A Novel Memory Type in *C. elegans* Exhibits Post-Training Consolidation

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Abstract

Memories are often categorized into types, reflecting their behavioural, anatomical and molecular diversity: these classifications both aid understanding of the differences among varieties of memory and help delineate the unifying cross-species principles underlying them. In the nematode worm *Caenorhabditis elegans,* we find that an associative memory of the pairing of the normally attractive odorant benzaldehyde and starvation depends on *de novo* transcription and translation, is independent of CREB, and is produced by massed training: a pattern which does not correspond to any of the well-characterized molecular categories of invertebrate memory. Further, as has been shown for many memories in vertebrates, but not previously in nematodes, we find that formation of this memory continues after removal of the stimuli initially causing it, and that it is labile to disruption through protein synthesis inhibition following training, but that inhibition of proteasomal activity does not extend the duration of the memory. Previous findings have implicated insulin pathway signalling as a key component of this benzaldehyde/starvation memory, however we find that the transcriptional activity required for the memory is likely to be independent of the transcription factors that function at the terminus of this pathway. These findings better characterize this model associative memory in relation to other invertebrate memory types and identify ways in which it both shares their traits and differs from them.

Introduction

Since at least the era of William James, researchers have understood the importance of systematically classifying the diverse phenomena of memory to understand how they function (James, 1890). Memories have been categorized along many different axes, including conceptual structure (non-associative and associative memory), stimuli and paradigm (fear conditioned

memory, conditioned taste avoidance memory), cognitive function (episodic memory, semantic memory, implicit memory, working memory), and by anatomical location (hippocampal memory, cerebellar memory, etc.).

Within neuroscience, one of the most influential ways of categorizing memories has been to divide them by the dissociable molecular mechanisms underlying them (and the corresponding perturbations disrupting these mechanisms). These molecular mechanisms have proven to be at least loosely correlated with the temporal sequence by which the memory develops and appear to be widely conserved, particularly in invertebrates. In the fruit fly *Drosophila melanogaster*, memories have been divided into short-term memory (STM), anesthesia-sensitive memory (ASM), anesthesia-resistant memory (ARM), and long-term memory (LTM) (Tully et al., 1994), with broadly similar divisions in *Aplysia* (Hawkins et al., 2006) (for a review of similar delineations in mammals, see Hernandez & Abel (2008)). Across all animals, most of the longest term memories require protein synthesis, as well as repeated training sessions (spaced training) rather than single training blocks (massed training) to form (Smolen et al., 2016), although there are prominent exceptions (Garcia et al., 1955).

Memory in the nematode worm *Caenorhabditis elegans* has been the subject of extensive research, and a broad assortment of genes have been identified, organized into several signalling cascades, which are required for various learning modalities. How these modalities relate to the categories of memory studied in other model invertebrates has not been clearly worked out: although types of memory are often treated as if they are broadly generalizable categories (at least within invertebrates), evidence for this remains lacking.

	Memory Type			
Memory Characteristic	**STM**	**ASM**	**ARM**	**LTM**
Protein Synthesis-Dependent	No (Tully et al., 1994)	No (Tully et al., 1994)	No (Tully et al., 1994)	Yes (Tully et al., 1994)
Spaced Training Required	No (Davis, 2011)	No (Tully et al., 1994)	No (Tully et al., 1994)	Yes (Tully et al., 1994)
Blocked by Cold Shock	Yes (Saitoe et al., 2005)	Yes (Tully et al., 1994)	No (Tully et al., 1994)	No (Xia et al., 1998)
Duration	< 2 h (Tully et al., 1990)	~ 7 h (Tully et al., 1994)	2-4 days (Tully et al., 1994)	> 7 days (Tully et al., 1994)
Genes Involved	*dunce, rutabaga* (Tully & Quinn, 1985)	*amnesiac* (Tully et al., 1994)	*radish* (Folkers et al., 1993)	CREB (Yin et al., 1994)

Table 1 Characteristics of memory types in *Drosophila melanogaster*.

To better understand how these categories of memories which were primarily developed from research in fruit flies and *Aplysia* apply in *C. elegans*, we characterized a model aversive memory in which the smell of benzaldehyde, which is normally attractive to the worm, becomes temporarily aversive following paired presentation of the odour with starvation (Nuttley et al., 2002). We try to orient this memory within the categories established in the *Drosophila* literature (**Table 1**) to understand the general principles, if any, underlying molecular memory types across animals.

Materials and Methods

Strains and General Methods

All experiments were performed using wild type N2 *Caenorhabditis elegans* unless otherwise stated. Worms were grown using standard techniques at 20°C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50. All behavioural experiments were done on synchronized populations of worms 52 h after release from L1 arrest and were

75 performed in an environmentally controlled room at 20 °C and less than 25% humidity. Worms
76 used in behavioural tests did not experience starvation between release from L1 arrest and
77 initiation of training. The *C. elegans* strains N2, CF1038 *daf-16(mu86)*, RB759 *akt-1(ok525),*
78 YT17 *crh-1(tz2)* and VC3149 *crh-2(gk3293)* were provided by the CGC, which is funded by
79 NIH Office of Research Infrastructure Programs (P40 OD010440). The 6x outcrossed CQ528
80 *pqm-1(ok485)* strain was a generous gift from Coleen Murphy. The double CREB mutant
81 UT1343 *crh-1(tz2); crh-2(gk3293)* was created using standard methods.

82 The *akt-1(mm200)* allele was generated by ethyl methanesulfonate mutagenesis (Brenner,
83 1974), and isolated from a mixed population following repeated selection for learning mutants in
84 an approach similar to that of Colbert & Bargmann (1995). *mm200* contains a single base pair
85 change resulting in an p.L199F substitution, and the originally isolated strain was outcrossed 4x
86 to give UT1306 *akt-1(mm200)*. The *akt-1* rescue strain was created by microinjection of the
87 fosmid WRM065aH03 into UT1306 to give UT1309.

