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A receptor tyrosine kinase plays separate roles in sensory integration and associative learning in *C. elegans*.

Abbreviated title: Differences in learning and sensory integration

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Conceptualization, G.S.W. and D.v.d.K.; Methodology, G.S.W. and D.v.d.K.; Formal Analysis, G.S.W. and S.F.; Investigation, G.S.W., V.W.T., E.P., D.M.M., and G.W.S.; Writing – Original Draft, G.S.W. and V.W.T.; Writing – Review and Editing, G.S.W., D.M.M., G.W.S., S.F., and D.v.d.K.; Funding Acquisition, D.v.d.K.; Resources, D.v.d.K.; Supervision, D.v.d.K.

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27 The authors declare no competing financial interests

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42 **Abstract**

43 Associative learning and sensory integration are two behavioral processes that involve
44 the sensation and processing of stimuli followed by an altered behavioral response to these
45 stimuli, with learning requiring memory formation and retrieval. We found that the cellular and
46 molecular actions of *scd-2* dissociate sensory integration and associative learning. This was
47 discovered through investigation of a *Caenorhabditis elegans* mutation (*lrn-2 (mm99)*) affecting
48 both processes. After mapping and sequencing, *lrn-2* was found to be allelic to the gene, *scd-2*.
49 *scd-2* mediated associative learning and sensory integration operate in separate neurons as
50 separate processes. We also find that memories can form from associations that are processed
51 and stored independently from the integration of stimuli preceding an immediate behavioral
52 decision.

53 **Significance Statement**

54 We show that the mutation *lrn-2*, a learning mutant derived from a random mutagenesis
55 screen is allelic to *scd-2*, a receptor tyrosine kinase. Differences in the role of *scd-2* provide the
56 first evidence for genetic, cellular, and behavioral dissociations of sensory integration and
57 associative learning in *C. elegans*. We show that *scd-2* uses different genetic and neuronal
58 pathways for its role in sensory integration versus associative learning. Furthermore, this
59 dissociation shows that sensory integration and associative learning are separate phenomena and
60 that memories can form from associations independent of initial sensory integration. This implies
61 memory formation can be separated from real-time sensory perception.

62 **Introduction**

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63 While *C. elegans* has a relatively small and simple nervous system in comparison to
64 mammals, it is capable of behavioral processes such as associative learning and sensory
65 integration (Wen *et al.*, 1997; Ishihara *et al.*, 2002; Giles *et al.*, 2006). Within *C. elegans*, these
66 two behavioral processes are seemingly similar. Associative learning involves the pairing of two
67 sensory stimuli, so that one becomes predictive of the other and leads to a persistent modified
68 behavioral response to the individual conditioned cues. Sensory integration involves the
69 processing of multiple sensory stimuli followed by a modified behavioral response in the
70 presence of the sensory stimuli; however, unlike associative learning, this response may not
71 require the retrieval of a memory. If there are further differences between sensory integration and
72 associative learning (other than presence or absence of memory formation), then such differences
73 may help illuminate the mechanisms of learning and memory function, and indeed how
74 associations are formed.

75 *lrn-2* (*mm99*) is an EMS (ethyl methanesulfonate, a mutagen that causes random point
76 mutations) derived *C. elegans* mutant with associative learning deficits across multiple
77 associative learning paradigms. Since these deficits include both olfactory and gustatory
78 learning, *lrn-2* is unlikely to be active in either olfactory or gustatory sensory neurons alone. It
79 was originally isolated in a screen for mutants with deficits in associating salt with starvation
80 (Wen *et al.*, 1997), but further research has shown *lrn-2* to have other deficits in associative
81 learning about olfactory cues such as diacetyl (Morrison *et al.*, 1999), and in occasion setting
82 (Law *et al.*, 2004). Here we reveal a deficit in the *lrn-2* mutant in another associative learning
83 paradigm that involves the pairing of the odor and pathogenicity of *Pseudomonas aeruginosa*
84 (PA14) bacteria (Zhang *et al.*, 2005). We have found that *lrn-2* has a deficit in learning about
85 PA14, and the use of this paradigm has led us to a connection between associative learning and

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86 sensory integration in *C. elegans* (Fig. 1A,B). The PA14 learning paradigm used for most of the
87 following experiments is modified from Zhang *et al*. (2005). by combining the training and
88 testing phases into a simpler assay (Fig. 1A), with comparable results to the original method
89 when the original method was employed in our hands (Fig 1B, C). While *lrn-2* has an increased
90 approach to PA14 after exposure during training, which could be the result of learning that PA14
91 has nutritive value, it does not learn that PA14 is pathogenic. This appetitive learning about
92 nutritive value may be masked in the wild type by aversive learning about pathogenicity. Since
93 *lrn-2* affects multiple associative learning paradigms it is likely that it is active downstream of
94 the sensory neurons. This differentiates it from many of the genes involved in benzaldehyde-
95 starvation associative learning, a *lrn-2* independent memory that apparently is stored in the
96 primary olfactory sensory neuron AWC (Pereira and van der Kooy, 2012). Associative learning
97 using pathogenic bacteria has been shown to require parts of the TGF-β pathway, specifically
98 *dbl-1,* which interacts with *sma-5* in the hypodermis (Zhang and Zhang, 2012). However, the
99 genetic and molecular mechanisms that connect pathogenic bacteria associative learning to other
100 paradigms affected by *lrn-2,* such as associative salt learning and diacetyl learning, have not
101 been studied previously.

102 **Materials and Methods**

103 **Strains**

104 Strains used in this paper include Bristol N2, UT2 *lrn-2 (mm99),* UT1320 *lrn-2 (mm99)*; *publ-*
105 *5::GFP,* JT249 *scd-2 (sa249)* V, RB783 *scd-2 (ok565)* V, TY3553 *scd-2 (y386)* V, FX3084 *scd-*
106 *2 (tm3084)* V, VC980 *fsn-1 (gk429)* III, ZM488 *fsn-1 (hp1)* III, ZM588 *fsn-1(hp1)* III; juls1
107 [*unc-25p::snb-1::GFP+lin-15(+)*] IV; *scd-2(ok565)* V, *pgcy-28.d::scd-2;pmyo-3::GFP,*

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108 UT1321 *pceh-2::scd-2;pmyo-2::mcherry*. Strains were grown at 20 degrees C under standard
109 conditions (Brenner, 1974).

110 **Sequence analysis and alignment**

111 Sequence reads were mapped to the *C. elegans* reference genome version WS230
112 (www.wormbase.org) using the short-read aligner BWA (Li and Durbin, 2009). Single-
113 nucleotide variants and small insertions and deletions were identified and filtered with the help
114 of the SAMtools toolbox (Li *et al*., 2009). Each variant was annotated with a custom-made Perl
115 script and gene information downloaded from WormBase.

116 **One hour Choice Assay**

117 PA14 and OP50 were grown overnight in LB medium and re-suspended at an absorbance
118 of 1.0 at 600nm. 25µL of each suspension was spotted on opposite sides (4 cm from the center)
119 of a 10 cm Petri dish filled with 30 mL of Nematode Growth Medium and allowed to air-dry for
120 2 hours. 1 µL of 10mM sodium azide was applied to each bacterial spot. 50-150 young adult
121 worms were then placed in the center of the dish and allowed to move freely for 1 hour. At 1
122 hour, worms at each bacterial spot were counted.

123 **Egg Laying Assay**

124 This assay was modified from Gardner *et al*. (2013). 50 µL of either OP50 or PA14 were
125 evenly spread on small plates and incubated at 37 degrees for 48 hours. Worms were grown from
126 eggs on OP50 for 48 hours at 23 C. Two worms were then placed on each plate, which was then
127 sealed and incubated at 20 degrees for 40 hours. For counting, adult worms were immersed in a
128 droplet of bleach solution and dissolved. Eggs produced per worm were counted as the number
129 of eggs retained after dissolving and the number of eggs laid on the plate added together.

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130 **Killing Assay**

131 Less than 100 young adult worms were placed on small plates containing either OP50 or
132 PA14 lawns on Nematode Growth Medium (NGM). The numbers of live worms were counted at
133 multiple time intervals. Worms that were moving, had a pumping pharynx, and were not sticks
134 were considered to be alive.

135 **The PA14 associative learning assay**

136 This assay is modified from Figure 1A of Zhang *et al.* (2005) to combine training and
137 testing into a single step. Age synchronized young adult worms that were raised on NGM with
138 OP50 at room temperature were used for this assay. 10 cm petri dishes were filled with 30 mL of
139 NGM. Once dry the plates were seeded with two bacterial lawns by pipetting 100 µL of OP50
140 and PA14 each obtained from overnight cultures grown in LB medium. These lawns were
141 allowed to grow for 48 hours before the commencement of the assay. 100-200 worms were
142 washed in M9, and the placed on the PA14 lawn via Pasteur pipette. Once the worms were dry,
143 the plates were sealed in Parafilm and left for the remainder of the assay (4 hours). The assay
144 plates were then refrigerated and scored later. The number of worms remaining on the PA14
145 lawn were counted and divided by the total number of worms on the plate to provide the
146 percentages seen in the assay figures.

