# C. elegans positive olfactory associative memory is a molecularly conserved behavioral paradigm

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## ARTICLE INFO

## ABSTRACT

While it is thought that short-term memory arises from changes in protein dynamics that increase the strength of synaptic signaling, many of the underlying fundamental molecular mechanisms remain unknown. Our lab developed a Caenorhabditis elegans assay of positive olfactory short-term associative memory (STAM), in which worms learn to associate food with an odor and can remember this association for over 1 h. Here we use this massed olfactory associative assay to identify regulators of C. elegans short-term and intermediate-term associative memory (ITAM) processes. We show that there are unique molecular characteristics for different temporal phases of STAM, which include: learning, which is tested immediately after training, short-term memory, tested 30 min after training, intermediate-term memory, tested 1 h after training, and forgetting, tested 2 h after training. We find that, as in higher organisms, C. elegans STAM requires calcium and cAMP signaling, and ITAM requires protein translation. Additionally, we found that STAM and ITAM are distinct from olfactory adaptation, an associative paradigm in which worms learn to disregard an inherently attractive odor after starvation in the presence of that odor. Adaptation mutants show variable responses to short-term associative memory training. Our data distinguish between shorter forms of a positive associative memory in C. elegans that require canonical memory pathways. Study of STAM and ITAM in C. elegans could lead to a more general understanding of the distinctions between these important processes and also to the discovery of novel conserved memory regulators.

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## 1. Introduction

Learning and memory allow animals to navigate, find food, and survive in a changing environment. In humans, memory declines with age, and memory deficits are often a hallmark of neurodegenerative disorders, such as Alzheimer's Disease (Hodges & Patterson, 1995; Salthouse, 1991). Therefore, an understanding of the molecular bases of different forms of memory is essential to develop treatments for memory loss.

Short-term memory lasts from minutes to hours, resulting from changes in synaptic strength mediated by modifications at the synapse in the appropriate neurons (Kandel, 2001). cAMP signaling and calcium signaling are required for short-term memory in many organisms (Hawkins, Kandel, & Bailey, 2006; Kandel, 2001). These pathways are activated in response to neurotransmitter receptor

activity, can modulate receptor activity, and are thought to activate synaptic proteins to facilitate memory formation and maintenance. Several kinases (Giese & Mizuno, 2013; Kandel, 2012) and synaptic cellular adhesion proteins (Cheng et al., 2001; Grotewiel, Beck, Wu, Zhu, & Davis, 1998) have also been identified as regulators of short and intermediate-term memory in higher organisms. However, downstream targets or parallel regulatory pathways remain largely unknown. Therefore, it is important to establish a model system in which regulation of short-term memory is conserved and new genetic regulators of short-term memory can be rapidly identified.

Caenorhabditis elegans has a simple nervous system comprised of just 302 neurons, and its stereotypic neural connections (the "connectome") have been mapped (Varshney, Chen, Paniagua, Hall, & Chklovskii, 2011; White, Southgate, Thomson, & Brenner, 1986). This small nematode can learn and form both associative and non-associative memories, lasting as long as 24 h (Ardiel & Rankin, 2010; Kauffman, Ashraf, Corces-Zimmerman, Landis, & Murphy, 2010). We developed a protocol that pairs a relatively neutral odor (butanone at a specific concentration) with food to create a positive association, resulting in strong attraction to the odor (Kauffman et al., 2010). Short-term associative memory (STAM) after one conditioning period decays by 2 h after training,

while long-term memory after spaced training (7 conditioning cycles) lasts more than 16 h (Kauffman et al., 2010). Importantly, because the worms display an <u>increased</u> response to odor after training, our positive associative assay allows clear distinction from non-associative forms of learning, such as habituation and adaptation, which repress behavioral response to a stimulant and therefore could be interpreted as negative associative learning.

We previously found that positive associative learning requires conserved receptors, and that long-term associative memory requires transcription as well as the activity of the Zn finger transcription factor CREB (Kauffman et al., 2010). Here we show that *C. elegans* STAM requires cAMP and calcium signaling pathways. We also find that protein translation is required at two different steps: it is first required during massed associative memory training to extend memory past 30 min, and subsequently after memory training to ensure proper decay of the associative memory, or forgetting. Intermediate-term memory requires translation but not transcription (Ashraf, McLoon, Sclarsic, & Kunes, 2006; Ghirardi, Montarolo, & Kandel, 1995); our results show that massed training results in both short-term (30 min) and intermediate-term (1 h) associative memory (STAM and ITAM). Our data show that shorter-term memory mechanisms in *C. elegans* are conserved with higher organisms and further establish *C. elegans* as a good model to study the genetic regulation of short-term and intermediate-term associative memory.

*C. elegans* has been shown to associate starvation with a myriad of sensory cues including olfactory, gustatory, thermosensory, and pathogenic cues (Colbert & Bargmann, 1995; Mohri et al., 2005; Saeki, Yamamoto, & Iino, 2001; Zhang, Lu, & Bargmann, 2005). Adaptation is a behavior in which worms display reduced responsiveness to an odor after a one-hour odor exposure in the absence of food (Colbert & Bargmann, 1995). Stetak, Horndli, Maricq, van den Heuvel, and Hajnal (2009) use the same odor/starvation conditioning paradigm to induce what they refer to as a negative associative short-term memory. Our massed associative memory assay also involves a one-hour exposure to an odor, but in the presence of food rather than the absence of food, resulting in a positive association between food and odor. Therefore, one hypothesis that we set out to test is whether STAM is simply a response occurring in the opposite direction of adaptation that requires the same molecular machinery. However, we find that mutants that are defective for adaptation have varying STAM/ITAM phenotypes, including prolonged, reduced, and normal memory, establishing positive olfactory STAM/ITAM as a distinct memory process in *C. elegans*.