88 *Statistical Analysis*

89 No statistical test was performed to predetermine sample sizes, which were set at 9 test
90 plates for all experiments. The mean chemotaxis index (C.I.) and the standard error of the mean
91 (SEM) were calculated using Excel. In most experiments with multiple independent variables,
92 comparisons between C.I. were made by two- or three-way ANOVA (as appropriate) performed
93 in GraphPad Prism, which were then followed by t-tests using Tukey's multiple comparison test.
94 In experiments (**Fig. 2B**, **Supplemental Fig. 1**) where a full factorial design was impractical,
95 precluding the use of a two-way ANOVA, t-tests alone were performed to test specific predicted
96 effects, and the results adjusted for multiple comparison using Bonferroni correction. Differences
97 were considered significant when the adjusted $p<0.05$. All figures depict data from three

98 replicates performed on three different days. All statistical data are provided in **Supplemental**
99 **Table 1**.

100 *CREB Phylogenetic Tree*

101 The phylogenetic tree of CREB proteins shown in **Fig. 3A** was produced with T-Coffee
102 sequence alignment (Notredame et al., 2000), Gblocks curation (Castresana, 2000), PhyML
103 phylogeny reconstruction (Guindon & Gascuel, 2003) and TreeDyn rendering (Chevenet et al.,
104 2006) using http://www.phylogeny.fr (Dereeper et al., 2008). Sequence labels were edited
105 following tree generation for clarity.

106 Protein isoforms used for analysis were *C. elegans* CRH-1a (NP_001022859.1), CRH-2a
107 (NP_740985.2), *Drosophila melanogaster* CREBA-PA (NP_524087.3), *Drosophila*
108 *melanogaster* CREBB-PF (NP_996504.1), *Aplysia californica* CREB1 (XP_012939791.1),
109 *Aplysia californica* CREB1 (NP_001191630.1), *Mus musculus* CREB1A (NP_598589.2), *Mus*
110 *musculus* ATF-2 isoform 1 (NP_001020264.1) and *Mus musculus* CREB3 (NP_001369747.1).

111 *Behavioural Tests*

112 Benzaldehyde/starvation experiments were conducted as per Nuttley et al. (2002).
113 Briefly, learning was tested by training 1000-1500 worms for 1 hour on 6 mL 10 cm parafilm-
114 sealed NGM agar plates without bacteria, with either 2 µL of 100% benzaldehyde placed on a
115 small parafilm square on the inside centre of the lid (trained condition), or a parafilm square
116 without benzaldehyde placed on the inside centre of the lid (naïve condition). Following training,
117 worms were rinsed off the plate with M9 and divided into three approximately equal groups,
118 which were then moved to the centre of three fresh 10 cm NGM testing plates, each with 1 µL of
119 1% benzaldehyde (Bnz) in ethanol on one side, and a second spot of 1 µL 100% ethanol (EtOH)
120 on the other. 1 µL of 1 M sodium azide was placed on top of each odorant to paralyze the worms

121 upon reaching it. The number of worms within 2 cm of each point of odorant, and elsewhere on
122 the plate, was counted after 1 hour, with any worms that remained where they were placed in the
123 centre of the plate and appeared dead or injured discounted (usually < 2%), and the chemotaxis
124 index calculated using the equation C.I.=((#Bnz)-(#EtOH))/(#Total Worms).

125 *Cycloheximide Treatment*

126 Experiments utilizing cycloheximide (Bioshop) to disrupt protein synthesis were
127 performed using 1 mL of 6 mg/mL cycloheximide dissolved in water. This was added to 5 mL of
128 NGM agar, which had been melted and allowed to cool to below 60 °C, for a final concentration
129 of 1 mg/mL in the training plates and/or testing plates unless otherwise stated.

130 For cycloheximide experiments which employed a gap between training and testing, after
131 being washed off training plates, worms were suspended in 3 mL of M9 buffer, with or without 1
132 mg/mL of cycloheximide, and agitated on a rocker for 1 hour.

133 *Bortezomib Treatment*

134 Worms were treated with 60 µg/mL bortezomib (Cell Signaling Technology) for 5 hours
135 prior to training, during training, and during a 1 hour gap between training and testing. Prior to
136 training, bortezomib was added to HT115 *E. coli* which had been grown overnight (to saturation)
137 at 37°C in agitated LB media, and then concentrated 3 times by centrifugation followed by
138 resuspension of the pellet in an appropriate volume of M9. During training and the forgetting
139 gap, bortezomib was added to M9 buffer along with 0.006% benzaldehyde, or M9 buffer alone,
140 respectively. Naïve controls were treated with bortezomib in M9 buffer alone during both
141 training and the forgetting gap. Tubes containing worms were agitated on a rocker throughout
142 the experiment. Bortezomib was prepared as a concentrated stock solution at 300 µg/mL in M9.

143 *α-amanitin/actinomycin D Treatment*

144 Worms were treated with 100 µg/ml α-amanitin (Sigma) or 200 µg/ml actinomycin D
145 (Sigma) for 4 hours prior to, and subsequently during, training. Prior to training, drugs were
146 added to HT115 *E. coli* which had been grown overnight at 37 °C in agitated LB media before
147 being concentrated 3 times by centrifugation followed by resuspension of the pellet in an
148 appropriate volume of M9. During training, drugs were combined in M9 buffer along with
149 0.006% benzaldehyde, or M9 buffer alone for naïve controls. Tubes containing worms were kept
150 agitated on a rocker throughout the experiment. α-amanitin was prepared as a concentrated stock
151 solution at 1 mg/mL in M9, while actinomycin D was dissolved at 222 µg/ml in M9.

152 *Cold Shock*

153 Worms were cold shocked in 3 mL ice-cold M9 buffer for 6 minutes immediately before
154 and/or 500 µL ice-cold M9 buffer immediately after training, as indicated. Controls conditions
155 which were not cold shocked were allowed to settle in 20 °C M9 buffer for 6 minutes, before
156 and/or after training. Training took place at 20 °C in M9 buffer containing 0.006%
157 benzaldehyde, or in M9 buffer alone for naïve controls. Tubes containing worms were agitated
158 on a rocker during training.