147 In Fig. 1B the assay included separate training and testing phases. Preparation was the
148 same as above until the plating of worms onto PA14 plates. In this case, training plates contained
149 a PA14 lawn only. 100-200 worms were washed in M9, and the placed on the PA14 lawn via
150 Pasteur pipette. Once the worms were dry, the plates were sealed in Parafilm and left for the
151 remainder of the assay (4 hours). Worms were then washed off these plates and placed on the

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152 centre of testing plates with 50 μL point sources of PA14 and OP50 on either side of the plate for
153 one hour. In naïve conditions, worms were placed on OP50 plates for 4 hours in lieu of PA14
154 training. These worms were then subjected to the same testing phase as PA14 exposed worms.

155 **Sensory integration and copper acetate memory experiments**

156 The sensory integration assay was performed as described in Figure 1A of Ishihara *et al.*
157 (2002). The assay testing for a learned response to copper (II) acetate is modified from those
158 methods. 10 cm NGM petri dishes used for the training phase were split into three groups. For
159 the paired condition, a barrier of 30 μL of 30 mM copper (II) acetate solution (in water) was
160 spread across the middle of the plate and the gradient was allowed to set for 18 hours. Then, age
161 synchronized, young adult worms washed in M9 were placed on one side of the copper barrier,
162 and a 2 μL droplet of 1:100 diacetyl was placed on the opposite side. Worms were dried with a
163 Kimwipe, the plate was sealed with Parafilm and left for 1 hour. In the control conditions, worms
164 were either exposed to a 30 μL drop of 30mM copper (II) acetate without diacetyl or a naïve
165 condition with no stimuli added to the plate. The testing phase used plates with a 30 μL drop of
166 30mM copper (II) acetate left for 18 hours. After the completion of the training hour, worms
167 were gently washed off the training plates with M9. Worms from all conditions were then
168 pipetted onto the centre of testing plates, dried with Kimwipes, sealed, and left for 1 hour. Plates
169 were then refrigerated and scored. Scoring for sensory integration was measured by counting the
170 numbers of worms on each half of the plates and dividing the number on the copper side by the
171 total number on each plate.

172 **Statistical Analyses**

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173 Statistical analyses were performed with Prism (Graphpad). The t-test was used for
174 comparisons of two variables, while experiments with multiple conditions were analyzed using
175 ANOVA with Bonferroni corrections for multiple comparisons. For experiments in which groups
176 have two independent variables for comparison, Two way ANOVA was used, with Tukey's test
177 for multiple comparisons. These analyses are summarized in Table 1.

178 Table 1. Statistical table

	Data Structure	Type of Test	Power (α=0.05)
a (Fig. 1A)	Normally distributed	Student's T-test	0.0005
b (Fig. 1B)	Normally distributed	One way ANOVA	<0.0001
c (Fig. 1E)	Normally distributed	One way ANOVA	0.0007
d (Fig. 1F)	Normally distributed	Two way ANOVA	Bacterial strain <0.0001 Worm strain 0.8379
e (Fig. 1G)	Normally distributed	Student's T-test	0.2824
f (Fig. 1H)	Normally distributed	One way ANOVA	0.0003
g (Fig. 1I)	Normally distributed	Two way ANOVA	Strain 0.8493 Time
h (Fig. 2A)	Normally distributed	One way ANOVA	<0.0001
i (Fig. 2B)	Normally distributed	One way ANOVA	<0.0001
j (Fig. 2C)	Normally distributed	One way ANOVA	<0.0001
k (Fig. 3A)	Normally distributed	One way ANOVA	<0.0001
l (Fig. 3B)	Normally distributed	One way ANOVA	<0.0001

m (Fig. 4A)	Normally distributed	One way ANOVA	0.0034
n (Fig. 4B)	Normally distributed	One way ANOVA	<0.0001
o (Fig. 4C)	Normally distributed	One way ANOVA	0.0074
p (Fig. 4D)	Normally distributed	One way ANOVA	<0.0001
q (Fig. 5A)	Normally distributed	Two way ANOVA	Worm strain 0.0217 Training cond. 0.0013
r (Fig. 5B)	Normally distributed	Two way ANOVA	Worm strain 0.0986
s (Fig. 5C	Normally distributed	Two way ANOVA	Training cond. 0.6410 Worm strain 0.1643 Training cond. <0.0001

Results

Mapping and identification of the *lrn-2* mutation locus

Zhang *et al*. (2005) showed that *C. elegans* forms associative memories about PA14's olfactory cues and pathogenicity, and the *lrn-2* deficit shown here in this assay (Fig. 1A, B, C) demonstrates that this form of associative learning has shared genetic requirements with salt associative learning, diacetyl associative learning, and sensory integration (Fig. 1D, E) (Wen *et al.*, 1997; Morrison *et al.*, 1999; Ishihara *et al.*, 2002). We first tested whether deficits in PA14 learning might be secondary to the effectiveness of PA14 pathogenicity. However, in both N2

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188 and *lrn-2*, PA14 exposure causes an equivalent decrease in egg production, indicating that both
189 strains are similarly affected by the pathogenicity of PA14 (Fig. 1F) (Gardner *et al.*, 2013). It is
190 still possible that the learning deficit in *lrn-2* is related to an inability to detect PA14
191 pathogenicity, despite being affected by it. N2 and *lrn-2* also have equivalent approach to both
192 PA14 and OP50 *E. coli* after one hour showing that they do not differ in baseline odour sensation
193 (Fig. 1G). There is no significant difference in the rate at which N2 and *lrn-2* worms are killed
194 by PA14 pathogenicity (Fig. 1H).

195 *lrn-2* emerged as a recessive loss-of-function mutation from a random mutagenesis
196 screen (Wen *et al.*, 1997; Morrison *et al.*, 1999). We determined the locus of the *lrn-2* mutation
197 within the genome in order to investigate further its function within associative learning
198 pathways. Preliminary snip-SNP mapping indicated that a portion of chromosome V was the
199 likely region of interest. Whole genome sequencing was performed on both *lrn-2* and the N2
200 reference strain, and after analysis of the sequence using SAMTools, a short list of non-silent,
201 non-synonymous mutations was found in the identified region of chromosome V (Li *et al.*,
202 2009). The only mutant gene that also was not found in the reference strain within the snip-SNP
203 targeted region was *scd-2*. The mutation is a missense mutation causing an A-P amino acid
204 change in the extracellular glycine rich region, which has been shown to be conserved in
205 *Drosophila* and human homologues of *scd-2*, and there is evidence in *Drosophila* that this region
206 is important for protein function (Lorén *et al.*, 2003). In tests to confirm *scd-2* as the site of the
207 *lrn-2* mutation, the *lrn-2* mutant failed to complement an *scd-2* mutant strain, and insertion of a
208 fosmid (WRM0614dF06) containing wild type *scd-2* into a *lrn-2* mutant background rescued
209 associative learning (Fig 2 A, B). The complementation test crossed *scd-2* (*ok565*) worms with a
210 glowing *lrn-2* strain. The glowing *lrn-2* strain was created by crossing *publ*-5::GFP worms in a

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211 wild type background into *lrn-2*, selfing the progeny, identifying which progeny displayed the
212 *lrn-2* learning phenotype in a PA14 assay, and confirming with sequencing for the mutation
213 identified previously by whole genome sequencing. Together, the complementation test and the
214 fosmid rescue confirm that the *lrn-2* mutation is located in the *scd-2* gene.

215 The *scd-2* gene codes for a receptor tyrosine kinase that is homologous to mammalian
216 anaplastic lymphoma kinase (ALK), which has been shown to affect mouse learning and
217 memory in a suppressive manner (Reiner *et al.*, 2008; Weiss *et al.*, 2012). Worms with mutations
218 in *scd-2* show deficits in sensory integration, and mutations in *hen-1*, the suggested ligand of
219 *scd-2,* are salt learning deficient (Ishihara *et al.*, 2002; Shinkai *et al.*, 2011).

220 As predicted, three other strains with separate *scd-2* mutant alleles (including a null
221 mutant with a large deletion (*ok565*)) have similar learning deficits to the *lrn-2* strain in the
222 PA14 associative learning assay (Fig. 2C). While worms carrying different *scd-2* alleles have
223 learning deficits of slightly varying magnitudes (Fig. 2C), this is likely caused by differences in
224 the functional severity of the mutated *scd-2* gene product. Similarly, *lrn-2* mutant worms show a
225 deficit in sensory integration much like *scd-2*(*sa249*), where the deficit is represented as the
226 inability to integrate opposing cues when presented with an attractive diacetyl odor point source,
227 sensed by AWA neurons, beyond an aversive copper acetate barrier, sensed by ASH neurons
228 (Fig. 1E) (Shinkai *et al.*, 2011). In order to examine whether the "*scd*" (suppression of
229 constitutive dauer) phenotype had an influence on PA14 associative learning, two other *scd*
230 strains were tested. Neither *scd-1* nor *scd-3* have notable deficits in learning about pathogenic
231 bacteria, indicating that the role of *scd-2* is likely not caused by processes related to a deficit in
232 dauer development (Fig. 2D).