## 2. Methods

### 2.1. Worm cultivation

*C. elegans* were cultivated at 20 °C on High Growth Media (HG) or Nematode Growth Media (NGM) seeded with OP50 *E. coli* using standard methods (Brenner, 1974). Animals were synchronized by hypochlorite treatment and tested for learning and memory at Day 1 of adulthood at room temperature.

### 2.2. Strains

Wild type: (N2 Bristol); mutant strains: KP1182 (*acy-1(nu329)*), KG518 (*acy-1(ce2)*), KG532 (*kin-2(ce179)*), KG744 (*pde-4(ce268)*), VC1052 (*unc-43(gk452)*), VC1408 (*magi-1(gk657)*), JH1270 (*nos-1(gv5)*), JK3022 (*fbf-1(ok91)*), CX20 (*adp-1(ky20)*), RB995 (*hpl-2(ok916)*), NL917 (*mut-7(pk204)*), and DR466 (*him-5(e1490)*) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). *egl-4(ky95)*; *him-5(e1490)* was a

kind gift from N. L'Etoile (University of California, San Francisco, San Francisco, CA).

### 2.3. Behavioral assays

#### 2.3.1. Short-term associative memory training

STAM assays were performed as previously described (Kauffman et al., 2010, 2011). Briefly, synchronized day 1 hermaphrodites were starved for 1 h in M9 buffer. Worms were then transferred to 6 cm NGM plates with 500 μL of OP50 and 2 μL of 10% butanone in ethanol on the lid and conditioned for 1 h. After conditioning, 100–500 worms from the trained population of worms were tested once for chemotaxis to butanone either immediately (0 h) or after being transferred to 10 cm NGM plates with 900 μL fresh OP50 for specified intervals before testing (30 min–4 h). Graphs display the average chemotaxis index at each time-point for 3 or more separate biological replicate experiments, with each experiment displayed as a pale colored curve behind this average line. Each experiment included at least 3 technical replicates per time-point, with the exception of one of four biological replicate experiments for *acy-1(nu329)* and *kin-2(ce179)* mutants that had only one technical replicate per time-point. Significance was calculated by comparing the experimental and control groups using two-way ANOVAs ($p \leqslant 0.05$ for significant experiments) followed by Bonferroni-corrected post hoc unpaired *t*-tests comparing the experimental and control at each time-point (Bonferroni-correction per *t*-test: $p_{bonf} \leqslant 0.05$).

#### 2.3.2. Chemotaxis assay

Chemotaxis assays were performed as previously described (Bargmann, Hartwieg, & Horvitz, 1993). Briefly, 100–500 day 1 adult worms were placed at the origin on a 10 cm NGM plate with butanone (1 μL 10% butanone in ethanol +1 μL NaN₃) and ethanol control (+1 μL NaN₃) equidistant from the origin. After 1 h., black and white images of each plate were taken using a Basler A fire wire camera (Basler AG, Ahrensburg, Germany) using "Measurement and Automation" software (National Instruments) to capture images. Images were analyzed using count_worms_v7.3 (Kauffman et al., 2011). The chemotaxis index = $[(n_{attractant}) - (n_{control})] / [(Total - n_{origin})]$.

#### 2.3.3. Drug treatments

200 μg/mL Actinomycin $D \geqslant 95\%$ (Sigma Aldrich, Saint Louis, Missouri) was added to M9 buffer for the final 30 min of starvation and added to S basal during conditioning along with 1:1000 Butanone and OP50 *Escherichia coli* bacteria that had been grown overnight. Cycloheximide $\geqslant 94\%$ (Sigma Aldrich, Saint Louis, Missouri) was added to NGM at 0.8 mg/mL. Plates were poured and solidified overnight at 4 °C, seeded with OP50 *E. coli*, and then used for Short-term associative memory training either during conditioning or after training. To ensure 1 h of drug treatment for 30 min conditioning, worms were added to cycloheximide plates with no food for the last 30 min of starvation followed by 30 min of conditioning on cycloheximide with OP50 and 10% butanone.

## 3. Results

### 3.1. C. elegans STAM and ITAM require conserved memory mechanisms

#### 3.1.1. Blocking translation, but not transcription, inhibits both memory maintenance and forgetting

We and others have shown that long-term memory in *C. elegans* requires both transcription and translation (Kauffman et al., 2010; Timbers & Rankin, 2011; Vukojevic et al., 2012), as is required for

long-term memory in other organisms. While transcription is not required for temporal phases other than long-term memory, protein translation is required for intermediate-term (1–3 h) phases of memory (Davis & Squire, 1984; Ghirardi et al., 1995; Hawkins et al., 2006; Jin, Kandel, & Hawkins, 2011; Michel, Green, Gardner, Organ, & Lyons, 2012; Parvez, Stewart, Sangha, & Lukowiak, 2005; Sutton, Masters, Bagnall, & Carew, 2001) and for what is termed negative associative short-term memory in *C. elegans* that may actually be intermediate-term memory (Hadziselimovic et al., 2014). As in our previously published short-term associative memory assay, several thousand day 1 adult worms are starved for 1 h, conditioned with food and 10% butanone for 1 h, and then held on food before subsets of the trained population are tested for memory using a chemotaxis assay at specific time-points after training (Fig. 1A) (Kauffman et al., 2010).

In order to assess the requirement for transcription and translation in *C. elegans* short-term associative memory, we used actinomycin D and cycloheximide, respectively, during conditioning or after training to inhibit these processes in wild-type day 1 adult worms (Fig. 1A). To test whether STAM requires transcription, we treated worms with 200 μg/mL actinomycin D during the final 30 min of starvation and in S Basal + OP50 and 1:1000 butanone during conditioning. We tested worms 30 min after training, allowing them to regain motility after submersion (Fig. 1A). While treatment with actinomycin D during spaced training was sufficient to inhibit long-term associative memory (Kauffman et al., 2010), we found that inhibiting transcription during training does not inhibit short-term memory formation or retention (Fig. 1B). This is consistent with our previous observation that short-term associative memory is not dependent on the cAMP Response Element-Binding Protein (CREB) transcription factor (Kauffman et al., 2010).

