 nucleotides in the core sequence (for example, wild-type COE site: T**CC**CNNGGGA >> COE MUT site: T**GG**CNNGGGA). Animals at the fourth larval (L4) stage were analyzed. N = 20 animals per transgenic line. **E:** The expression of an *ddr-2*^{prom}*::gfp* reporter is severely affected in SABV neurons of *unc-3* mutants (Quantification in **G**, N = 25). Animals were analyzed at the fourth larval stage (L4). **F:** A schematic representation of the *ddr-2* locus that shows the presence of a COE motif ~ 1.5kb upstream of the gene. The *ddr-2*^{prom}*::gfp* reporter contains 3kb of *cis*-regulatory information upstream of the *ddr-2* locus.

Table S1 – Related to Figure 2: Summary of rescue analysis on *unc-3(e151)* mutants.

Table S2 – Related to Figure 3F: Quantification of mutational analysis on *madd-4 cis*-regulatory region.

Table S3 – Related to Figure 6: COE motifs are found in all *unc-3*-dependent, SAB-expressed genes

Table S4 – Related to Figure 6: Quantification of *cis*-regulatory analysis on UNC-3 target genes.

Table S5 – Related to Figure 4. Thirty putative terminal identity genes with reported expression in VNC motor neurons based on WormBase.

Table S6: Transgenic lines generated or used for this study.

Table S1. Summary of rescue analysis on *unc-3 (e151)* mutants.

Rescuing lines used	Genotype	SABD AChR clustering	SABV SYD-1 clustering
None	*N2 wild type*	+++	+++
None	*unc-3(e151)*	+	–
*otEx5569-72 [**unc-3 fosmid** clone WRM0622bH08]*	*unc-3(e151); otEx5569*	+++	+++
	unc-3(e151); otEx5570	+++	+++
	unc-3(e151); otEx5571	+++	+++
	unc-3(e151); otEx5572	+++	+++

Notes:
Acetylcholine receptor (AChR) clustering was assessed using the *kr208 (unc-29::TagRFP)* knock-in strain. SYD-1 clustering was assessed using the *juIs40 (syd-1^{prom}::SYD-1::GFP)* transgenic strain. At least 20 animals per genotype were evaluated.

– : indicates no SYD-1 signal at the SABV innervation zone.
+ : indicates very poor AChR or SYD-1 clustering at the SAB synapses in all animals examined.
+++ : indicates wild-type AChR or SYD-1 clustering at the SAB synapses in at least 90% of the animals examined.

Table S2. Quantification of mutational analysis on *madd-4 cis*-regulatory region.

madd-4L	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
WRM0626CA02 fosmid-based reporter	100	90	100	0	100	*krEx1067*	20
	100	97.1	100	0	100	*krEx1068*	35
2882 bp::gfp	100	97.1	100	0	100	*sEx14990*	35
734 bp::gfp	82	84.6	100	0	100	*otEx4946*	39
	82.5	92.5	100	0	100	*otEx5410*	40
734 bp COE1 MUT ::gfp	0	0	6.7 #	0	100	*otEx4950*	30
	0	0	100 #, ^	0	100	*otEx4951*	30
	10	0	100 #	0	100	*otEx5607*	30

madd-4B	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
WRM0626CA02 fosmid-based reporter	94.3	91.4	100	100	100	*krEx1069*	35
	94.3	88.6	100	100	100	*krEx1070*	35
4366 bp::gfp	90	100	100	100	100	*otEx5601*	40
	96.4	89.1	100	100	100	*otEx5602*	55
	93.8	100	100	100	100	*otEx5603*	48
4366 bp COE5 MUT ::gfp	82.3	82.3	100	100	100	*otEx5680*	17
	93.3	66.7	100	100	100	*otEx5681*	15
4366 bp COE2,3&5 MUT ::gfp	76.7 #	100	N. D	N. D	100	*otEx5682*	30
	56 #	96	N. D	N. D	100	*otEx5683*	25
	100 #	96.7	N. D	N. D	100	*otEx5684*	30
4366 bp COE2,3,4 &5 MUT ::gfp	84 #	100	N. D	N. D	100	*otEx5685*	25
	90 #	90	N. D	N. D	100	*otEx5686*	30
	90 #	96.6	N. D	N. D	100	*otEx5687*	30
	60 #	93.3	N. D	N. D	100	*otEx5688*	30

Notes:
* : indicates expression in SABVL and/or SABVR.
# : indicates faint expression in the respective neurons in at least 50% of the animals.
N : number of animals scored per transgenic line.
^ : indicates significantly fewer cells when compared to control construct.
N. D: Not determined.