233 **FSN-1 acts upstream of SCD-2 in sensory integration, but not in associative learning.**

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234 The F-Box gene *fsn-1* regulates synapse formation, as part of a SCF ubiquitin ligase
235 complex, and SCD-2 is a downstream target of FSN-1 (Liao *et al.*, 2004; Li *et al.*, 2011). FSN-1
236 has an intracellular interaction with SCD-2, likely through ubiquitination, causing SCD-2 down-
237 regulation and preventing development of abnormal synapse morphology at the neuromuscular
238 junction (Liao *et al*., 2004). Li *et al.* (2011) showed that *scd-2* also plays a role in sensory
239 integration and found that FSN-1 suppresses SCD-2's effect on sensory integration. We also
240 confirmed that the *fsn-1;scd-2* double mutant has a deficit in sensory integration similar to *scd-2*
241 alone (Fig. 3A). We predicted that SCD-2 could have a similar relationship to FSN-1 in
242 associative learning. However, the results of testing *fsn-1 (hp1)*, *scd-2 (ok565)*, and double
243 mutants (*fsn-1;scd-2)* in the PA14 associative learning assay shows that the double mutants
244 demonstrate wild type learning (Fig. 3B). This differs from the double mutant's sensory
245 integration phenotype (Fig. 3A) and indicates that even though the *fsn-1* single mutant does not
246 have a deficit, there is an interaction between FSN-1 and SCD-2 that is revealed by the double
247 mutant. This interaction rescues wild type learning even though there is a mutation in *scd-2*
248 present. Thus, the role of FSN-1, as part of the SCF ubiquitination complex, has a different
249 interaction with SCD-2 in associative learning, and this difference in the interaction between
250 these proteins provides evidence for a dissociation of the molecular pathways governing sensory
251 integration and associative learning.

252 ***scd-2* expression is required in different neurons for sensory integration and associative**
253 **learning**

254 The site of activity for *scd-2* in sensory integration has been found to be within the AIA
255 interneuron, since expression of wild type *scd-2* in AIA (under the AIA specific *gcy-28.d*
256 promoter (*pgcy-28.d::scd-2;pmyo-3::GFP*) within a *scd-2* mutant background) is sufficient to

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257 rescue wild type sensory integration (Fig. 4A) (Shinkai *et al.*, 2011). If the roles of *scd-2* in
258 sensory integration and associative learning are part of the same process, then it could be
259 expected that AIA interneurons are similarly important for learning, as the mechanism would be
260 shared. However, the differential role of *fsn-1* in mediating sensory integration, as opposed to
261 associative learning, suggests that *scd-2* mediated learning and memory may require other sites
262 of action. If *scd-2* has independent roles in sensory integration and associative learning, it would
263 not necessarily require AIA expression for learning. Using the same rescue strain as the previous
264 sensory integration rescue experiment, which expresses *scd-2* exclusively in AIA, we tested
265 whether AIA expression was sufficient to rescue associative learning using the PA14 pathogenic
266 bacteria assay (Shinkai *et al.*, 2011). Expression of wild type *scd-2* in AIA was not sufficient to
267 rescue learning in an *scd-2* mutant background (Fig. 4B). This suggests that the site of *scd-2*
268 activity in associative learning and memory formation is in neurons other than AIA. Two
269 remaining candidate neurons with endogenous expression are PVT and NSM (Zhang *et al.*,
270 2014). NSM neurons are serotonergic and are involved in sensation of bacteria, and control of
271 foraging behaviors, both of which affect PA14 learning (Zhang *et al.*, 2005; Rhoades *et al.*,
272 2018). Expression of wild type *scd-2* in NSM using the *ceh-2* promoter in an *scd-2* mutant
273 background was not sufficient to rescue sensory integration (Fig. 4C), and indeed sensory
274 integration is AIA mediated. However, expression of wild type *scd-2* in NSM does lead to a
275 rescue of associative learning when trained to PA14 (Fig. 4D), which indicates that wild type
276 *scd-2* expression in NSM is sufficient for associative learning. Therefore, wild type *scd-2* must
277 be expressed in different neurons for sensory integration (AIA) and associative learning (NSM),
278 showing that these two phenomena are also dissociated at a cellular level.

279 **Training to copper acetate and diacetyl produces an *scd-2* independent memory**

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280 The gene *scd-2* plays roles in both sensory integration and associative learning—roles
281 that may be part of two independent processes that can be dissociated at the genetic and cellular
282 levels. Indeed, sensory integration involves the sensation of two stimuli and an immediate
283 behavioral choice in the presence of both stimuli. This description of sensory integration differs
284 from associative learning only in the lack of the persistence of the behavioral change (in the form
285 of a memory) with associative learning. If associative learning and sensory integration are truly
286 independent processes, an organism should be able to learn whilst sensory integration is blocked,
287 and vice versa. Furthermore, the same sensory cues that lead to a sensory integration decision,
288 could simultaneously form an associative memory. In order to investigate if there was a memory
289 formed by presentation of two cues to test for sensory integration, a modified version of the
290 diacetyl-copper sensory integration assay that includes separate training and testing phases was
291 used. This would demonstrate whether diacetyl and copper form an associative memory while
292 the worm is integrating these two cues. During training, N2 and *scd-2* worms were exposed to
293 diacetyl and copper, as in the sensory integration assay (Fig. 1D) for one hour, along with naïve
294 and copper only control groups. Both strains were then tested on plates with a point source of
295 copper acetate for one hour and their chemotaxis responses were recorded. When trained in
296 presence of both attractive diacetyl and aversive copper acetate, worms form a memory of the
297 association between these stimuli (Fig. 5A). This is shown by the attenuation of copper aversion
298 for conditioned worms in the testing phase when compared with single stimulus trained animals
299 (Fig. 5A). When tested to diacetyl instead of copper, there is no change in diacetyl approach,
300 implying that there is a ceiling effect (Fig. 5B). Furthermore, this learned response to copper
301 requires simultaneous presentation of both copper and diacetyl, since there is no learning when
302 presented sequentially (Fig. 5C). This indicates that copper approach reflects an associative

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303 memory. Since both N2 and *scd-2* worms are able to learn about copper acetate, this memory
304 must be separate from *scd-2* mediated memories and it must not require *scd-2* mediated sensory
305 integration in order to associate copper acetate and diacetyl. Even though an *scd-2* mutation
306 often leads to a deficit in learning, *scd-2* worms are able to form a memory when copper and
307 diacetyl are paired. This is possible because there are multiple learning circuits for different
308 types of memories in worms; *scd-2* mutant worms are able to pair benzaldehyde and starvation,
309 and can successfully learn about mechanosensory stimuli (Nuttley *et al*., 2002). Similarly, not
310 every gene involved in benzaldehyde starvation learning plays a role in *scd-2* mediated PA14 or
311 salt learning. This is a clear dissociation of sensory integration and associative learning at the
312 level of behavior, suggesting that memories formed by pairing copper acetate and diacetyl are
313 formed independently of the sensory integration of the same two stimuli as measured by the
314 sensory integration assay.

315 **Discussion**

316 The results of our experiments suggest that wild type *scd-2* plays independent roles in
317 associative learning and sensory integration. While the complete genetic and cellular circuits for
318 these two processes are not fully known, they both appear to use some of the same genetic
319 machinery within independent circuits. Associative learning requires a persistent alteration of the
320 behavioral response that can be observed during testing after the initial pairing of cues and in the
321 presence of the conditioned cue alone. This persistent response is caused by the formation of a
322 memory that can be repeatedly accessed when cues are presented at testing. Sensory integration
323 also a change in behavioral response, but this response is an immediate decision after cue
324 exposure, not a persistent learned choice. It is conceivable that sensory integration is a necessary
325 early step in processing sensory information that leads to an associative memory. If this were the

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326 case, we would expect to see *scd-2* acting in the same pathways for both processes, but our data
327 suggests that *scd-2* operates in independent genetic and cellular pathways. We describe this
328 difference as a dissociation because it is these data concerning the difference in *scd-2* activity
329 that identify the independence of the genetic and cellular circuits involved.

330 Associative memories that require *scd-2* cover a variety of sensory modalities (Wen *et*
331 *al.*, 1997; Ishihara *et al.*, 2002), but remain separate from AWC primary olfactory neuron
332 mediated associations such as benzaldehyde/starvation pairing (Nuttley *et al*., 2002), and
333 memories formed during the initial integration of diacetyl and copper acetate. Worms with *scd-2*
334 mutations do not have known deficits in chemosensation, mechanosensation, thermosensation,
335 nor in non-associative learning (Rankin *et al.*, 1990; Morris *et al.*, 1997; Mori, 1999). Further,
336 the present data show that associative memories of a copper and diacetyl pairing can be present
337 in the same *scd-2* mutants that are unable to undergo sensory integration of copper and diacetyl
338 nor PA14 associative learning (Fig. 5A, see below). Indeed, the present work provides evidence
339 that sensory integration and associative learning are dissociable at the genetic, cellular, and
340 behavioral levels. These data suggest that there are deeper differences between sensory
341 integration and associative learning in *C. elegans* than simply the formation of separate
342 memories. Not only are sensory integration and associative learning dissociated, but also learned
343 associations can be formed through a process completely independent from sensory integration
344 that must be occurring simultaneously and in parallel. With *scd-2* orthologs present in other
345 species, including mammals (Morris *et al.*, 1997), it is possible that this dual role in dissociated
346 processes is conserved across taxa, providing opportunity to investigate the function of
347 mammalian learning and memory genes in this genetically tractable nematode model.