We then tested whether protein translation is required for short-term associative memory. We treated worms with 0.8 mg/ml cycloheximide in growth medium during or after STAM training (Fig. 1A). Worms treated with cycloheximide during conditioning had a small reduction in learning ($p = 0.046$) that may be due to the stress of the treatment (Fig. 1C). However, 30 min memory was normal. 1 h memory was significantly reduced compared to untreated controls. These data suggest that memory 30 min after training is a translation-independent short-term memory, and that by 1 h after training *C. elegans* have formed an intermediate-term memory, since intermediate-term memory requires translation but not transcription (Ghirardi et al., 1995). (For continuity, we will continue to refer to our training as "STAM training", although the same training also induces ITAM.) We also tested whether conditioning worms for only 30 min would result in a fully translation-independent memory, since we had previously shown that 30 min of conditioning is enough to maximize learning (Kauffman et al., 2010). We treated worms for 1 h with cycloheximide, as in our initial experiment, by treating for the last 30 min of starvation followed by the full 30 min of conditioning. Interestingly, we found that duration of memory after 30 min of conditioning was equivalent to that of 1 h of conditioning, and 1 h memory was dependent on translation (Sup. Fig. 1).

Unexpectedly, worms treated with cycloheximide after training showed increased chemotaxis at both at 2 and 4 h, after untreated worms have lost their attraction to butanone; in fact, memory is still close to 0 h post-training levels after 4 h, suggesting a decrease in forgetting (blue trace, Fig. 1D). (The full duration of memory for *C. elegans* treated after cycloheximide training could not be tested due to the impairment of worm motility after 4+ hours of cycloheximide exposure.) Forgetting a negative associative memory also requires translation (Hadziselimovic et al., 2014). Taken together, these data indicate that forgetting is an actively regulated process that requires protein translation.

### 3.1.2. Conserved genetic pathways regulate STAM and ITAM

We and others have shown that *C. elegans* associative learning is dependent on AMPA type receptors (Kauffman et al., 2010; Lau, Timbers, Mahmoud, & Rankin, 2013; Vukojevic et al., 2012), as in other organisms (Cui et al., 2011; Li, Roberts, & Glanzman, 2005; Sanderson et al., 2008; Zamanillo et al., 1999). In order to identify genes that are required for memory, we examined calcium signaling genes downstream of receptor activity, specifically calcium-activated CaMKII and components of the cAMP pathway that are required for learning and memory in many organisms (Hawkins et al., 2006; Squire & Kandel, 2008). cAMP activates Protein Kinase A (PKA) and modulates memory in a concentration-dependent manner in *Drosophila* (Feany, 1990). Adenylate cyclase catalyzes the conversion of ATP to cAMP and is required for short-term memory in mice, *Drosophila*, and *Aplysia* (Davis, Cherry, Dauwalder, Han, & Skoulakis, 1995; Hawkins et al., 2006; Wang, Phan, & Storm, 2011). Protein Kinase A is activated by cAMP and is required for memory in many organisms (Li, Tully, & Kalderon, 1996; Michel et al., 2012; Nguyen & Woo, 2003; Squire & Kandel, 2008). We found that loss-of-function mutants of adenylate cyclase, *acy-1(nu322)*, have reduced chemotaxis compared to WT, but can learn to associate butanone and food. Memory after training is significantly reduced by 30 min compared to wild-type worms (Fig. 2A). To take the naïve chemotaxis index (CI) of *acy-1(nu322)* mutants into account, we calculated the learning index after training (Learning Index = $CI_{after training} - CI_{naïve}$) (Sup. Fig. 2). We find that memory at 1 h after training is still significantly reduced in *acy-1(nu322)* mutants compared to WT. *acy-1(ce2)* gain-of-function mutants have normal naïve chemotaxis, but reduced 30 min and 1-h memory (Fig. 2B). Taken together, these data indicate that cAMP signaling is required for STAM and ITAM. *kin-2(ce179)* mutants are mutated in the PKA inhibitory domain and have increased PKA activity; *kin-2(ce179)* mutants have reduced one-hour memory compared to WT (Fig. 2C). cAMP phosphodiesterase (PDE), is required for memory in *Drosophila* (Dudai, Jan, Byers, Quinn, & Benzer, 1976). However, *pde-4(ce268)* mutants have normal short-term associative memory (Sup. Fig. 3) though this may be due to the partial loss-of-function of available mutants. $Ca^{2+}$/cal-modulin-dependent protein kinase II (CaMKII) is required for memory in many organisms (Ashraf et al., 2006; Giese, Fedorov, Filipkowski, & Silva, 1998; Hawkins et al., 2006) and required for forgetting adaptation in *C. elegans* (Inoue et al., 2013). Mutants of the *C. elegans* CaMKII homolog *unc-43(gk452)* have reduced memory by 1 h. (Fig. 2D).

In addition to cAMP and calcium signaling pathways, short-term memory requires changes at the synapse (Grotewiel et al., 1998; Hawkins et al., 2006; Stetak et al., 2009). We have previously shown that the AMPA receptor subunit GLUR1 homolog, *glr-1*, is required for learning (Kauffman et al., 2010). Membrane-associated guanylate cyclase (MAGUK) proteins regulate receptor distribution at the synapse in many organisms, including *C. elegans* (Deng, Price, Davis, Mori, & Burgess, 2006; Dobrosotskaya, Guy, & James, 1997; Hirao et al., 1998; Stetak et al., 2009), and MAGI-1 regulates GLR-1 and is required for *C. elegans* negative associative memory (Stetak et al., 2009). We tested the role of MAGI-1 in our STAM assay, and interestingly, found that *magi-1(gk657)* mutants have positive short-term associative memory that is similar to wild-type's (Fig. 2E), indicating that negative and positive associative memories in *C. elegans* may be regulated differently at the synapse.