159 *Heat Shock*

160 Worms were transferred to NGM agar plates with a lawn of *E. coli* OP50 which had been
161 pre-warmed to 37 °C for 2 hours, and subsequently heat shocked at this temperature for 45
162 minutes in an air incubator immediately prior to training.

163 **Results**

164 *Benzaldehyde/Starvation Associative Memories are Protein Synthesis-Dependent*

165 We first attempted to determine whether a model associative memory in which
166 benzaldehyde is paired with starvation, resulting in subsequent aversion to benzaldehyde, is
167 dependent on protein synthesis. Application of the chemical protein synthesis inhibitor
168 cycloheximide during both initial training to avoid benzaldehyde, and during subsequent testing
169 for benzaldehyde preference, revealed a strong effect of protein synthesis inhibition in
170 preventing the memory, with only mild effects on naïve approach to benzaldehyde (**Fig. 1A**).
171 Decreasing the concentration of cycloheximide resulted in a modestly weaker learning deficit
172 while fully eliminating the naïve approach deficit (**Supplemental Fig. 1**).

173 To determine when protein synthesis inhibition was acting to inhibit the memory, we
174 applied cycloheximide selectively during training, during testing, and during both training and
175 testing, and evaluated the strength of the resultant memory. To our surprise, cycloheximide was
176 capable of partially inhibiting the memory during either training or testing, with the strongest
177 inhibition resulting when it was present during both (**Fig. 1B)**. This suggested that protein
178 synthesis was required even after removal of the training stimuli for complete learning to take
179 place.

180 *Cycloheximide Inhibits Consolidation Rather than Recall*

181 We reasoned that the inhibition of memory we observed when cycloheximide was given
182 during testing could be due to impaired chemotaxis during testing, impaired memory
183 consolidation following training, or impaired memory retrieval. Impaired chemotaxis was
184 inconsistent with the larger absolute chemotaxis indices we observed in the group receiving
185 cycloheximide during both training and testing compared to those seen in the no drug group. To