Table S3. COE motifs are found in all *unc-3*-dependent, SAB-expressed genes				
Category	**Gene**	**COE motifs** (+/– strand)	**Motif location (relative to ATG)**	**Conserved COE motif #**
Acetylcholine pathway genes	***unc-17/cha-1***	ggaatggtccccggggagctgt (–)	–4209–4187	Yes
	cho-1	aaacggtctccagggagagaaa (–)	–5023–5001	Yes
Neurotransmitter receptors	***glr-4***	tagtattcccaacggatcttcg (+)	–3190–3167	Yes
		tgatcttcccttgggagttcat (+)	–195–172	Yes
	glr-5	taagactcaccagtgaaatata (+)	–4701–4679	N. D
	exp-1	ttatgttccgccgagttacaggc (+)	–3167–3144	No
		tctcctccctcaagggattacta (–)	–523–500	Yes
		agcgcctccccggaaggcaatgt (+)	–390–367	Yes
		taaaagtcccaatggaacattac (+)	–262–249	Yes
Ion channels	***del-1***	aacgagtccctggtgagccaca (+)	–2573–2551	Yes
		accaactcccacaagtattccg (+)	–954–932	No
		ttcgctgtcccttgggagatat (–)	–123–101	No
	unc-8	tttgtgtccccatagaagtttt (+)	+121+143	No
		cccatatccccatggaaggctt (+)	+381+403	No
		tgggagttccccgaagtgaagc (+)	+1507+1529	No
		caatgatcccaagagaggccaa (+)	+1625+1647	Yes
		agttgatccctcaggaagagga (+)	+1656+1678	Yes
Synaptogenic genes	***madd-4L***	tttggttctcctgggacctcct (–)	–2571–2559	Yes
	madd-4B	tgaagttacccatgggagtttat (–)	–1176–1153	Yes
		atcgtctcccgcgggtttaaaag (+)	–709–685	No
		atgtggtccccagataaattgga (+)	–570–547	No
		tgcggttcccttgagactttgaa (+)	+2175+2198	No

Notes:

#: Indicates that the COE motif is conserved in at least three other nematode species.

N. D: Not determined.

SEQUENCE LOGO FOR COE MOTIF

The sequence logo (shown above) was generated by combining the 22 COE motifs of the eight SAB-expressed genes presented in Supplementary Table 3. The web tool enoLOGOS was used (http://www.benoslab.pitt.edu/cgi-bin/enologos/enologos.cgi).

Table S4. Quantification of *cis*-regulatory analysis on UNC-3 target genes.

unc-17	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
3249 bp::gfp	82.5	82.5	100	0	100	*vsIs48*	40
1001 bp::yfp	96	88	100	0	100	*otEx5691*	25
	100	92	100	0	100 ^	*otEx5689*	25
	96	76	100	0	100 ^	*otEx5690*	25
1001 bp COE MUT ::yfp	0	0	N.D	0	100 ^	*otEx5715*	25
	4	4	N.D	0	100 ^	*otEx5716*	25
	0	0	N.D	0	100 ^	*otEx5717*	25

cho-1	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
cho-1 fosmid::yfp "intact" COE motif	100	100	100	0	100	*otIs321*	20
	100	85	100	0	100	*otIs323*	20
	100	100	100	0	100	*otIs534*	20
cho-1 fosmid::yfp "mutated" COE motif	15	15	0	100	100	*otEx6066*	20
	5	15	0	100	100	*otEx6067*	20
4880 bp::yfp	0	0	60 #,^	0	100	*otEx5718*	20
	0	0	0	0	100	*otEx5719*	20
	0	0	45 #,^	0	100	*otEx5720*	20
280bp:yfp	92	92	100	0	100 ^	*otEx5722*	25
	96	100	100	0	100 ^	*otEx5721*	25
	72	100	100	0	100 ^	*otEx5723*	25
280bp COE MUT ::yfp	15 #	15 #	N.D	0	100 ^	*otEx5724*	30
	24 #	28 #	N.D	0	100 ^	*otEx5725*	25
	12 #	12 #	N.D	0	100 ^	*otEx5726*	25