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348 The transcription factor FSN-1 has an established role in regulating synapse formation at
349 the neuromuscular junction, and *fsn-1* mutants display both abnormal synapse morphology (Liao
350 *et al.*, 2004) and enhanced sensory integration (Li *et al.*, 2011). Experiments with double mutants
351 of *fsn-1* and *scd-2* show an *scd-2*-like sensory integration deficit (Fig. 3A) (Li *et al.*, 2011).
352 While this relationship regulates both sensory integration and the neuromuscular junction, the
353 roles of SCD-2 and FSN-1 in associative learning about pathogenic bacteria must be part of an
354 independent pathway because the double mutant does not produce a deficit in PA14 associative
355 learning. Since *fsn-1;scd-2* double mutants are phenotypically similar to *fsn-1* alone in
356 associative learning about pathogenic bacteria, there must be a different interaction between
357 FSN-1 and SCD-2 compared with their activity during sensory integration. If these two proteins
358 did not interact in PA14 learning, the double mutant would be expected to show a deficit similar
359 to *scd-2* alone; the *fsn-1* mutation somehow masks the *scd-2* learning deficit (Fig. 3A, B). There
360 is further evidence of separate *fsn-1* dependent and independent pathways containing shared
361 components from previous research. The insulin-like protein, INS-6, acting through the DAF-2
362 receptor has an antagonistic relationship with FSN-1 in which reduced insulin signalling rescues
363 the *fsn-1* abnormal synapse morphology phenotype (Hung *et al.*, 2013). Furthermore, INS-6
364 plays a role in enabling associative learning about pathogenic bacteria by repressing transcription
365 of *ins-7* (Chen *et al.*, 2013). Thus, wild type *ins-6* plays a role in synapse formation that is
366 phenotypically similar to the role of wild type *scd-2*, but its role in associative learning may not
367 be through negative regulation of FSN-1. The effect of *ins-6* on learning also happens through a
368 different mechanism than its antagonistic relationship with *fsn-1* in sensory integration; this
369 further supports the notion of that sensory integration and associative learning are independent
370 processes with some shared genetic components. While a connection between *scd-2* and *ins*-6

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371 remains unknown, we have found in the present results that *scd-2* could have a similar
372 relationship with to *fsn-1* as *ins-6*, in which sensory integration appears to require *fsn-1*
373 suppression but associating pathogens with odor operates independently.

374 Expression of wild type *scd-2* from fosmid injection employing the endogenous promotor
375 rescues both sensory integration and associative learning, but the individual cells necessary for
376 each process must be established. We have shown that targeted expression of wild type *scd-2*,
377 under an AIA interneuron specific promoter, while sufficient for rescue of sensory integration in
378 an *scd-2* mutant background, is not sufficient for rescue of associative learning (Fig. 4A,B).
379 However, wild type *scd-2* expression in NSM did lead to a rescue of the associative learning
380 phenotype in the mutant, so NSM neurons are the cellular location of *scd-2* activity during PA14
381 associative learning (Fig. 4D). NSM neurons endogenously express wild type *scd-2* (Mckay *et*
382 *al.*, 2003; Zhang *et al.*, 2014), and are serotonergic, located in the head, play a role in sensation
383 of bacteria, and mediate foraging behavior (Avery *et al*., 1993; Rhoades *et al.*, 2018). There also
384 is evidence that NSM regulates attractive food related response to bacteria, which could indicate
385 that the present findings concerning the *scd-2* mutation are caused by the inability to properly
386 identify whether PA14 or OP50 are the more attractive or nutritious bacteria (Zhang et al., 2005).
387 These features of NSM are all consistent with the features of PA14 learning, since learning about
388 PA14 requires serotonin, uses bacteria as an unconditioned stimulus, and leads to modified
389 foraging behavior when worms no longer approach PA14. The difference in cellular localization
390 of *scd-2* expression in sensory integration and associative learning provides another dissociation
391 of these two processes at the circuit level. While *scd-2* is involved in both processes, these data
392 indicate that *scd-2* expression and activity are required in separate neurons in two dissociated
393 circuits that have differing genetic interactions with *fsn-1*. While neuronal circuits for sensory

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394 integration have been proposed previously (Ishihara *et al.*, 2002; Shinkai *et al.*, 2011), the
395 neuronal circuit for PA14 learning is less complete. ADF has been shown to be important for
396 serotonergic activity, *ins-6* and *ins-7* have been shown to communicate through URX and RIA,
397 *dbl-1* activity requires release from AVA to ASI or hypodermal cells (Zhang and Zhang, 2012;
398 Chen *et al.*, 2013), and we now know that *scd-2* also plays a role in NSM. Since the wild type
399 ligand of *scd-2*, *hen-1* can still rescue sensory integration even when expressed in neurons that
400 do not endogenously express it; *hen-1* may not need to be expressed in neurons that synapse
401 directly with NSM for *scd-2* learning (Ishihara *et al.*, 2002). Thus, it is possible that detection of
402 PA14 odors by AWC (Ha *et al.*, 2010) leads to HEN-1 release from AIY, which travels to SCD-
403 2 receptors in NSM, causing an learned alteration of foraging behavior. Nevertheless, how this
404 part of the proposed neuronal circuit connects with the URX, ADF and AVA neurons will
405 require further study.

406 The existence of multiple associative learning circuits, both *scd-2* independent and
407 dependent, provides an opportunity to investigate whether sensory integration plays a role in the
408 initial sensory cue processing step in memory formation. *scd-2* does not have deficits in all forms
409 of associative learning, so the finding that pairing copper acetate and diacetyl leads to the
410 formation of an *scd-2* independent memory (Fig. 5A) is not remarkable on its own. However, the
411 evidence that an *scd-2* independent memory can be formed after training to copper acetate and
412 diacetyl cues, despite a deficit in integrating those cues, suggests that the formation of such a
413 memory uses a mechanism of sensory association that is independent of the decision-making
414 process during sensory integration. Therefore, sensory integration of opposing stimuli is not the
415 process by which the cues are associated to form memories, and associative learning must be
416 occurring both simultaneously and independently of sensory integration. While the initial

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417 associative mechanism used to form copper acetate and diacetyl associative memories is still
418 unknown, the dissociation at the level of sensory integration and associative learning is
419 consistent with the dissociations of these two behavioral processes at the circuit and molecular
420 levels.

421 In worms, if associations can be formed independently of sensory integration, then there
422 must be a difference in how a memory is represented versus how integrated stimuli are
423 represented within the worm nervous system, much like the differential processing of contextual
424 cues in mammals (Skinner *et al.*, 1994; Good *et al.*, 1998). One possibility is that sensory
425 integration leads to a blending of two stimuli in a single representation that leads to an
426 appropriate motor response, while associative learning encodes an association between the
427 separate representations of two stimuli. For example, integration of copper and diacetyl would
428 create a temporary blended representation that is interpreted as “copper tastes different and better
429 mixed with diacetyl”, and the worm’s motor response would depend on salience of these cues
430 when presented together. Since a sensory integration response is not memory dependent and only
431 functions in the presence of both stimuli, it can be predicted that the worm is responding to a
432 temporary perceived representation of a blended stimulus. Since associative memories can be
433 retrieved after training by a single associated cue without required simultaneous presentation, it
434 is likely that the worm processes associative learning as two separately stored but paired cues
435 instead of a blended representation. Memory recall could activate a representation of the
436 conditioned stimulus that co-activates a separate representation of the unconditioned stimulus
437 and triggers the appropriate behavioral response. Such an engram preserves the associative
438 information learned during training, while allowing post-training retrieval without the presence
439 of both stimuli simultaneously.

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440 Sensory integration and associative learning, while both involving detection and
441 processing of sensory cues followed by a behavioral choice, are dissociated at three distinct
442 levels. Differences in the FSN-1 interaction with SCD-2 dissociate these processes through their
443 molecular mechanisms. Activity of SCD-2 is required in different cells for sensory integration
444 and associative learning, indicating a dissociation of the neuronal circuitry behind these
445 processes. The ability to form *scd-2* independent associative memories without *scd-2* mediated
446 sensory integration indicates that these processes are also functionally dissociated at the
447 behavioral level. This dissociation suggests that wild type *scd-2* is an important regulator of
448 multiple processes. *scd-2* also has been shown to regulate forgetting of memories dependent on
449 AWA sensory neurons, while our findings show an *scd-2* mediated independent regulation of
450 memory formation of AWC based PA14 learning (Kitazono *et al.*, 2017) The independent
451 importance of wild type *scd-2* in forgetting, sensory integration, and associative learning shows
452 that it is an important gene to study for further understanding of nematode behavior. While the
453 details of the molecular, cellular, and behavioral mechanisms behind sensory integration and
454 associative learning are not fully known, *scd-2* provides evidence that they are two independent
455 behavioral processes governing *C. elegans* behavior. Understanding the nature of such
456 dissociations may be able to inform how learning and memory occur both within the nematode,
457 and potentially, across taxa.