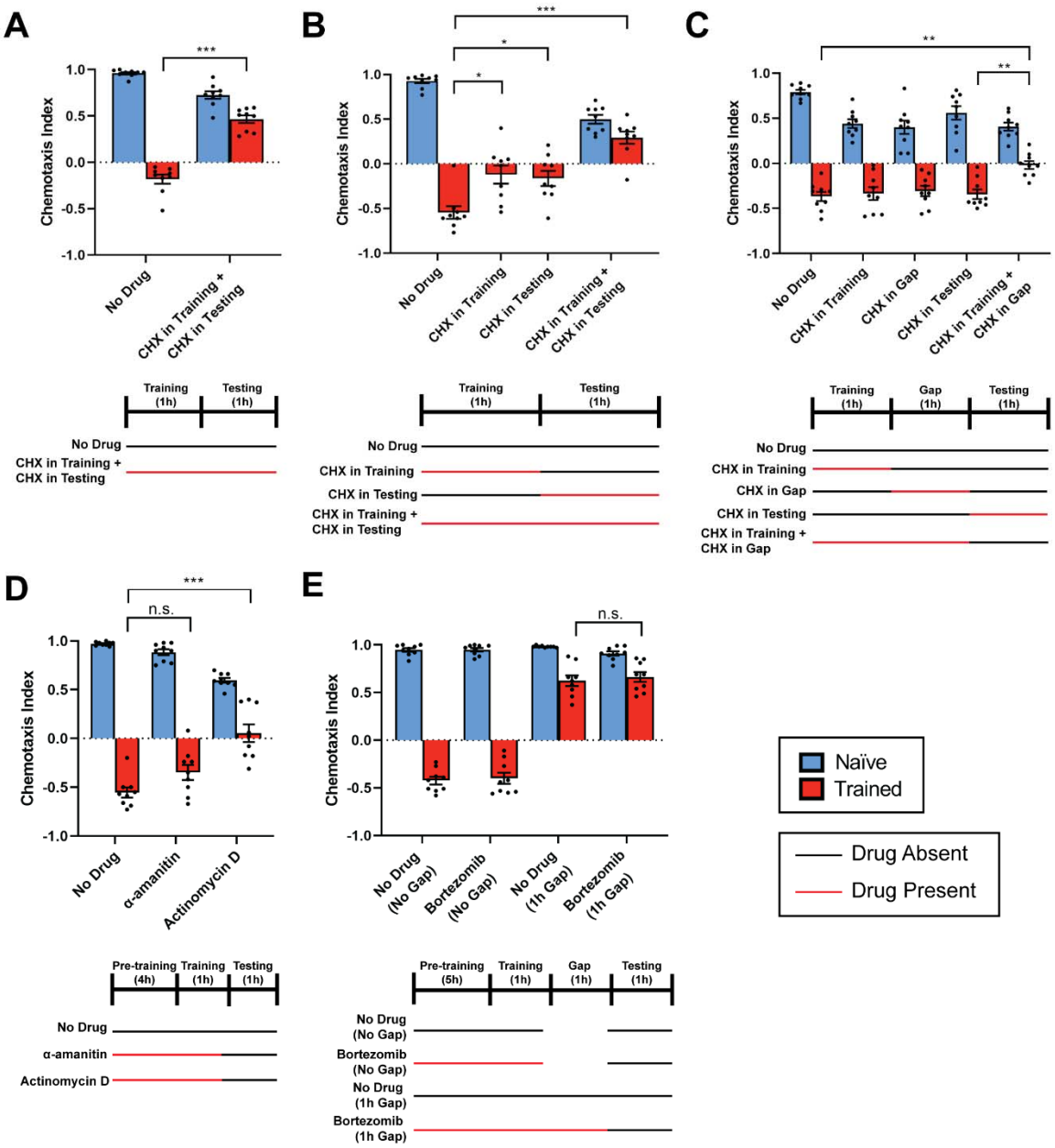

Figure 1 Benzaldehyde/Starvation Memory is Translation- and Transcription-Dependent. **A)** Chemotaxis of wild type animals to a point of benzaldehyde after benzaldehyde/starvation training, with and without cycloheximide in training and testing plates. **B)** Chemotaxis of wild type animals to a point of benzaldehyde after benzaldehyde/starvation training, with cycloheximide absent, present during training, during testing, and during training and testing. **C)** Chemotaxis of wild type animals to a point of benzaldehyde after benzaldehyde/starvation training and a 1 hour consolidation gap, with cycloheximide absent, present during training, present during the gap, present during testing or present during training and the gap. **D)** Chemotaxis of wild type animals to a point of benzaldehyde following benzaldehyde/starvation training in α-amanitin or actinomycin D, after 4h pre-exposure to the drug. **E**) Chemotaxis of wild type animals to a point of benzaldehyde after benzaldehyde/starvation training following 5 hours of proteasome inhibition by bortezomib, with and without a 1 h forgetting period after training.

196 distinguish between the remaining two possibilities, we added a 1 h post-training gap between
197 training and testing for consolidation to occur in, reasoning if the observed memory inhibition
198 was due to impaired consolidation, cycloheximide given during this gap and during training (but
199 not during testing) would inhibit consolidation, resulting in a memory deficit, while
200 cycloheximide given during testing alone would have no effect. Conversely, if the memory
201 inhibition was caused by impaired recall, cycloheximide during testing alone should retain its
202 inhibitory effects. We find that cycloheximide during testing has no effect on memory given a 1
203 h post-training gap for consolidation to occur in, suggesting that it impairs memory consolidation
204 and that the effect of the drug during recall is in fact due to an extended period of memory
205 formation that begins during training and continues after its end (**Fig. 1C**).

206 *Benzaldehyde/Starvation Memories are Transcription-Dependent*

207 We next wondered whether benzaldehyde/starvation associative memories in *C. elegans*
208 were dependent on *de novo* transcription, in addition to translation. We employed the
209 transcriptional inhibitors α-amanitin and actinomycin D to inhibit transcription during training,
210 and find that actinomycin D can inhibit the memory to a comparable extent as cycloheximide. α-
211 amanitin was found to also inhibit memory to a lesser extent, but this was not statistically
212 significant after adjustment for multiple testing (**Fig. 1D**).

213 *Proteasome Inhibition does not Extend Benzaldehyde/Starvation Memory Duration*

214 Our finding that inhibition of protein translation impairs benzaldehyde/starvation
215 memory led us to wonder if inhibition of the proteasome might, conversely, prolong the duration
216 of the memory by extending the lifetime of the relevant translated proteins, inhibiting an
217 endogenous memory decay process. To test this hypothesis, we exposed worms to the small
218 molecule proteasome inhibitor bortezomib for 5 hours prior to training, during training, and then

219 during a 1 hour gap between training and testing: 6 hours of exposure to bortezomib at this
220 concentration has previously been shown to be sufficient to inhibit the *C. elegans* proteasome
221 (Melo & Ruvkun, 2012). To maximize our ability to see an increase in memory retention, we
222 trained worms in liquid, exploiting an earlier chance observation that duration of the memory is
223 shorter when worms are trained in liquid media than on agar plates.

224 We find that bortezomib-mediated inhibition of the proteasome does not result in
225 extension of the benzaldehyde/starvation memory (**Fig. 1E)**, suggesting that forgetting of this
226 memory in the worm was not mediated by the proteasome.

227 *Heat Shock Impairs Benzaldehyde/Starvation Memory*

228 Inhibition of protein synthesis by heat shock has been demonstrated in yeast (Lindquist,
229 1981), *Drosophila* (Lindquist, 1981), mammalian cell culture (Shalgi et al., 2013) and *C. elegans*
230 (Snutch & Baillie, 1983), and has been suggested to be mediated by pausing of translation
231 elongation (Shalgi et al., 2013). Although not often used for classification of memories in flies
232 and *Aplysia sp.*, heat shock has been used to impair memory in *C. elegans* (Beck & Rankin,
233 1995; Rose & Rankin, 2001), presumably through inhibition of protein synthesis. We find that
234 heat shock prior to training partially blocks the benzaldehyde/starvation memory, with no effect
235 on naïve approach to benzaldehyde (**Fig. 2A**).

236 *Benzaldehyde/Starvation Memory is Resistant to Cold Shock*

237 In *Drosophila* (Quinn & Dudai, 1976), the slug *Limax flavus* (Yamada et al., 1992) and
238 the snail *Lymnaea stagnalis* (Sangha et al., 2003), post-training cold shock is capable of
239 disrupting the consolidation of one type of memory into another, and this effect has been