exp-1	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
3480 bp::gfp	75	100	100	0	100	*wyIs75*	20
792 bp:: TagRFP	72.7	90.9	100	0	100	*otEx5727*	22
	90	90	100	0	100	*otEx5728*	20
	81.8	100	100	0	100	*otEx5729*	22

del-1	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
2634 bp::gfp	100	4	100	0	100	*otEx5732*	25
	100	0	100	0	100	*otEx5730*	25
	100	10	100	0	100	*otEx5731*	20
1827 bp::gfp	100	0	100	0	100	*otEx5733*	25
	100	12	100	0	100	*otEx5734*	25
	96	0	100	0	100	*otEx5735*	25
1827 bp COE2 MUT ::gfp	100	0	100	0	100	*otEx5736*	30
	100	0	100	0	100	*otEx5737*	25
	96	0	100	0	100	*otEx5738*	25
1827 bp COE3 MUT ::gfp	0	0	100 #,^	0	100	*otEx5739*	25
	0	0	100 #,^	0	100	*otEx5740*	25
	0	0	100 #,^	0	100	*otEx5741*	25
1827 bp COE2&3 MUT ::gfp	0	0	100 #,^	0	28 #	*otEx5742*	25
	0	0	100 #,^	0	8 #	*otEx5743*	25
	0	0	100 #,^	0	12 #	*otEx5744*	25

glr-4	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
4.9 kb::gfp	100	100	88	0	100	*akEx32*	25
538 bp:: TagRFP	90	95	30 ^	0	100 ^	*otEx5745*	20
	100	100	10 ^	0	100 ^	*otEx5746*	20
	95	95	0	0	100 ^	*otEx5747*	20
538 bp COE1 MUT ::TagRFP	0	0	80 #	0	100 ^	*otEx5750*	20
	0	0	80 #	0	100 ^	*otEx5748*	20
	0	0	75 #	0	100 ^	*otEx5749*	20
938 bp:: TagRFP	0	0	95	0	100 ^	*otEx5751*	22
	4.5	0	90.9	0	100 ^	*otEx5752*	20
	0	0	100	0	100 ^	*otEx5753*	20
938 bp COE2 MUT ::TagRFP	0	0	0	0	100 ^	*otEx5754*	20
	0	0	0	0	100 ^	*otEx5756*	20
	0	0	0	0	100 ^	*otEx5755*	20

unc-8	**Percentage (%) of animals with expression in:**						
	SABVL/R*	SABD	VNC MNs		Head neurons	Transgenic line	N
			ACh MNs	GABA MNs			
2010 bp:: mCherry	95.6	82.6	100	N. D	100	*otEx5773*	23
	95.4	81.8	100	N. D	100	*otEx5774*	22

Notes:
* : indicates expression in SABVL and/or SABVR.
# : indicates faint expression in the respective neurons.
N : number of animals scored per transgenic line.
^ : indicates significantly fewer cells (less than half) expressing the reporter when compared to longer (*unc-17, cho-1, glr-4*) or control (*del-1*) reporter construct.
N. D: Not determined.