Together, these data indicate that *C. elegans* STAM requires cAMP signaling, and ITAM requires both cAMP and CaMKII signaling, as is required for short and intermediate-term memory in other organisms.
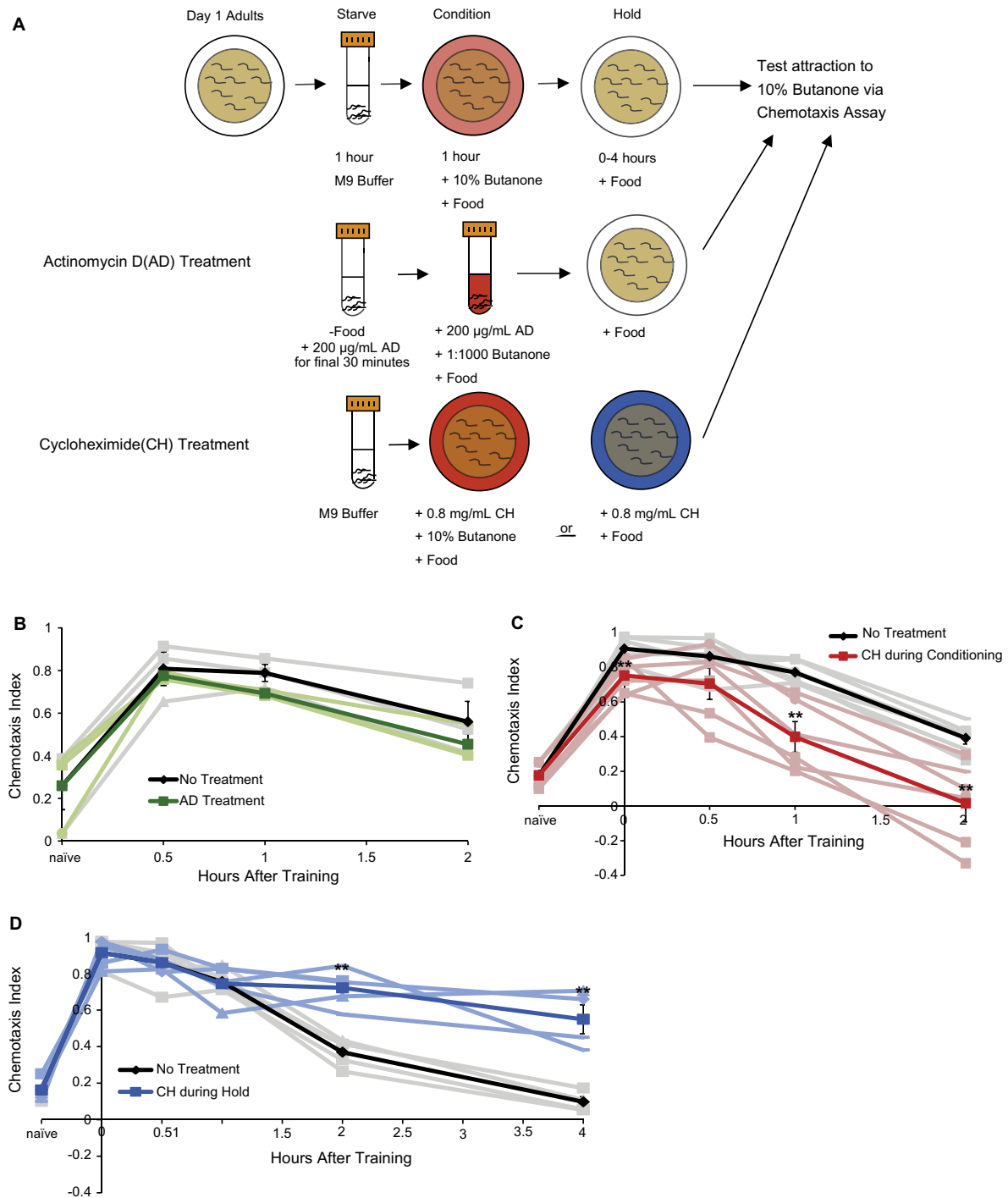
**Fig. 1.** *C. elegans* positive olfactory short-term associative memory (STAM) requires translation but not transcription. (A) Illustration of short-term associative memory assay and changes for drug treatment protocols. For Actinomycin D (AD) treatment, worms were starved in M9 with 200 µg/mL AD added during the final 30 min of starvation then conditioned in OP50 in S-Basal with 200 µg/mL AD and 0.1% butanone. Untreated worms were tested using the same protocol without AD. For Cycloheximide (CH) treatment, 0.8 mg/mL CH was added to nematode growth media (NGM) when plates were poured. OP50 was grown overnight on NGM plates with or without drug and worms were treated either during the STAM conditioning period or during the holding period after training. Otherwise the protocol followed the normal STAM assay. (B) There is no change in memory retention with Actinomycin D treatment. Worms were either not treated (black), or treated during starvation and conditioning (green). $n = 3$ experiments (pale lines). (C) Cycloheximide treatment during training causes a slight decrease in learning (red; $p_{bonf} = 0.046$), at at 1 h ($p_{bonf} = 0.007$), but 30-min memory does not require translation. Chemotaxis index of treated worms at 2 h was also significantly