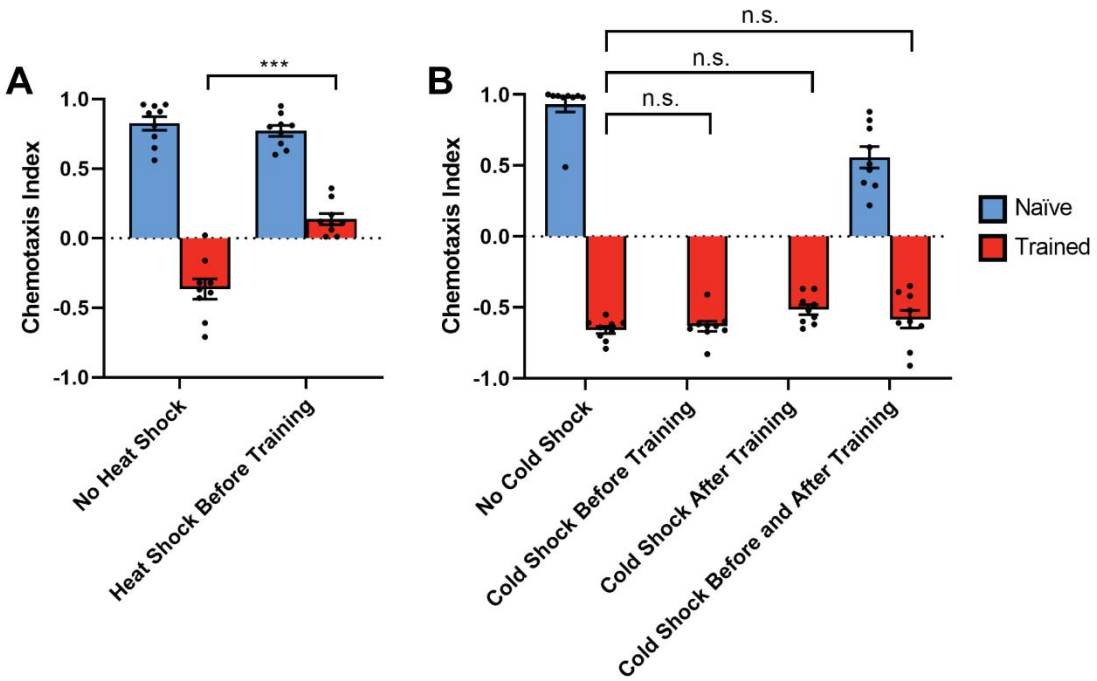

Figure 2 Heat Shock, but not Cold Shock, Inhibits the Benzaldehyde/Starvation Memory. **A)** Chemotaxis of wild type animals to a point of benzaldehyde after benzaldehyde/starvation training, with and without heat shock immediately before training. **B)** Chemotaxis of wild type animals to a point of benzaldehyde without cold shock, and with cold shock given before, after, and before and after training.

suggested to be mediated by cooling-induced disruption of protein synthesis (Takahashi et al., 2013). In *Drosophila*, anesthesia-resistant memory is behaviourally differentiated from anesthesia-sensitive memory by resistance to disruption by cold shock. While some memories in *C. elegans* can be disrupted by cold shock (Morrison & van der Kooy, 1997; Nishijima & Maruyama, 2017), long-term tap habituation and long-term 1-nonanol/food appetitive learning have been reported to be cold shock-resistant (Aamodt, 2006, p. 49; Nishijima & Maruyama, 2017). To determine whether the benzaldehyde/starvation model associative memory is sensitive to cold shock, we first evaluated various durations of cold shock to determine the maximum duration we could subject worms to ice-cold M9 for without severely hindering subsequent chemotaxis, and found that 6 minutes of cold shock resulted in only a minor deficit in chemotaxis (**Supplemental Fig. 2**). This duration of cold shock is far longer than that required to

256 disrupt memory in analogous worm paradigms (Nishijima & Maruyama, 2017), suggesting that it
257 should be sufficient to reveal cold shock disruption if possible in our paradigm.

258 We previously determined that cold shock of appetitive food/salt memories in *C. elegans*
259 had distinct effects when performed before and after training (Morrison & van der Kooy, 1997).
260 Therefore, to determine whether this benzaldehyde/starvation memory was similarly sensitive to
261 cold shock, we tested the effects of cold shock before, after, and both before and after training.
262 We find that, contrary to food/salt memory but consistent with long-term tap habituation and 1-
263 nonanol/food memory, the benzaldehyde/starvation memory was cold shock-resistant under all
264 tested conditions (**Fig. 2B**).

265 *CREB is Dispensable for the Benzaldehyde/Starvation Memory*

266 Our finding that transcription and translation are required for formation of the
267 benzaldehyde/starvation memory prompted us to investigate candidate transcription factors
268 which might mediate memory formation. The basic region/leucine zipper (bZIP) transcription
269 factor CREB1 has been shown to be necessary for long-term memories in diverse organisms,
270 including *Aplysia* (Dash et al., 1990; Kaang et al., 1993), *Drosophila* (Yin et al., 1994), mice
271 (Bourtchuladze et al., 1994) and in some paradigms *C. elegans* (Amano & Maruyama, 2011;
272 Dahiya et al., 2019; Timbers & Rankin, 2011).

273 In most animals, CREB proteins exist as a diversified family, with the majority of
274 members not having any role in memory. While the *C. elegans* genome contains two genes
275 encoding for CREB family members, *crh-1* and *crh-2,* only the protein product of *crh-1* clusters
276 with known positive regulators of memory function in other animals (**Fig. 3A**). To determine
277 whether the benzaldehyde/starvation memory requires CREB, we tested a strain carrying the *crh-*
278 *1(tz2)* allele. We find that *crh-1(tz2)* mutants exhibit slightly impaired naïve approach to

279 odorants, as has been reported previously (Dahiya et al., 2019), but see no evidence of a
280 benzaldehyde/starvation learning deficit (**Fig. 3B**), consistent with our previous observations on
281 these mutants in more complex memory paradigms (Merritt et al., 2019). Since the *crh-1(tz2)*
282 allele deletes most of the bZIP domain of *C. elegans* CREB1 **(Fig. 3C)**, and the allele results in
283 the elimination of CREB as detected by anti-phospho-CREB antibody (Kimura et al., 2002), it is
284 a presumptive null, and we therefore conclude that the benzaldehyde/starvation memory is CRH-
285 1-independent.

286 While homology and behavioural evidence suggested that CRH-1 was the most likely
287 CREB family candidate involved in this memory, it remained possible that the CRH-2 was the
288 relevant CREB protein. We therefore tested a strain carrying the *crh-2(gk3293)* allele, which
289 eliminates the entire CRH-2 bZIP domain and is therefore a probable null, but found no evidence
290 of a learning deficit. A double mutant strain carrying mutations in both *crh-1* and *crh-2* shows no
291 additive phenotype over the *crh-1* mutant strain, suggesting that our failure to observe a learning
292 deficit was not due to genetic redundancy **(Fig. 3D)**.