Table S5. Thirty putative terminal identity genes with reported expression in VNC motor neurons based on WormBase				
Gene	**Category**	**Transgene**	**Expression**	
			SAB MNs	Comments
acr-2	*Acetylcholine receptor*	*juIs14*	*No*	
acr-5	*Acetylcholine receptor*	*wdEx60*	*No*	
acr-16	*Acetylcholine receptor*	*wdEx419*	*No*	
trp-1	*TRP channel*	*kyIs123*	*No*	
nca-1	*Cation channel*	*sEx15028*	*No*	
rig-4	*IGCAM*	*otEx223*	*No*	
max-1	*Axon guidance molecule*	*juIs140*	*No*	
casy-1	*Transmemebrane protein*	*sIs10330*	*No*	
nra-4	*Nicotinic receptor associated*	*sIs10437*	*N. D*	Broad expression *
nspd-9	*Nematode specific peptide*	*sEx12386*	*No*	
srab-12	*GPCR, Serpentine receptor*	*sIs12174*	*No*	
klp-20	*Kinesin-like*	*sIs11790*	*N. D*	Broad expression *
twk-6	*Potassium channel*	*sIs12531*	*N. D*	Broad expression *
gpa-7	*G protein*	*sIs11457*	*No*	
nlg-1	*Neuroligin*	*sIs13247*	*No*	
egl-19	*Calcium channel*	*sEx10744*	*No*	
slo-2	*Potassium channel*	*sEx10749*	*No*	
flp-18	*FMRF-like neuropeptide*	*sEx11142*	*No*	
rgs-9	*Regulator of G protein*	*sEx11487*	*N. D*	Broad expression *
klp-13	*Kinesin-like*	*sEx11796*	*No*	
klp-4	*Kinesin-like*	*sEx11799*	*No*	
unc-129	*TGFbeta-like*	*evIs82B*	*Yes*	
klp-8	*Kinesin-like*	*sEx11857*	*No*	
cca-1	*Calcium channel*	*sEx14060*	*No*	
scd-2	*Receptor tyrosine kinase*	*sEx14933*	*No*	
ddr-2	*receptor tyrosine kinase*	*sIs10576*	*Yes*	
anat-1	Arylalkylamine N-AcetylTransferase	*sEx15588*	*No*	
glb-18	*Globin-related*	*sEx15931*	*N. D*	Broad expression *
srsx-30	*Serpentine receptor*	*sEx15999*	*No*	
tkr-3	*Tachykinin receptor*	*sEx11537*	*No*	

Notes:

Asterisk (*) indicates that the reporter gene displayed very broad expression in the nervous system. Genes with broad expression in the nervous system were not pursued further because – as shown in Figure 4D-E – such genes are likely not to be regulated by *unc-3*. N. D, not determined.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***C.elegans* strains**. Worms were grown at 20^{o}C or 25^{o}C on nematode growth media (NGM) plates seeded with bacteria (*E.coli* OP50) as a food source as previously described [S1]. Mutant alleles used in this study: LGI: *madd-4 (kr270)* [S2], *madd-4 (kr249)* [S2], *unc-29 (kr208::tagRFP)* [S3], *unc-13 (e51).* LGIV: *unc-8 (e15lb145).* LGV: *snb-1(js124)*. LGX: *unc-3 (e151), unc-3 (kr268), del-1(ok150), unc-18(e234).* Details on all reporter strains used in this study are provided in Table S6.

Generation of transgenic reporter animals. Fosmid recombineering was done as previously described [S4], using the following fosmids: *cho-1* - WRM0613dC12, *unc-3* - WRM0622bH08, and *madd-4* – WRM0626CA02. For *unc-3* and *cho-1* fosmid reporters, an SL2 spliced, nuclear localized *gfp* (for *cho-1)* or *mChOpti* (for *unc*-3) coding sequence was engineered at the C-terminus of the respective locus, as previously described [S4]. These fosmids were injected as complex arrays [S4]. The generation of the fosmid-based *madd-4* translational reporters is described in [S2].

Reporter gene fusions for *cis*-regulatory analysis described in Figure 3 and 6 were made using a PCR fusion approach [S5]. Genomic fragments were fused to *gfp*, or *yfp*, or *tagrfp* coding sequence, which was followed by the *unc-54 3' UTR.* The TOPO XL PCR cloning kit was used to introduce the PCR fusion fragments into the pCR-XL-TOPO vector (Invitrogen). Mutagenesis was performed using the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene). PCR fusion DNA fragments were injected into young adult *pha-1(e2123)* hermaphrodites at 50ng/µl using *pha-1* (pBX plasmid) as co-injection marker (50ng/µl).