below that of controls (black; $p_{bonf} = 0.023$). $n = 6$ experiments. (D) Cycloheximide treatment after training (blue) shows that translation is required for normal forgetting ($p_{bonf} = 0.001$ at 2 h. $p_{bonf} = 0.010$ at 4 h). $n = 5$ experiments. Error bars represent the mean ± SEM. Asterisks indicate significance as determined by an unpaired student *T*-test with a Bonferroni correction for multiple comparisons.
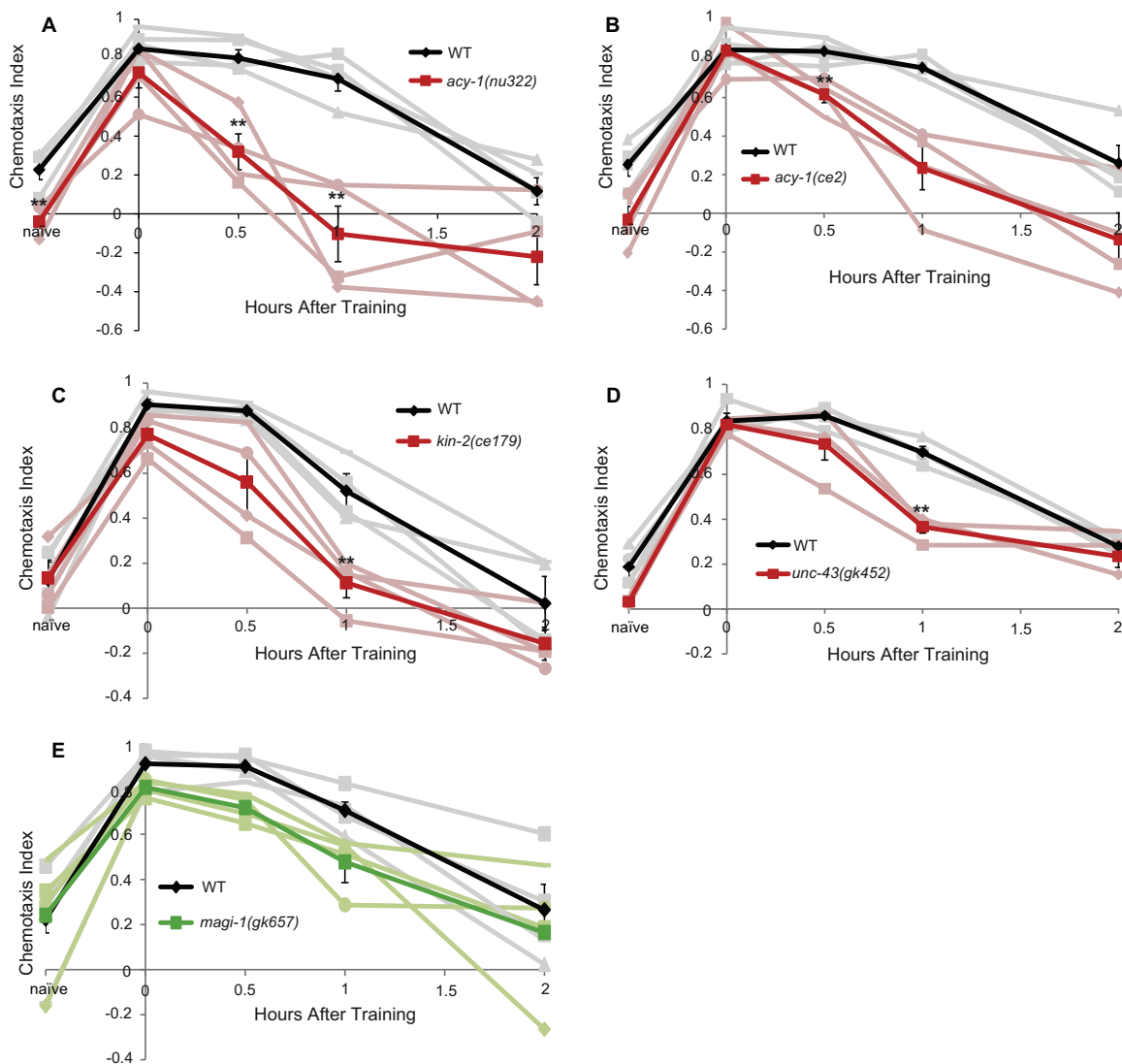
**Fig. 2.** *C. elegans* STAM requires canonical memory genes. (A) *acy-1(nu329)* mutants (red) have impaired naïve chemotaxis as compared to wild type (black) ($p_{bonf}$ = 0.025). *acy-1(nu329)* can learn but impaired memory by 30 min. WT ($p_{bonf}$ = 0.012 at 30 min. and $p_{bonf}$ = 0.011 at 1 h). n = 4 experiments. (B) *acy-1(ce2)* gain-of-function mutants (red) have reduced 30 min. and 1 h. memory as compared to WT (black) ($p_{bonf}$ = 0.031 at 30 min. and $p_{bonf}$ = 0.021 at 1 h). n = 4 experiments. (C) *kin-2(ce179)* mutants (red) have reduced 1 h. memory as compared to wild-type (black) ($p_{bonf}$ = 0.018). n = 4 experiments. (D) *unc-43(gk452)* mutants (red) have reduced 1 h. memory as compared to WT (black) ($p_{bonf}$ = 0.0007). n = 4 experiments. (E) *magi-1(gk657)* mutants (red) are not significantly different from WT (black) n = 4 experiments. Error bars represent the mean ± SEM. Asterisks indicate significance as determined by an unpaired student *T*-test with a Bonferroni correction for multiple comparisons.