293 *DAF-16 Regulated Transcriptional Targets May Constitute the Necessary Translated Proteins*

294 Previous work from our group and others (Cheng et al., 2022; C. H. A. Lin et al., 2010;
295 Tomioka et al., 2006) has described a key role for insulin signalling in olfactory and gustatory
296 learning in *C. elegans*. The extensive transcriptional changes known to take place downstream of
297 insulin signalling (Murphy, 2006) suggested a straightforward explanation: could the
298 requirement we observed for transcription in the benzaldehyde/starvation learning paradigm
299 result from insulin signalling-mediated changes in gene expression?

300 Canonical regulation of transcription through the insulin signalling pathway is mediated
301 by two downstream transcription factors with opposing regulatory effects: DAF-16

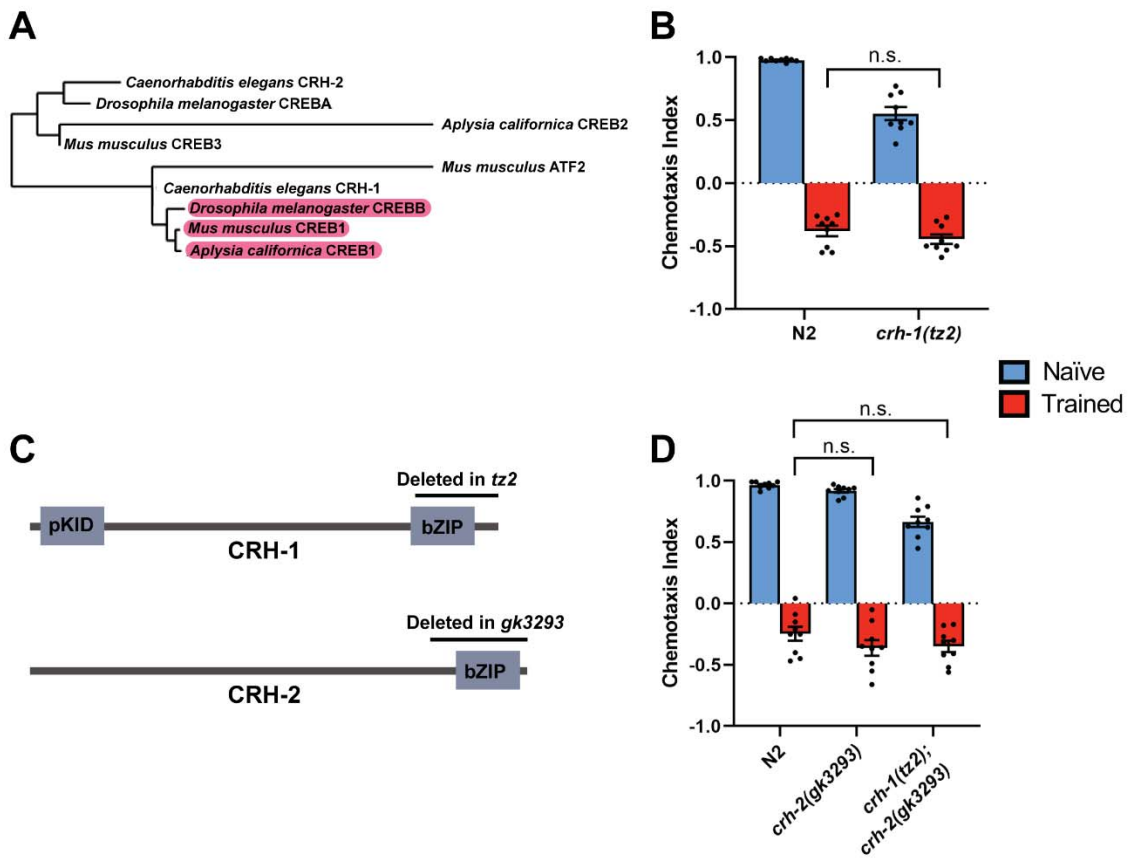

Figure 3 CREB is Dispensable for Benzaldehyde/Starvation Memory. **A)** Phylogenetic tree of CREB family members, with proteins implicated as positive regulators of learning highlighted in pink. **B)** Chemotaxis of wild type and *crh-1* mutant animals to a point of benzaldehyde after benzaldehyde/starvation training. **C)** Structure of *C. elegans* CRH-1 and CRH-2 proteins, with deletions tested indicated above. **D)** Chemotaxis of wild type, *crh-2* mutant and *crh-1;crh-2* double mutant animals to a point of benzaldehyde after benzaldehyde/starvation training.

(Schuster et al., 2010) and PQM-1 (Tepper et al., 2013). DAF-16 is excluded from the nucleus by phosphorylation by the kinase AKT-1 when the insulin signalling pathway is active (Paradis & Ruvkun, 1998), but in the absence of upstream signalling enters the nucleus to transcriptionally activate target genes (class I genes). PQM-1 is excluded from the nucleus by nuclear DAF-16, and so only in the presence of insulin signalling is it able to enter the nucleus to transcriptionally activate its targets (class II genes) (Tepper et al., 2013).

We first sought to clarify whether the entire canonical insulin signalling pathway was required for benzaldehyde/starvation associative learning. Previous work has shown a

316 requirement for the sole insulin receptor *daf-2*, its ligand *ins-1* and the downstream kinase *age-1*
317 in this paradigm (C. H. A. Lin et al., 2010). Since in the canonical pathway the final step before
318 transcription factor involvement is phosphorylation of the serine/threonine kinase AKT-1, we
319 first tested mutants in *akt-1* to confirm the necessity of the pathway up to the point of
320 transcriptional regulation. We find that *akt-1* mutant animals exhibit a severe abrogation of
321 learning (**Fig. 4A**) which can be rescued by wild type *akt-1* (**Supplemental Fig. 3**). This
322 confirmed that the requirement for the insulin signalling pathway extends at least as far in the
323 pathway as *akt-1.*

324 Since AKT-1 activity results in the nuclear localization of PQM-1 (via nuclear exclusion
325 of DAF-16), we reasoned that PQM-1 was the downstream transcription factor most consistent
326 with our observed effects of transcriptional inhibition. We therefore tested *pqm-1* loss of function
327 mutant animals (Tepper et al., 2013) for learning deficits, but to our surprise found that this
328 strain exhibited only an extremely weak learning deficit (**Fig. 4B**). *daf-16* null animals (K. Lin et
329 al., 1997) were also capable of learning (**Fig. 4C**), however they showed a mild deficit in naïve
330 approach to benzaldehyde similar to that previously reported in this strain (Tomioka et al., 2006).
331 This impaired approach made it difficult to conclusively rule out a simultaneous impairment of
332 learning comparable to *akt-1* mutants, since the loss of trained aversion to benzaldehyde we
333 observed could be due to either a genuine failure to learn or a movement disorder.