Immunocytochemistry. N2 wild-type and *unc-3(e151)* strains were grown at 25oC on nematode growth media (NGM). We followed an immunocytochemical staining procedure described previously [S6]. In brief, worms were prepared for staining following the freeze-crack procedure and they were subsequently fixed in ice-cold acetone (5 min) and ice-cold methanol (5 min). Worms were transferred using a Pasteur pipette from slides to a 50mL conical tube that contained 40mL 1XPBS. Following a brief centrifugation (2min, 3000rpm),

worms were pelleted and 1xPBS was removed. Next, the worms were incubated with 300 μl of blocking solution (1XPBS, 0.2% Gelatin, 0.25% Triton) for 30min at room temperature (rolling agitation). Following removal of the blocking solution, worms were incubated over-night with a mouse RIM2 antibody which recognizes the *C.elegans* UNC-10 protein [1:10 dilution in PGT solution (1xPBS, 0.1% Gelatin, 0.25% Triton), Developmental Studies Hybridoma Bank – University of Iowa]. Next, the primary antibody solution was removed and worms were washed five times with washing solution (1XPBS, 0.25% Triton). Worms were incubated with an Alexa Fluor 594 donkey anti-mouse IgG secondary antibody (1:1000 in PGT solution, A-21203, Molecular Probes) for 3 hours at room temperature. Following 5 washes, worms were mounted on a glass slide and examined at an automated fluorescence microscope (Zeiss, AXIO Imager Z1 Stand). The immunostaining presented in Figure 7 was performed using the antibodies and a protocol described previously [S2].

Single molecule RNA fluorescent *in situ* hybridization (FISH). Populations of *wdIs5 [unc-4^{prom}::gfp]* and *unc-3(e151); wdIs5* animals were synchronized using a standard egg preparation protocol. After hatching, animals were placed on NGM plates with OP50 bacteria as a food source for 6 hours. Animals were collected at the L1 stage and the single molecule mRNA FISH protocol was followed as previously described [S7]. RNA probes for *del-1, unc-8, snb-1,* and *unc-10* transcripts were designed using the Stellaris Probe Designer website (Biosearch Technologies).

Statistical analysis. For results shown in Figures 2, 4 C-D, 7, S1, and S3 C-D, statistical analysis was performed using the Student's *t*-test (tail 2, type 2). Values are expressed as mean ± standard deviation (s.d) or standard error of the mean (sem) as indicated in each figure legend. Differences with p value < 0.001 were considered significant. For Figures S3 E-G and S5, we used Wilcoxon rank-sum to test the differences between the wild type (N2 strain) and mutant strains [*unc-3 (e151), del-1 (ok150), unc-8 (e15lb145)].* We also performed the normal distribution Student's *t*-test (unpaired samples with unequal variance). q values for Wilcoxon test are shown. For results shown in Figures 3, 4G, S4, S6 and S7G we performed Fisher's exact test (two-tailed). Data graphs presented as notBoxPlot in Figures 2H-J and 7B,E,G, I were generated using MATLAB

(http://www.mathworks.com/matlabcentral/fileexchange/26508-notboxplot-alternative-to-box-plots).

Microscopy. Worms were anesthetized using 100mM of sodium azide (NaN_3) and mounted on 5% agarose on glass slides. Images shown in Figure 2 D, E, G, Figure 3, Figure 4, Figure 5, Figure 7, and Figures S1, S3, S4, S6 and S7 were taken using an automated fluorescence microscope (Zeiss, AXIO Imager Z1 Stand). Acquisition of several z-stack images (each ~1 μm thick) was performed with the Micro-Manager software (Version 3.1) [S8]. Representative images are shown following max-projection of 2-10 μm Z-stacks using the maximum intensity projection type. Image reconstruction was performed using Image J software [S9].

Image quantification: Quantification of L-AChR fluorescence (shown in Figure 2F) at the SAB innervation zone in the *unc-29 (kr208::tagRFP)* knock-in background was performed as previously described in [S2]. Fluorescence levels were calculated along a 55 μm region of the SAB axons, which includes all SAB NMJs. We ran a Kruskall-Wallis nonparametric test followed by a Dunn's post test, using the GraphpadPrism software. To quantify the intensity of fluorescence associated with presynaptic varicosities shown in Figure S3B, the images corresponding to each axon were projected (Z projection, Max Intensity), rotated and cropped around the axon. On the channel corresponding to the presynaptic marker (*jsIs42*, GFP), following background substraction (radius = 4px) and convolution with a circle of diameter 5px, the image was segmented using the MaxEntropy method and the mask obtained was used on the postsynaptic channel (UNC-29::TagRFP) to measure the mean postsynaptic fluorescence intensity associated with each presynaptic varicosity. The mean fluorescence intensity per varicosity was averaged on each axon, then the mean and s.e.m. of the mean intensity per axon were computed for each genotype and plotted on Figure S3B.