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544 FIGURES:

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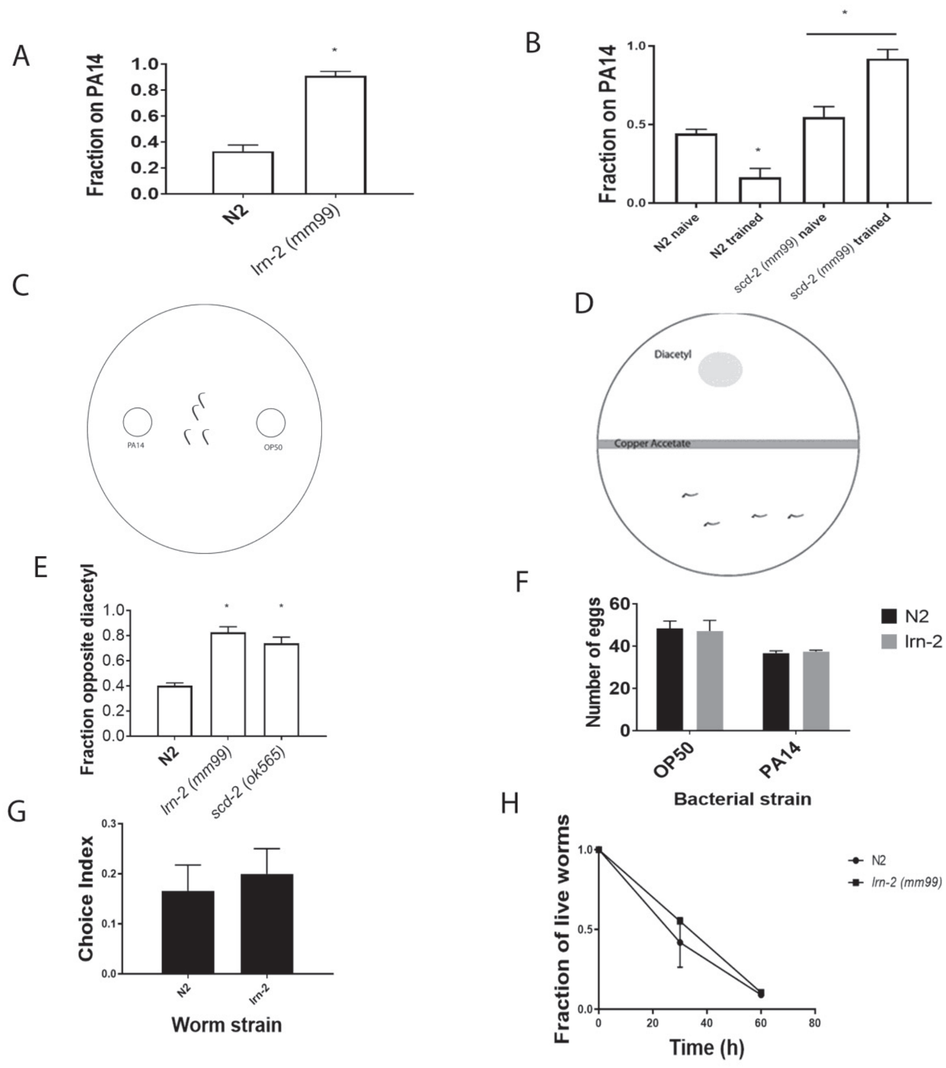

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547 Figure 1. The *lrn-2* mutation displays both associative learning and sensory integration deficits.

548 A) This assay is modified from Zhang *et al*. (2005), Figure 1A. It simplifies the procedure by
549 combining the testing and training phases into a single plate. When assayed for
550 associative learning by pairing odor and pathogenicity of PA14, *lrn-2* mutants did not
551 learn to leave the pathogenic lawn, while N2 worms made the association and move to
552 the safer OP50 bacteria. Learning was compared to wild type N2 using a Student's T test.
553 * $p<0.05$; $n\geq3$ plates; means ± SEMs.

554 B) When assayed for associative learning, using the original method in Figure 1A of Zhang
555 *et al*. (2005) that includes separate training and testing trials, *lrn-2* mutants still did not
556 learn a negative association with the pathogenic lawn (one-way ANOVA ($F_{(3,14)}$ =
557 27.54; $p < 0.0001$) with Bonferroni correction (* $p<0.05$); $n\geq5$ plates; means ± SEMs),
558 while N2 worms made the association and chose the safer OP50 bacteria when presented
559 with point sources of OP50 and PA14 and compared with naïve worms that were not
560 exposed to PA14 during training. This is comparable to the modified assay in 1A. *lrn-2*
561 mutants have an increased attraction to PA14 after training, which may be the result of
562 appetitive learning that is masked by aversive learning in wild type. Furthermore, this
563 deficit may be caused by an inability to properly detect the pathogenicity of PA14 in the
564 mutant.

565 C) The experimental set up for the testing phase of the PA14 assay. After exposure to PA14
566 for four hours, worms are transferred to a new plate, in which they are placed between
567 two point sources of bacteria. One side of the plate has a point of PA14 and the other side
568 has a point of OP50. Worms in the middle can crawl to either side depending one
569 learning.

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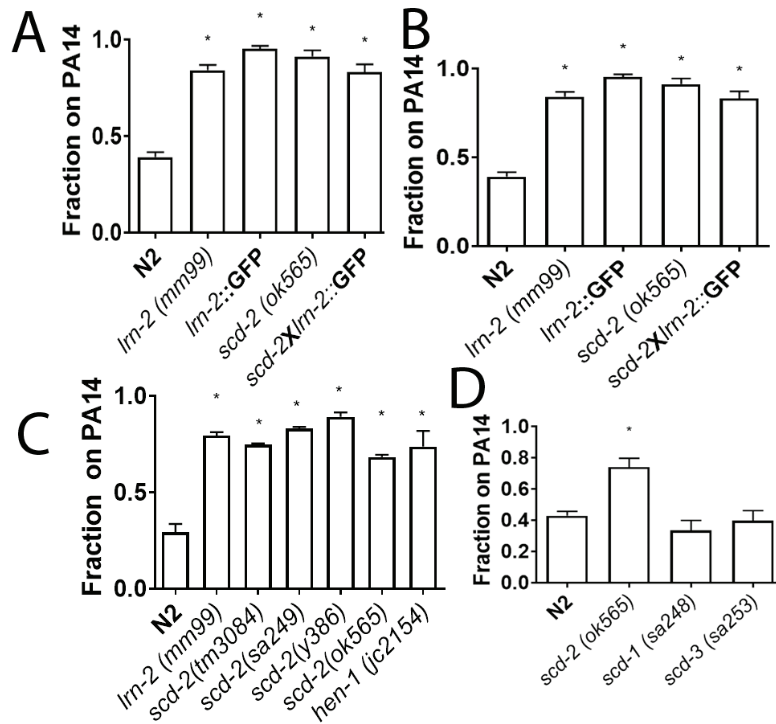
570 D) The experimental set up for the sensory integration assay, based on Figure 1A of Ishihara
571 *et al*. (2002). A Petri dish of NGM with a barrier of Copper (II) acetate down the middle
572 and a droplet of diacetyl on one side is used. Worms are placed opposite the diacetyl spot
573 and after one hour, worms on each side of the copper barrier are counted. Worms that are
574 able to integrate two opposing sensory cues and cross the aversive barrier to reach
575 diacetyl are considered to have normal sensory integration.
576 E) Both *lrn-2 (mm99)* and *scd-2 (sa249)* failed to integrate copper and diacetyl cues, as they
577 crossed the aversive copper barrier to reach an attractive diacetyl odor less than wild type
578 N2 (one-way ANOVA ($F_{(2, 6)} = 30.83$; $p < 0.001$) with Bonferroni correction (* $p<0.05$);
579 $n \geq 3$ plates; means ± SEMs).
580 F) N2 and *lrn-2* both produced similarly fewer eggs on PA14 after 40 hours compared to
581 N2. Numbers of eggs produced (both laid and retained in the gonad over the 40 hour
582 assay) were analyzed using a two-way ANOVA. There was a significant main effect of
583 bacterial strain ($F_{(1,24)} = 87.02$; $p < 0.0001$; $n \geq 6$ worms; means ± SEMs), but no
584 significant effect of worm strain nor any significant interaction, showing that N2 and *lrn-*
585 *2* worms showed similar suppression of egg laying in response to food deprivation.
586 G) Given a choice between *P. aeruginosa* (PA14) and OP50 *E. coli*, both N2 and *lrn-2*
587 mutants showed a similar naïve preference for PA14 after one hour (Student's t-test. n.s.,
588 not significant; $n \geq 6$ plates; means ± SEMs), despite its pathogenicity. Positive
589 chemotaxis index indicates PA14 approach.
590 H) Both N2 and *lrn-2* worms die at a similar rate when exposed to pathogenic bacteria
591 (PA14), therefore it is unlikely that the difference in learning is caused by a resistance to

592 PA14 pathogenicity. Using two way ANOVA, there was no significant effect of time or

593 strain.



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596 Figure 2. *scd-2* is the locus of the *lrn-2* mutation.

597 A) The F1 progeny of a cross between *scd-2 (RB783)* and a GFP tagged *lrn-2* strain showed

598 that these mutations do not complement, and that the cross progeny still have the mutant

599 PA14 learning deficit compared to wild type N2 using a one-way ANOVA ($F_{(4,35)}$ =

600 59.50; $p < 0.0001$) with Bonferroni correction (* $p<0.0001$); n≥7 plates; means ± SEMs.