334 **Discussion**

335 *Protein Synthesis-Dependent Consolidation Continues After Training*

336 Our data suggest that withdrawal of protein synthesis inhibition after completion of
337 training allows for partial formation of the benzaldehyde/starvation memory. When training is

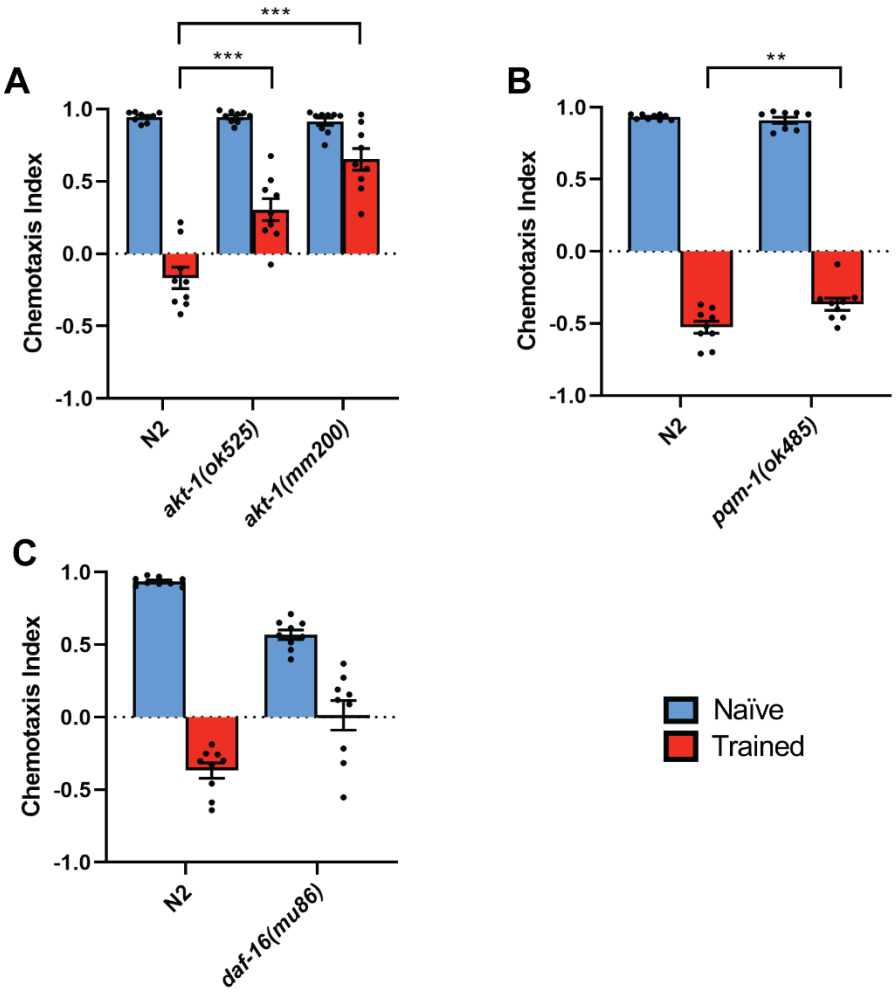

Figure 4 Benzaldehyde/Starvation Learning Depends on Insulin Signalling but not Canonical Insulin Signalling Transcription Factors. **A)** Chemotaxis of wild type and *akt-1* mutant animals to a point of benzaldehyde after benzaldehyde/starvation training. **B)** Chemotaxis of wild type and *pqm-1* mutant animals to a point of benzaldehyde after benzaldehyde/starvation training. **C)** Chemotaxis of wild type and *daf-16* mutant animals to a point of benzaldehyde after benzaldehyde/starvation training.

immediately followed by testing, inhibition of protein synthesis during either phase results in significant abrogation of memory, and inhibition of protein synthesis during both results in nearly total loss of memory. When a gap period is interspersed between training and testing phases, however, preventing protein synthesis during testing or training individually no longer impairs memory: memory formation is only blocked, and even then not fully, when protein synthesis is inhibited during both training and the gap period. This pattern of results suggests that

349 there is a wide temporal window for protein synthesis-dependent memory formation to occur in,
350 and further, that there is no necessary requirement for protein synthesis during memory recall.

351 Post-training consolidation of memory has been extensively studied in mammals, flies
352 and *Aplysia*, primarily in the context of long-term memories. Consolidation in these organisms
353 can be disrupted by protein synthesis inhibition, with a critical period of a few hours post-
354 training generally constituting the window during which protein synthesis inhibitors are effective
355 (Flexner et al., 1965; Goelet et al., 1986; Wu et al., 2017) except during episodes of subsequent
356 reconsolidation (Nader et al., 2000). Our data support a similar mechanism in formation of the
357 benzaldehyde/starvation memory, whereby external stimuli set in motion memory formation
358 processes which continue after their removal.

359 *The Identity of Gene Products Requiring Translation Remains Unclear*

360 Many genes have been implicated in *C. elegans* memory, and in principle translation of
361 any of them might be required during learning. Our chemical inhibition data suggest that *de novo*
362 transcription and translation of previously inactive genes is required during learning. This
363 constrains the range of possible genes to those which are not normally transcribed and translated
364 in the relevant cell type, and to protein coding genes specifically (ruling out, for example, *odr-1*-
365 targetting endo-siRNAs (Juang et al., 2013)).