Automatic worm tracking: Automatic worm tacking analysis was performed as previously described [S10]. Transgenic *juIs40; kyEx5161* animals at the young adult stage (day 1 of adulthood) were placed on NGM plates containing 10mM histamine and OP50 bacteria as food source. As a control, transgenic *juIs40; kyEx5161* animals at the young adult stage (day 1 of adulthood) were placed on NGM plates containing OP50 bacteria as food source but no histamine. Histamine plates were prepared as previously described in [S11]. Animals were allowed to lay eggs and the progeny reached the L4 stage at 20^{o}C. Automatic tracking was performed at ~ 25oC (room temperature) with Worm Tracker 2.0 (WT2), which uses a mobile camera to track and record individual worms and 5 min videos were generated. Analysis of the tracking videos was performed as previously described [S10] and three features that describe head movement are shown in Figure S3 E-G (positive backward head bend mean, positive backward head crawling amplitude, absolute backward foraging speed).

Wild-type (N2) and mutant [*unc-3 (e151), del-1 (ok150), unc-8 (e15lb145)]* animals were always kept at 20^{o}C. Animals were picked when they reached the fourth larval stage (L4) and moved to a (NGM) plate seeded with bacteria (*E.coli* OP50) till the second day of adulthood. Then, each animal was placed onto an NGM plate that contained at its center a single drop (50ul) of E.coli OP50 bacterial culture that served as food source and attracted the worm at the center of the plate. Automatic tracking was performed at ~ 22oC (room temperature) with Worm Tracker 2.0 (WT2), which uses a mobile camera to track and record individual worms and 5 min videos were generated. Analysis of the tracking videos was performed as previously described [S10] and three features that describe head movement are shown in Figure S5 (absolute head bend mean, absolute foraging amplitude, absolute foraging speed).

SAB neuronal silencing using the histamine chloride channel 1 (HisCl1) system. Transgenic animals carrying the HisCl1 gene under the control of the *unc-4* promoter (kyEx5161 [unc-4^{prom}::HisCl1::SL2::mCherry, elt-2^{prom}::mCherry]) were crossed with transgenic animals carrying a SAB presynaptic marker (*juIs40 [syd-1^{prom}::SYD-1::GFP])*. Transgenic *juIs40; kyEx5161* animals at the young adult stage (day 1 of adulthood) were placed on NGM plates containing 10mM or 20mM histamine and OP50 bacteria as food source. As a control, transgenic *juIs40; kyEx5161* animals at the young adult stage (day 1 of adulthood) were placed on NGM plates containing OP50 bacteria as food source but no

histamine. Histamine plates were prepared as previously described in [S11]. Animals were allowed to lay eggs and the progeny reached the L4 stage at 20°C. Transgenic *juIs40; kyEx5161* animals were mounted on a slide and the SAB innervation zone was photographed. The SAB presynaptic puncta (SYD-1::GFP) were quantified using an automated fluorescence microscope (Zeiss, AXIO Imager Z1 Stand). Transgenic *juIs40; kyEx5161* animals were kept at 20°C at all times.

Bioinformatic analysis. To predict the binding site (COE motif) for the transcription factor UNC-3 in the *cis*-regulatory region of all SAB-expressed, *unc-3*–dependent genes described here, three bioinformatic tools were used: (a) the MatInspector program from Genomatix (http://www.genomatix.de) [S12], (b) TargetOrtho, a phylogenetic footprinting tool to identify TF binding sites [S13] and (c) FIMO (Find Individual Motif Occurrences) [S14], a software tool which is part of the MEME (Multiple EM for Motif Elicitation) suite software toolkit (http://meme.nbcr.net/meme/). To use FIMO, we first generated a position weight matrix for the COE motif based on the UNC-3 binding site information from 15 *bona fide unc-3*-dependent genes that we previously described [S15]. These 15 genes contained 36 COE motifs, which were uploaded to MEME along with a background Markov model file with background intergenic nucleotide frequencies of A: 0.325 C: 0.175 G: 0.175 T: 0.325.

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