### 3.2. C. elegans STAM and adaptation are molecularly distinct behavioral paradigms

Adaptation is a plastic response in which *C. elegans* learn to ignore the presence of odors persisting in their environment (Colbert & Bargmann, 1995). Many of the molecular mechanisms underlying this behavioral response have been previously elucidated using a negative associative assay that pairs odor with starvation to induce adaptation (Colbert, Smith, & Bargmann, 1997; Juang et al., 2013; Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009; L'Etoile et al., 2002). Adaptation to butanone and benzaldehyde requires AWC[on], the sensory neuron required for sensing butanone (Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009). Because our short-term associative memory assay tests *C. elegans'* response to butanone after 1 h of butanone exposure with food as opposed to without food, as in adaptation studies, and butanone is also sensed by AWC[on], one hypothesis is that adaptation and our short-term associative memory use the same molecular machinery. If this were true, we would expect that

mutants that cannot adapt to butanone would extend memory after STAM training.

To test this hypothesis, we examined adaptation mutants. Loss-of-function mutations in *nos-1*, *fbf-1*, *egl-4*, *mut-7*, *hpl-2*, and *adp-1* result in the inability to adapt to an attractive odor after exposure (Colbert & Bargmann, 1995; Juang et al., 2013; Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009; L'Etoile et al., 2002). FBF-1, a Pumilio homolog, facilitates the translation of EGL-4, a cyclic GMP-dependent protein kinase (Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009). Both *fbf-1(ok91)* and *egl-4(ky95)*, which is mutated only at the site of FBF-1 binding, have prolonged memory after STAM training, decaying by 4 h instead of 2 h (Fig. 3A and B). Similarly, *adp-1(ky20)*, which was identified in a screen for adaptation mutants but remains uncloned (Colbert & Bargmann, 1995), displayed extended memory after STAM training that decays by 4 h (Fig. 3C). Thus, *fbf-1*, *egl-4*, and *adp-1* mutants all extend STAM, as would be expected of a mutant that cannot adapt.

By contrast, *nos-1(gv5)*, a mutant of one of three *C. elegans* Nanos homologs, has reduced memory by 30 min as compared to
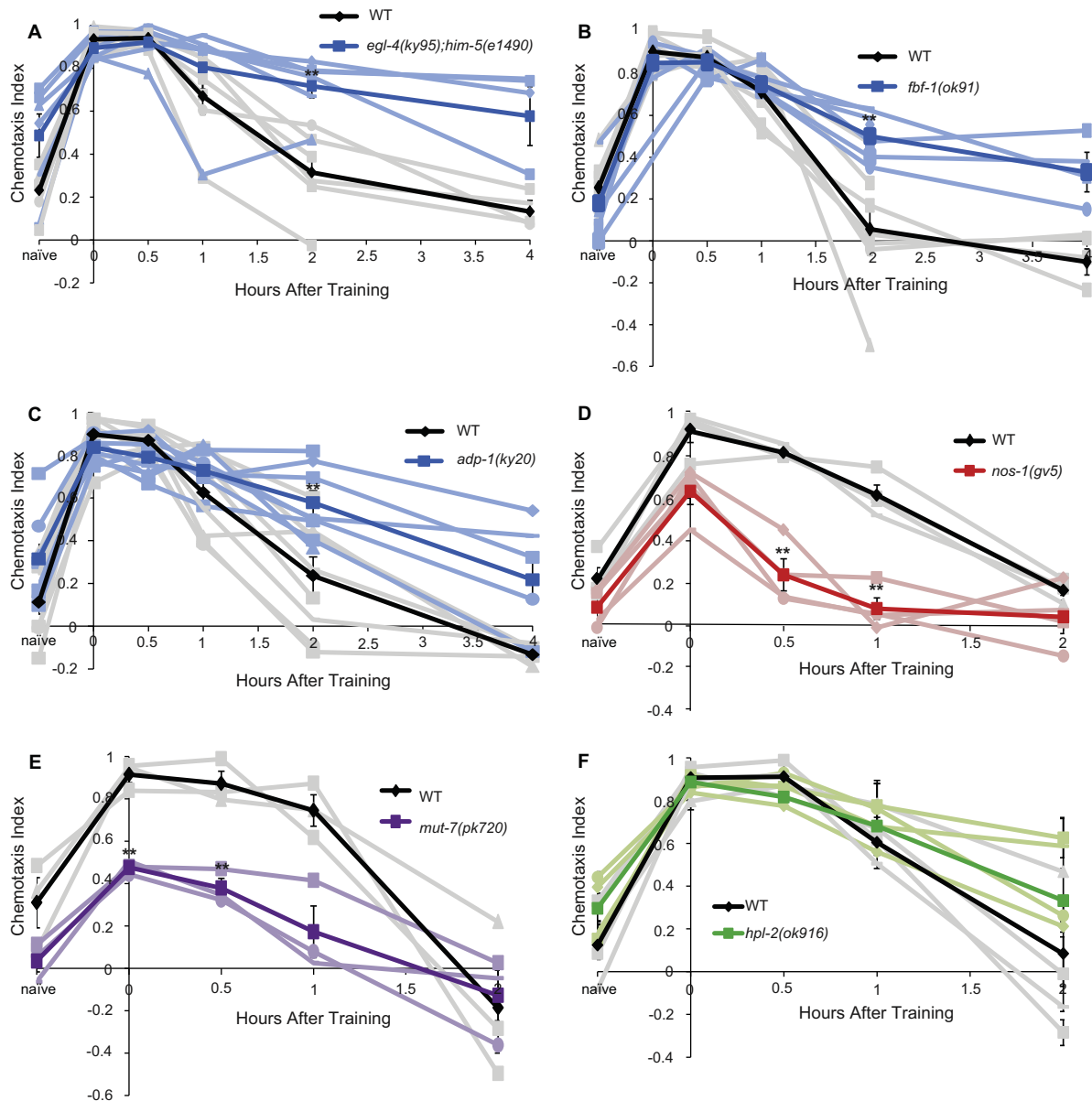
**Fig. 3.** *C. elegans* STAM and Adaptation are molecularly distinct behavioral paradigms. (A) *egl-4(ky95); him-5(e1490)* mutants (blue) have extended memory compared to WT (black) ($p_{bonf}$ = 0.013 at 2 h). $n$ = 3 experiments for naive-2 h. and 3 experiments for naive-4 h. (*egl-4(ky95)* mutants were also mutant for *him-5*, but *him-5(e1490)* has normal STAM (Sup. Fig. 4)) (B) *fbf-1(ok91)* mutants (blue) have extended memory compared to WT (black) ($p_{bonf}$ = 0.036 at 2 h) $n$ = 4 experiments for naïve-2 h and 3 experiments for naïve-4 h. (C) *adp-1(ky20)* mutants (blue) have extended memory compared to WT (black) ($p_{bonf}$ = 0.037 at 2 h) $n$ = 3 experiments for naïve-2 h. and 5 experiments for naïve-4 h. (D) *nos-1(gv5)* mutants (red) have no memory by 30 min after training ($p_{bonf}$ = 0.001 at 30 min. and $p_{bonf}$ = 0.001 at 1 h). $n$ = 4 experiments. (E) *mut-7(pk720)* mutants (purple) have reduced learning and memory as compared to WT (black) ($p_{bonf}$ = 0.002 at 0 h. and $p_{bonf}$ = 0.013 at 30 min). $n$ = 3 experiments. (F) *hpl-2(ok916)* mutants (green) have normal memory compared to WT (black). $n$ = 5 experiments. Error bars represent the mean ± SEM. Stars indicate significance as determined by an unpaired student *T*-test with a Bonferroni correction for multiple comparisons.