366 The key role played by insulin signalling during benzaldehyde/starvation associative
367 learning, and its well-studied termination in DAF-16- and PQM-1-mediated transcriptional
368 changes, make it an appealing mechanism to explain our drug inhibition effects, however the
369 data we present here suggest it is unlikely to be the causative mechanism. Our present findings,
370 in conjunction with the previous identification of *ins-1*, *daf-2* and *age-1* as required genes (C. H.
371 A. Lin et al., 2010), support the necessity of components of the insulin signalling pathway as far

372 downstream as AKT-1, but *pqm-1* mutants exhibit only extremely small learning deficits relative
373 to those caused by transcriptional or translational inhibition. *daf-16* mutant animals are more
374 ambiguous because of a fairly severe naïve approach deficit, however their learning deficit is still
375 weaker than that caused by our chemical manipulations. A requirement for *daf-16*-mediated
376 transcription is also mechanistically inconsistent with a requirement for transcription, since *akt-1*
377 mutations result in the constitutive nuclear localization of DAF-16, but result in abrogation of
378 learning. These findings suggest an insulin signalling-independent role of transcription in the
379 benzaldehyde/starvation memory, and further, that the role of the insulin signalling pathway in
380 learning is not mediated by its canonical transcriptional regulators DAF-16 and PQM-1.
381 Evidence for DAF-16-independent roles of the insulin signalling pathway in learning has
382 previously been identified in the worm (Tomioka et al., 2006), and highly paradigm-specific
383 roles for various components of the pathway have been suggested (Nagashima et al., 2019).

384 Research in other learning modalities in *C. elegans* has shown that regulation of
385 experience-dependent plasticity in synaptic size, thought to be a mechanism of learning, requires
386 translation of the ARP2/3 complex, and that this is negatively regulated by the pro-forgetting
387 protein MSI-1 (Hadziselimovic et al., 2014). This represents a second potential mechanism by
388 which transcriptional and translational inhibitors may impair memory in *C. elegans*.

389 Our finding that inhibition of the proteasome does not extend memory duration is
390 consistent with both a mechanism in which protein synthesis is necessary for formation of the
391 memory engram but does not itself constitute it, or with a proteasome-independent mechanism of
392 engram decay (for example, free cellular proteases). Genes mediating active processes of
393 forgetting in *C. elegans* have been identified (Arai et al., 2022; Bai et al., 2022; Hadziselimovic

394 et al., 2014; Inoue et al., 2013; Teo et al., 2022), however none have encoded proteins involved
395 in protein decay, consistent with our findings.

396 *Benzaldehyde/Starvation Memory Exhibits Characteristics of Several Invertebrate Memory*
397 *Types*

398 In fruit flies and *Aplysia*, memories produced by a single training session (massed
399 training) are generally independent of transcription, translation and the transcription factor
400 CREB, while repeated training sessions (spaced training) give rise to long-term memories
401 dependent on transcription, translation and CREB (**Table 1**). The *C. elegans* memory we
402 describe here does not clearly correspond to any of these: it depends on transcription and
403 translation, is independent of CREB, and is produced through a single massed training session.

404 Two very different interpretations of these results can be imagined. It could be that the
405 memory described here is a genuinely independent class of memory with different behavioural
406 and molecular requirements to those of other well-studied invertebrate memories. Alternatively,
407 it may be that the behavioural manifestation of this memory is mediated by an overlapping suite
408 of memory classes which independently correspond to the memory types seen in flies and
409 *Aplysia*, but which in conjunction manifest as a blended memory mechanism. However, since
410 this latter interpretation would at the very least require postulating a protein synthesis-dependent
411 memory produced by massed training as one of these constituent components, the requirement
412 for a novel class of memory cannot be fully avoided, and so we favour the former interpretation
413 as more parsimonious.

414 Massed training protocols in the worm have been reported to be either independent
415 (Amano & Maruyama, 2011; Nishijima & Maruyama, 2017) or dependent (Stein & Murphy,
416 2014) on *de novo* protein translation, depending on the protocol. While it is conceptually

417 possible that some apparently massed protocols involve cryptic spaced training, it is difficult to
418 see ways by which this could occur in ours: food is uniformly absent on the agar plate (or in
419 liquid media) during training, and the worms are exposed to the smell through the gas phase,
420 which is ubiquitously present. Our findings, in conjunction with a similar report of protein
421 synthesis-dependent massed learning using a different paradigm (Stein & Murphy, 2014),
422 challenge the view that protein synthesis-dependent memory necessarily requires spaced
423 training: in *C. elegans,* requirement for spaced training appears instead to depend on the exact
424 conditioned and/or unconditioned stimuli employed.

425 None of the manipulations we describe here fully prevent memory. It is possible that the
426 small fraction of retained memory in our experiments represents a (potentially short-term)
427 memory process which is independent of translation and transcription. Since experiments in a
428 similar paradigm found that only 50% of protein synthesis was inhibited by a concentration of
429 cycloheximide three times greater than the one we use (Amano & Maruyama, 2011), however, at
430 least for our experiments utilizing cycloheximide, limited drug efficacy is a more likely
431 explanation.

432 Learning from your mistakes is a valuable skill for all animals, and for most animals
433 there are many different mistakes which might profitably be learned from. Our findings suggest
434 that memory types across invertebrates, and even different memories within a single species,
435 may exhibit fewer unifying principles than is commonly appreciated, at least as revealed by the
436 systems of classification which have been influential in flies and *Aplysia*. This both highlights
437 the importance of considering the specific paradigm and species in question when reasoning
438 about the mechanisms of memory processes, and draws attention to the diversity of ways in

which evolution has provided mechanisms to change behaviour in response to a dangerous world.

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Competing interests

Authors declare no competing interests.

Data and materials availability

All data is available in the main text or the supplementary materials.

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