601 This indicates that the *lrn-2* mutation is likely located in the *scd-2* gene.

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602 B) A fosmid containing wild type *scd-2* was expressed in a *lrn-2 (mm99)* background.
603 When tested for PA14 learning the worms expressing the fosmid and the *myo-*
604 *2::mCherry* co-injection marker showed a rescue of N2 like learning. The rescue
605 phenotype was not significantly different when compared to wild type N2 using a one-
606 way ANOVA ($F_{(4,27)} = 18.47$; $p < 0.0001$) with Bonferroni correction (* $p<0.01$; $n \geq 4$
607 plates; means ± SEMs). Non-glowing worms were counted as not containing the fosmid
608 and show a learning deficit. These data suggest that expression of *scd-2* can alleviate the
609 mutant deficit, further indicating that it is the locus of the *lrn-2* mutation.
610 C) Four *scd-2* mutant alleles containing both point mutations and deletions replicated the
611 deficit in learning to avoid pathogenic bacteria displayed by *lrn-2*. Learning in *lrn-2* was
612 reduced in comparison to wild type N2 worms as shown by a one-way ANOVA ($F_{(5, 9)} =$
613 76.74; $p < 0.0001$) with Bonferroni correction for multiple individual comparisons (*
614 $p<0.01$, n.s., not significant); $n \geq 3$ plates; means ± SEMs.
615 D) Other suppressor of constitutive dauer mutants, *scd-1* and *scd-3* do not show deficits in
616 associative learning about PA14 (one way ANOVA $F_{(3,13)} = 13.18$; $p < 0.001$with
617 Bonferroni correction. * $p<0.01$, n.s., not significant; $n \geq 3$ plates; means ± SEMs). This
618 suggests that the learning deficit is not caused by the dauer related phenotype alone.

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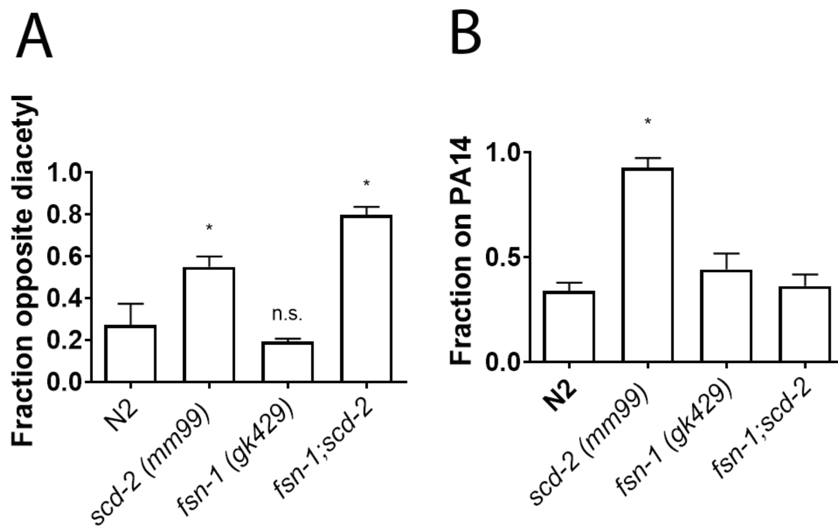

621

622 Figure 3. There is a dissociation between associative learning and sensory integration at the
623 genetic level.

624 A) When tested in the diacetyl and copper assay for sensory integration, *fsn-1;scd-2* double
625 mutants showed a deficit similar to *scd-2* single mutants compared to wild type N2 (one-
626 way ANOVA ($F_{(3,12)} = 21.29$; $p < 0.0001$) with Bonferroni correction (* $p<0.05$, n.s.,
627 not significant); n≥4 plates; means ± SEMs).
628 B) The *fsn-1;scd-2* double mutants did not show a deficit in learning about pathogenic
629 bacteria; instead the double mutants had a similar learned response to N2 or *fsn-1 (hp1)*
630 single mutants (one-way ANOVA ($F_{(5,22)} = 15.32$; $p < 0.0001$) with Bonferroni
631 correction (* $p<0.05$, n.s., not significant); n≥4 plates; means ± SEMs). This suggests that
632 SCD-2 and FSN-1 have a different interaction in associative learning compared to
633 sensory integration.

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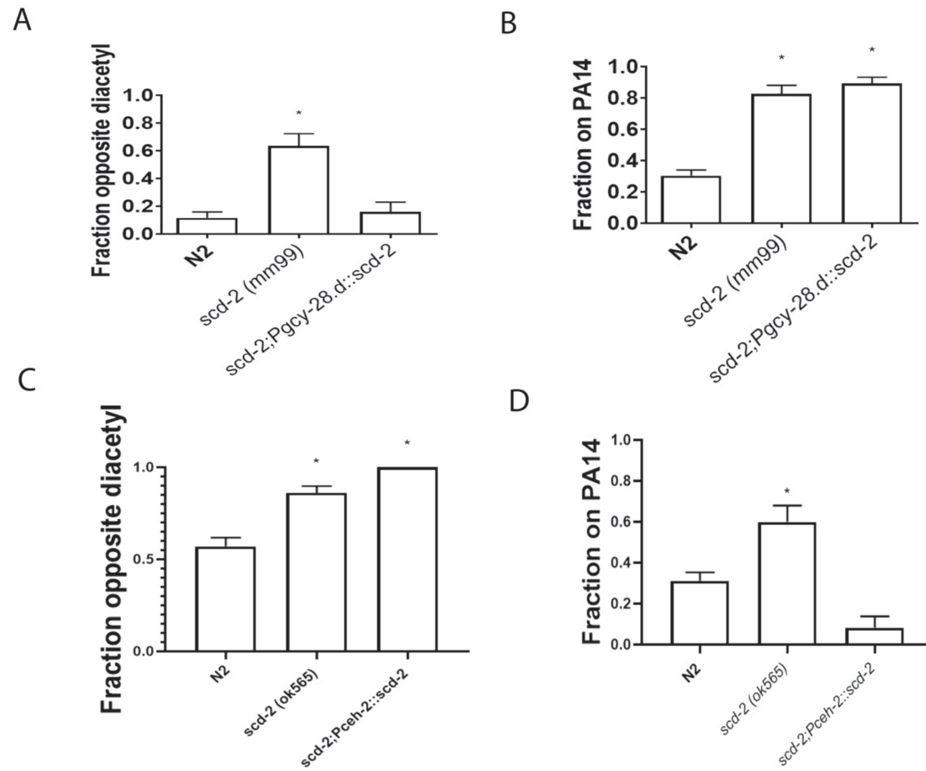

Figure 4. AIA neurons are necessary for sensory integration and NSM neurons are necessary for associative learning.

A) *pgcy-28.d::scd-2;pmyo-3::GFP* worms expressed wild type *scd-2* in AIA interneurons within an *scd-2* mutant background. Expression in AIA rescued the worm's ability to integrate sensory cues by crossing an aversive copper barrier to reach an attractive odorant. These data replicate the results originally found by Shinkai *et al*. (2011).

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641 Integration was compared to wild type N2 using a one-way ANOVA ($F_{(2,6)} = 16.86$; $p < 0.005$) with Bonferroni correction (* $p<0.0001$); n≥8 plates, means ± SEMs.

643 B) Expression of wild type *scd-2* in AIA did not rescue associative PA14 learning. This indicates that the learning deficit seen in *scd-2* mutants does not require AIA expression, unlike sensory integration (one-way ANOVA ($F_{(2,23)} = 52.6$; $p < 0.0001$) with Bonferroni correction (* $p<0.05$, n.s., not significant); n≥3 plates; means ± SEMs).

647 C) *Pceh-2::scd-2;Pmyo-2::mcherry* worms expressed wild type *scd-2* in NSM neurons within an *scd-2* mutant background. Expression in NSM did not rescue the ability to integrate sensory cues and cross an aversive copper barrier to reach an attractive diacetyl spot. These results are consistent with the findings that AIA expression is sufficient for sensory integration. Integration was compared to wild type N2 using a one-way ANOVA ($F_{(3,10)} = 7.203$; $p < 0.01$) with Tukey's test for multiple comparisons. (* $p<0.05$); n≥4 plates, means ± SEMs.

654 D) Expression of wild type *scd-2* in NSM neurons within an *scd-2* mutant background rescued associative learning about PA14. This indicates that the role of *scd-2* in PA14 learning requires expression in NSM. Learning was compared to wild type N2 using one-way ANOVA ($F_{(3,24)} = 17.17$; $p < 0.0001$) with Tukey's test for multiple comparisons (* $p<0.05$); n≥7 plates; means ± SEMs).

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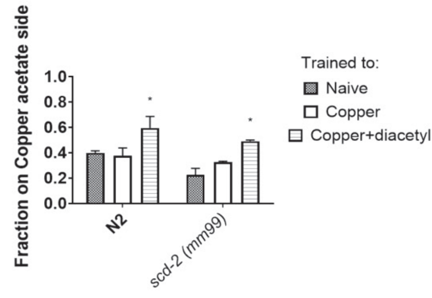

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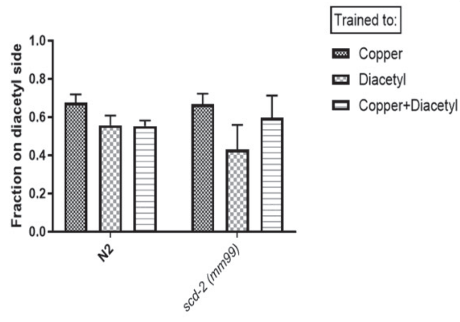

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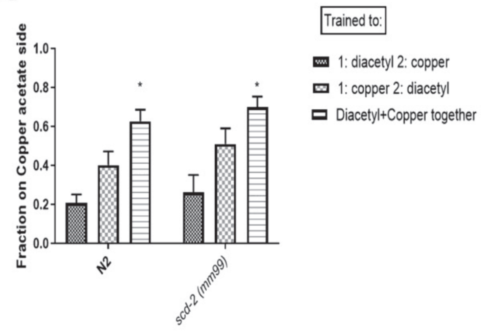

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661 Figure 5. *scd-2* mutants can form an associative memory independent of *scd-2* mediated
662 sensory integration.