wild-type (Fig. 3D). Adaptation requires EGL-4 translocation into the nucleus, where it phosphorylates MUT-7 and HPL-2 and regulates the expression of *odr-1*, a transmembrane guanylyl cyclase required for normal olfaction (Bargmann et al., 1993; Juang et al., 2013). After STAM training, *mut-7(pk20)* mutants of the RNAse III homolog have reduced learning and memory ability (Fig. 3E). Mutants of heterochromatin protein 1 homolog *hpl-2(ok196)* have normal learning and memory (Fig. 3F). Together, these data indicate that disrupting genes required for adaptation causes varying STAM phenotypes. While FBF-1, EGL-4, and ADP-1 may have overlapping functions between adaptation and STAM, NOS-1, HPL-2, and MUT-7 play different roles in adaptation and STAM, suggesting that the two paradigms are at least partially distinct.

## 4. Discussion

While many learning and memory paradigms have been described and several genes that regulate these plastic processes have been identified, many aspects of the process remain unknown. We previously developed a novel *C. elegans* positive olfactory short-term associative memory assay. Here we show that STAM does not require gene transcription, but that protein translation is required for longer memory after training and for forgetting. When we inhibited translation during training, memory was established, but was impaired by 1 h post-training. We have therefore identified a form of *C. elegans* intermediate-term associative memory. Though intermediate-term memory is a less well-defined

phase of memory, translation is required for later memory time-points after massed training in *Drosophila*, as well as for intermediate-term memory in *Aplysia* (Ashraf et al., 2006; Hawkins et al., 2006). Recently, a non-translation dependent intermediate-term memory had been identified for habituation in *C. elegans* and a translation-dependent negative-associative memory has been identified but was not characterized as intermediate-term memory (Hadziselimovic et al., 2014; C. Li et al., 2013). Furthermore, we found that protein translation is necessary for normal forgetting. Little is known about the process of forgetting in any organism. Previously, forgetting has been considered a passive process, possibly related to basal phosphatase deactivation of kinases that establish short-term memory (Jonides et al., 2008; Wixted, 2004). However, recent experiments in both flies and *C. elegans* suggest that forgetting involves active processes, including remodeling of the actin cytoskeleton at the synapse (Hadziselimovic et al., 2014; Shuai et al., 2010). Forgetting of a negative benzaldehyde odor association in *C. elegans* is regulated by an UNC-43-activated MAP kinase pathway in AWC (Inoue et al., 2013). However, in our positive associative assay, *unc-43* is required for memory maintenance, which may mask any potential role in forgetting.

We also show that as in higher organisms, *C. elegans* uses the conserved cAMP and calcium signaling pathways for the formation and maintenance of short-term and intermediate-term memory. Interestingly, our data show that both loss-of-function and gain-of-function adenylate cyclase (*acy-1*) mutants have reduced memory, suggesting that, as in *Drosophila*, a balanced (or adjustable) level of cAMP is required for normal memory. In addition to adenylate cyclase, ITAM requires PKA and CaMKII activity. Though the PKA regulatory subunit mutant, *kin-2*, has an ITAM defect, STAM may be affected as it is in other organisms (Dudai, 1988; Kandel, 2012) by knocking down the catalytic domain of PKA, *kin-1*, an experiment we have not been able to do due to the essential requirement of *kin-1*.

In *C. elegans*, CaMKII regulates neuronal cell fate and, through a separate pathway, regulates forgetting after adaptation (Chuang & Bargmann, 2005; Inoue et al., 2013). In higher organisms, different CaMKII alpha and beta mutations specifically affect behaviors from learning to long-term memory (Giese & Mizuno, 2013), and CaMKII autophosphorylation is essential for learning after massed training in mice (Irvine, Vernon, & Giese, 2005). Further research could determine whether our CaMKII mutant intermediate-term memory defect is specific to our positive olfactory associative assay, the particular CaMKII isoforms involved, and/or *C. elegans* behavior.

Our behavioral assay has allowed us to determine the temporal points at which particular gene products affect different phases of positive associative memory after massed training (Fig. 4A and B). Specifically, we find that *mut-7*, *casy-1*, *glr-1*, and *hen-1* (Kauffman et al., 2010), are required for learning, while *acy-1 and nos-1* are required for STAM. *kin-2*, *acy-1* and *unc-43* are required for ITAM, and finally, *egl-4* and *fbf-1* are required for forgetting.

The fact that forgetting requires protein translation is also interesting to consider in the context of the relationship between STAM and adaptation. We found that a mutation in the 3′UTR of the *egl-4* Protein Kinase G that inhibits its binding to translational activator FBF-1 (Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009) inhibits forgetting, as does deletion of *fbf-1*. These genes are uniquely co-expressed in the AWB and AWC neurons (Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009), so forgetting may occur in the sensory neuron through translation of EGL-4. The memory inhibitor MSI-1 has been found to reduce synapse size after negative STAM training through its translational repression of the ARP2/3 complex in command motor neurons downstream of the sensory neuron, and translation is required for forgetting a negative short-term associative memory (Hadziselimovic et al., 2014). Taken together, these data suggest that forgetting is an active process involving translation, possibly of multiple proteins that alter cellular machinery in both the sensory and command motor neurons.

Adaptation involves starvation-induced desensitization to a previously attractive odor after long-term exposure. Since our assay involves a 60-min exposure to butanone in the presence rather than the absence of food, we tested whether STAM and
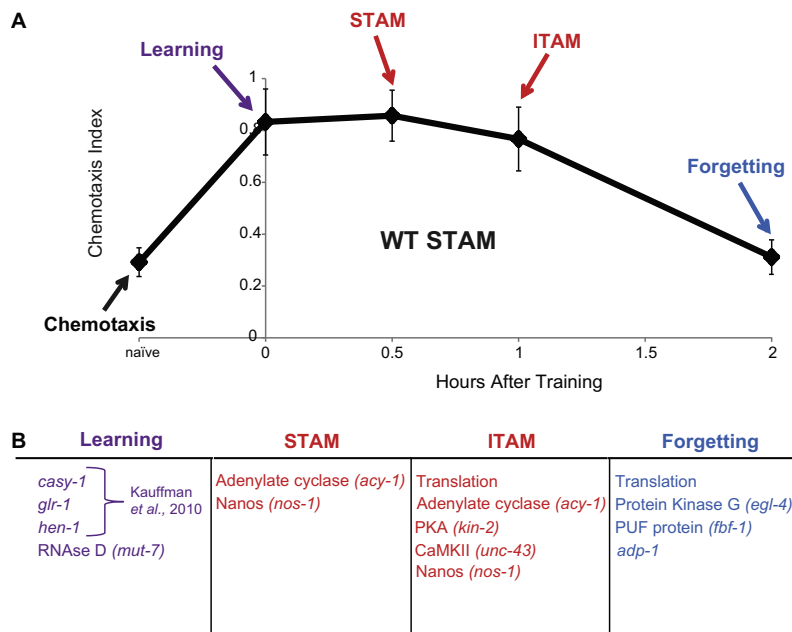


**Fig. 4.** Molecular regulation of phases of memory after massed training. (A) Schematic of normal shorter-term associative memory phases. Before training, a test is run to determine naïve attraction to butanone (chemotaxis ability). Following training, learning is immediately tested. Short-term memory (STAM) is tested 30 min following training, and Intermediate-term memory (ITAM) at 1 h. Forgetting is tested 2 h after training when wild-type memory has decayed. (B) Genes and molecular processes that regulate each phase of memory based on our current data and our previously published learning data (Kauffman et al., 2010).

**Table 1**
Adaptation and STAM phenotypes for mutants tested in both paradigms.

| Mutant | Homolog | Protein Cellular Localization | Adaptation Phenotype | STAM Phenotype |
|--------|---------|-------------------------------|----------------------|----------------|
| egl-4(ky95) | PKG | Cytoplasm | - | + |
| fbf-1(ok91) | PUF Protein | Cytoplasm | - | + |
| adp-1(ky20) | Uncloned | Unknown | - | + |
| nos-1(gv5) | Nanos | Unknown | - | - |
| mut-7(pk720) | RNAse III | Nucleus and Cytoplasm | - | - learning |
| hpl-2(ok916) | HPI | Nucleus | - | WT |

(−) reduced compared to WT. (+) increased compared to WT (Colbert & Bargmann, 1995; Juang et al., 2013; Kaye, Rose, Goldsworthy, Goga, & L'Etoile, 2009; L'Etoile et al., 2002).

adaptation are regulated by the same molecular mechanisms. Adaptation results in part from the translation of EGL-4, mediated by FBF-1, and subsequent EGL-4 translocation into the nucleus, where it phosphorylates HPL-2 and MUT-7, which then reduce the levels of *odr-1* guanylyl cyclase (Juang et al., 2013; Kaye et al., 2009). While all of the adaptation mutants have defective adaptation to AWC-sensed odors (Juang et al., 2013; Kaye et al., 2009; L'Etoile et al., 2002; Lee et al., 2010), we found here that they have varying STAM phenotypes (Table 1). The Nanos homolog mutant *nos-1* could learn, but could not remember, while *adp-1* had impaired forgetting. The cytoplasmic components of the adaptation pathway, *egl-4* and *fbf-1*, have forgetting defects, as one might have expected if STAM and adaptation are the same process. However, HPL-2, a nuclear component of the adaptation pathway, had normal memory when mutated, suggesting that it is only required for adaptation, not STAM. MUT-7, a nuclear component of the adaptation pathway that is expressed in both the cytoplasm and nucleus, is required for learning after STAM training, so its role in memory is not clear. Our data suggest that memory after massed training may only share the cytoplasmic components of the adaptation pathway, and that EGL-4 may have unidentified targets outside of the nucleus that regulate forgetting. Therefore, STAM and adaptation are separate but related learning and memory paradigms. Since adaptation is either non-associative or a result of a negative association (Pereira & van der Kooy, 2012), STAM that is formed after a positive association may be regulated differently by similar pathways. Indeed, Insulin/IGF-1-like signaling differentially regulates negative vs. positive associative learning and memory (Kauffman et al., 2010; Stein & Murphy, 2012). By testing adaptation mutants, we were able to identify conserved genes that have not previously been known to regulate associative memory (*nos-1*) and forgetting (*egl-4* and *fbf-1*) in higher organisms, though their mechanisms of action remain to be studied.

Here we have identified many genetic regulators of STAM and ITAM, including several conserved regulators, and we have determined when these regulators are temporally required. Importantly, we have established a positive olfactory associative paradigm that tests a conserved form of shorter-term memory. It is complicated to rule out possible habituation or adaptation defects in associative memory mutants using negative olfactory associative assays; a positive associative assay avoids these complications, allowing more precise determination of the temporal requirements of associative memory. Together, our data provide a molecular framework for future studies of short-term memory formation, maintenance, and forgetting.

### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2014.07.011.

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