663 A) When N2 and *scd-2* were tested for an associative memory in response to copper acetate
664 post diacetyl and copper training, both strains showed an attenuated aversion to copper
665 acetate. "Fraction on copper acetate side" refers to the fraction of worms on the side of
666 the testing plate that had a point source of copper acetate. This represents the fraction of
667 worms that are attracted to copper acetate. Learning was analyzed using a two-way
668 ANOVA; there was a main effect of worm strain ($F_{(1,12)} = 6.954$; $p < 0.05$), a main effect
669 of training condition ($F_{(2,12)} = 12.08$; $p < 0.05$), but no significant interaction. Tukey's
670 test for multiple comparisons was performed as a *post hoc* analysis (* $p<0.05$ compared
671 to naïve conditions, n.s., not significant); n≥3 plates; means ± SEMs. These data indicate
672 that while testing for sensory integration using diacetyl and copper, the worms learn that
673 copper is associated with diacetyl, and thus decrease their aversive response to copper.
674 Since *scd-2* was still able to learn, despite deficits in PA14 associative learning, this
675 memory is formed by a separate mechanism from associative PA14 learning.
676 B) Testing to diacetyl instead of copper does not show learning perhaps due to a ceiling
677 effect. N2 and *scd-2 (mm99)* worms were trained to associate diacetyl and copper, then
678 approach to a point source of diacetyl was measured. However, diacetyl remained highly
679 attractive across control and trained conditions. There is no significant difference
680 between groups.
681 C) N2 and *scd-2 (mm99)* worms were exposed to diacetyl for one hour, followed by copper
682 for on hour (and vice versa), and then tested to a copper (II) acetate point source. Copper
683 (II) acetate and diacetyl were also presented together for one hour in a paired condition

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684 following one hour on a plate with no odorants. These data indicate that diacetyl and
685 copper (II) acetate should be presented simultaneously in order for a learned association
686 to form. Learning was analyzed using a two-way ANOVA; there was a main effect of
687 condition ($F_{(2,41)} = 19.73$; $p < 0.0001$). Tukey's test for multiple comparisons was
688 performed as a *post hoc* analysis (* $p<0.05$ compared to naïve conditions); n≥9 plates;
689 means ± SEMs. The slight difference between the two conditions in which copper and
690 diacetyl were presented separately was not found to be statistically significant.

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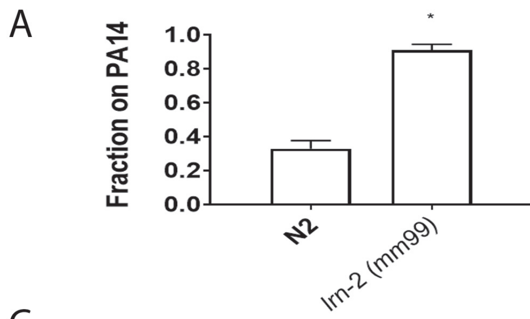

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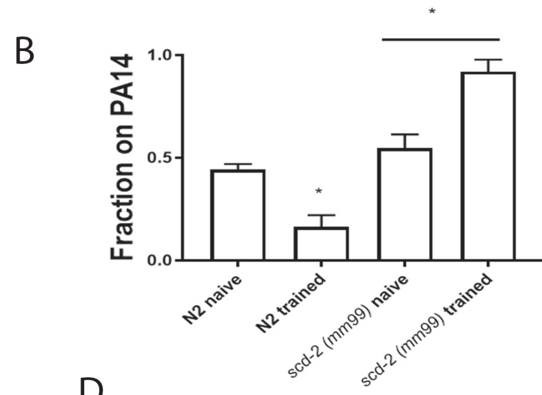

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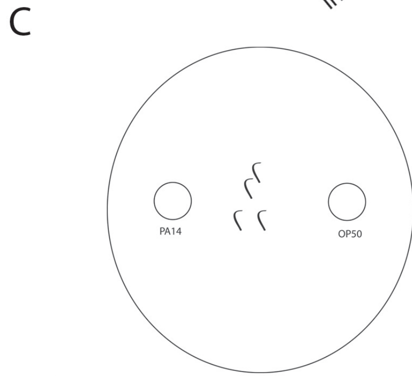

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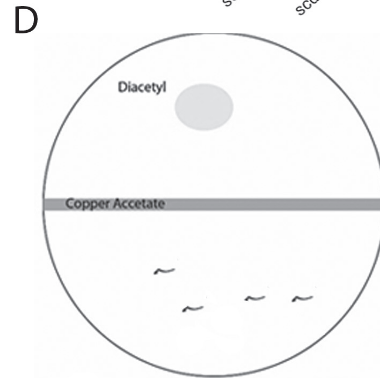

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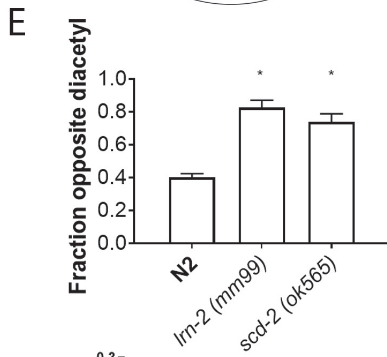

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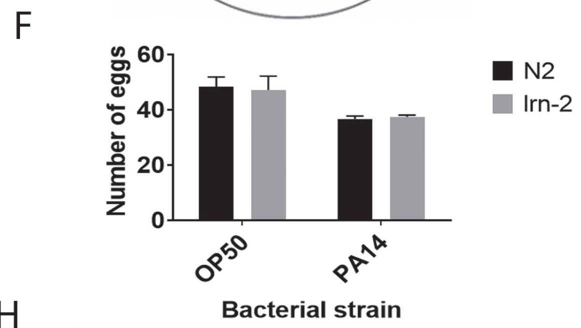

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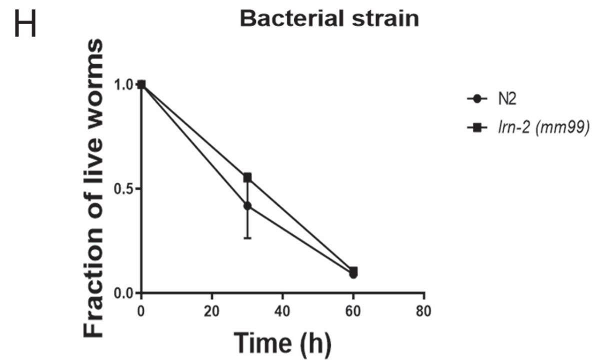

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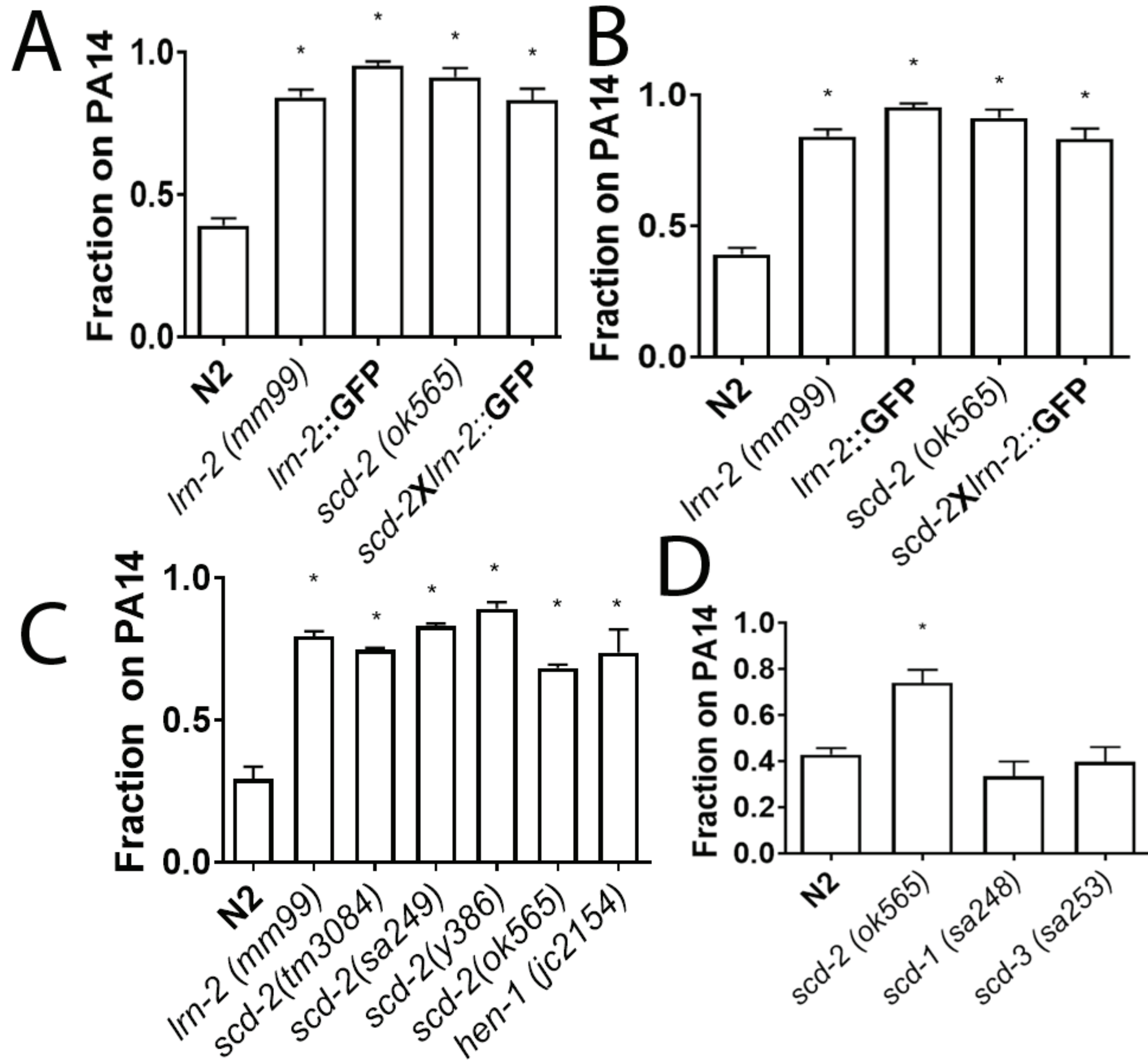

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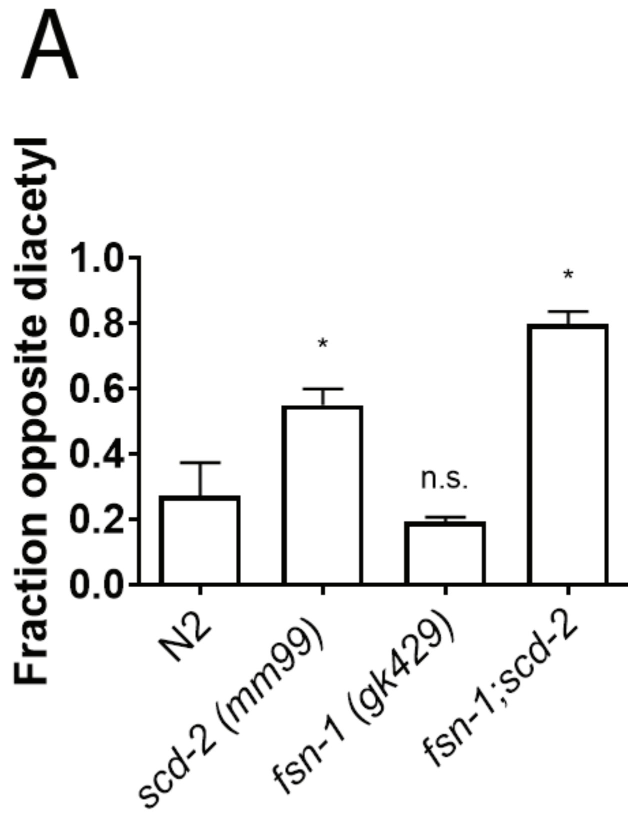

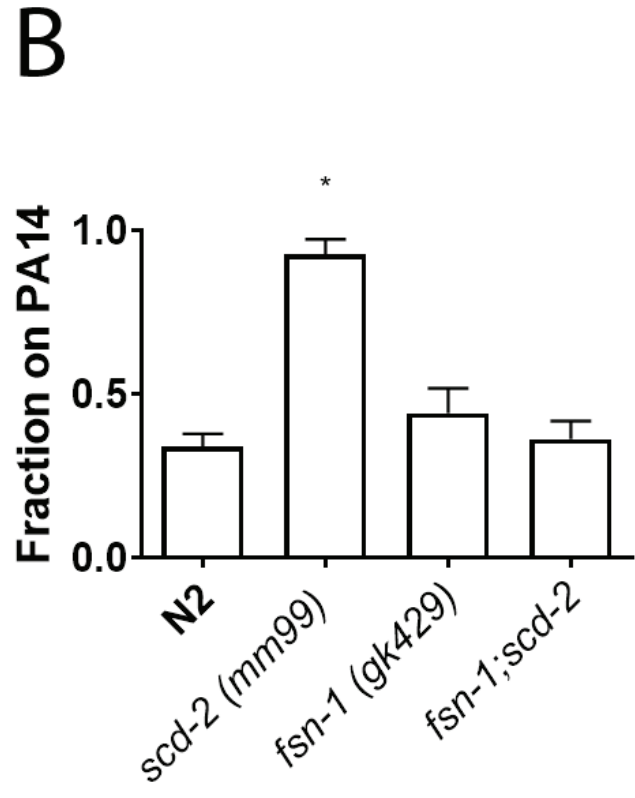

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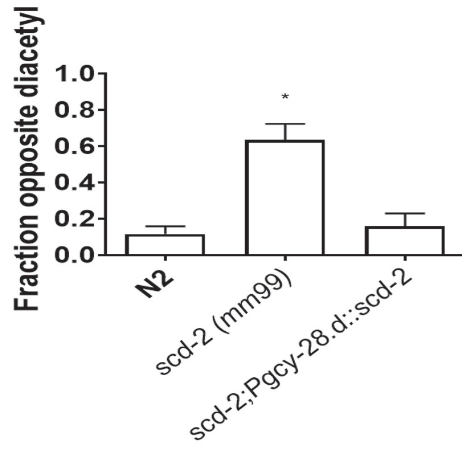

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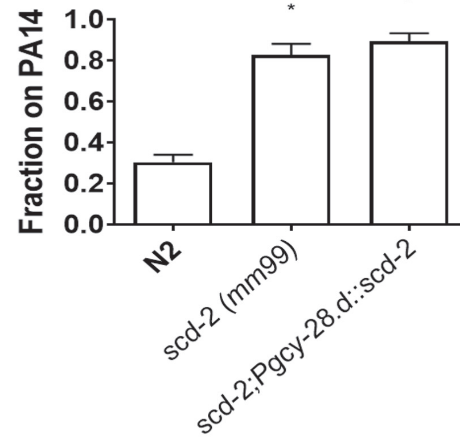

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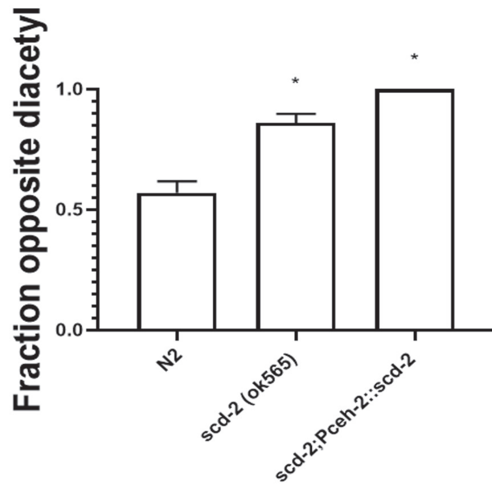

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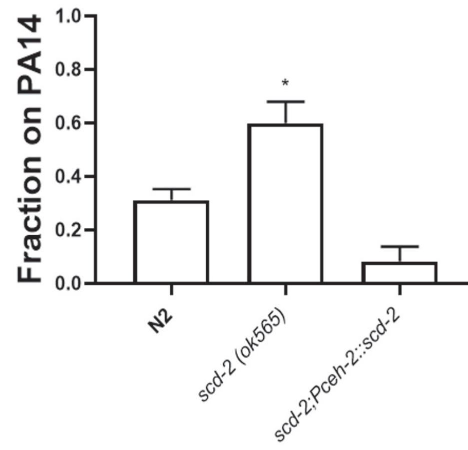

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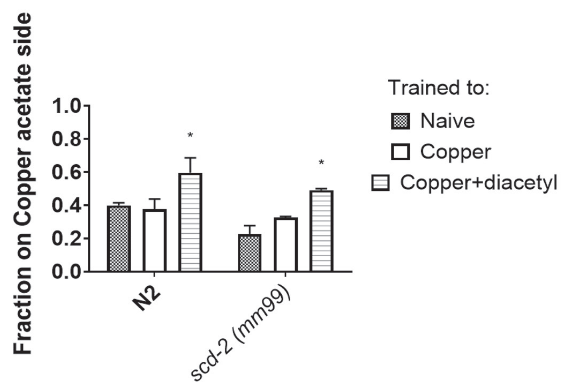

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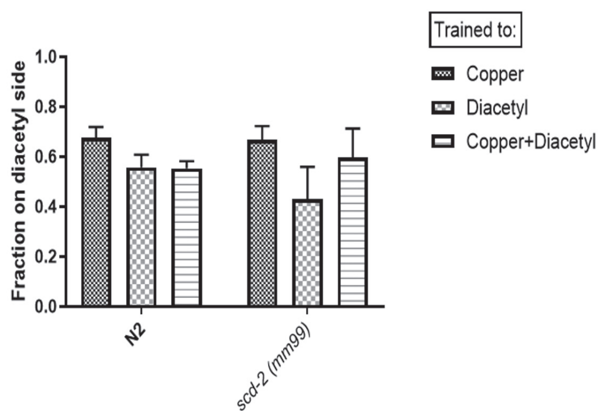

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