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RESEARCH REPORT

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Mutations in the guanylate cyclase *gcy-28* neuronally dissociate naïve attraction and memory retrieval

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Abstract

The molecules and mechanisms that are involved in the acquisition, storage, and retrieval of memories in many organisms are unclear. To investigate these processes, we use the nematode worm *Caenorhabditis elegans*, which is attracted naïvely to the odorant benzaldehyde but learns to avoid it after paired exposure with starvation. Mutations in the receptor-like guanylate cyclase GCY-28 have previously been thought to result in a behavioral switch in the primary chemosensory neuron AWC^{ON}, from an attractive state to an aversive (already-learned) state. Here, we offer a different interpretation and show that GCY-28 functions in distinct neurons to modulate two independent processes: naïve attraction to AWC^{ON}-sensed odors in the AWC^{ON} neuron, and associative learning of benzaldehyde and starvation in the AIA interneurons. Consequently, mutants that lack *gcy-28* do not approach AWC^{ON}-sensed odors and cannot associate benzaldehyde with starvation. We further show that this learning deficit lies in memory retrieval, not in its acquisition or storage, and that GCY-28 is required in AIA for sensory integration only when both AWC neurons (ON and OFF) are activated by chemical stimuli. Our results reveal a novel role of GCY-28 in the retrieval of associative memories and may have wide implications for the neural machineries of learning and memory in general.

KEYWORDS
associative learning, *C. elegans*, chemosensation, olfaction, sensory integration

1 | INTRODUCTION

Information regarding the innate odor preferences of many animals is encoded in chemosensory neurons (Marella et al., 2006; Troemel, Kimmel, & Bargmann, 1997; Zhao et al., 2003). These olfactory responses, however, can be modified by various processes, including adaptation, habituation, sensitization, and associative learning (Colbert & Bargmann, 1995; Lin et al., 2010; Rankin, Beck, & Chiba, 1990; Torayama, Ishihara, & Katsura, 2007). For example, animals can learn to avoid naïvely attractive odorants after they have been paired with an aversive stimulus (Nuttley, Harbinder, & van der Kooy, 2001).

To better understand the behavioral changes driven by learning and memory, we used the simple model organism *Caenorhabditis elegans*, a nematode worm in which all synaptic connections between its 302 neurons are known (White et al., 1986). *Caenorhabditis elegans* detects olfactory stimuli using primary chemosensory neurons located in the pharynx as bilateral pairs, including AWA, AWB, AWC, ASH,

Abbreviations: bt, butanone; bz, benzaldehyde; *C. elegans*, *Caenorhabditis elegans*; C.I., chemotaxis index; cGMP, cyclic guanosine monophosphate; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; n.s., not significant; NGM, nematode growth medium; pd, 2,3-pentanedione; SEM, standard error of the mean; TRPV, transient receptor potential vanilloid; WT, wildtype.

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and ADL (Bargmann, 2006). AWA and AWC are responsible for sensing attractive chemicals, while AWB, ASH, and ADL mediate avoidance of repulsive odorants (Bargmann, Hartwieg, & Horvitz, 1993; Bargmann & Mori, 1997). These neurons sense chemicals using seven-transmembrane G protein-coupled receptor (GPCR) olfactory receptors, which also exist and function similarly in mammalian olfaction (Buck & Axel, 1991; Robertson & Thomas, 2006). However, unlike the chemosensory system in mammals, in which one neuron expresses only one receptor, each sensory neuron in *C. elegans* detects several chemicals by expressing more than one olfactory receptor gene. Downstream of GPCRs, sensory transduction occurs in one of two ways depending on the type of cell. Using guanosine triphosphate (GTP), guanylate cyclases synthesize the secondary messenger cyclic guanosine monophosphate (cGMP), which subsequently signals through either cGMP-gated channels (AWB and AWC) or Transient Receptor Potential Vanilloid (TRPV) channels (AWA, ASH, and ADL) (Bargmann, 2006; Tobin et al., 2002).

gcy-28 encodes a receptor-like guanylate cyclase, a single-spanning transmembrane protein with an extracellular ligand binding domain, a transmembrane domain, and intracellular protein kinase-like and guanylate cyclase domains. It is expressed in AWC^{ON}, where it is localized to axons and regulates naïve odor preferences. Deficits in naïve attraction toward odorants in mutants that lack *gcy-28* have been reported (Tsunozaki, Chalasani, & Bargmann, 2008). Previous studies also have shown that GCY-28 mediates the integration of sensory signals in AIA interneurons (Shinkai et al., 2011). Sensory integration occurs when animals are required to make a behavioral choice between conflicting cues, such as the attractive stimulus diacetyl and the aversive stimulus copper (Ishihara et al., 2002). Shinkai et al. (2011) have shown that GCY-28 and the receptor tyrosine kinase SCD-2 function to mediate the integration of these alternative cues in AIA, where they converge. AIA is proposed to be important for the mutual inhibition of opposing cues, but not for the transduction of each cue. However, limited information is available for the molecular nature of the interaction between GCY-28 and SCD-2.

In this study, we investigated the molecular mechanisms of associative learning whereby an attractive conditioned stimulus is paired with an aversive unconditioned stimulus. Our experiments rely on the ability of wildtype *C. elegans* to learn that benzaldehyde, an innately attractive odor sensed by the pair of AWC neurons, AWC^{ON} and AWC^{OFF}, becomes aversive after pairing it with starvation (Nuttley et al., 2001). Various learning mutants, such as *ins-1(nj32)*, have been characterized previously. Although these worms are attracted naïvely to benzaldehyde and will migrate toward a point source of low concentrations of this chemical, they are unable to associate it with food deprivation (Lin et al., 2010; Tsui & van der Kooy, 2008).

Here, we reveal a novel role of GCY-28 in the benzaldehyde/starvation learning pathway and propose that it functions both in AWC^{ON} to mediate naïve benzaldehyde attraction and separately in AIA during memory retrieval to mediate the resolution of different signals coming from AWC^{ON} and AWC^{OFF} following sensory stimulation. We offer an alternative explanation for the chemosensory behaviors of *gcy-28* mutants, which naïvely avoid normally attractive AWC^{ON}-sensed odorants and were interpreted by Tsunozaki et al. (2008) to have a pre-conditioned phenotype as if they already had learned to associate these odorants with an adverse event. Our data suggest that *gcy-28* mutants have a learning deficit instead of an already-learned phenotype.

2 | MATERIALS AND METHODS

2.1 | Strains

In this study, we used the strains wildtype Bristol (N2) (Brenner, 1974), *gcy-28(ky713) I* (CX713), *gcy-28(qj4)*, *gcy-28(tm2411) I* (FX02411), *gcy-28(tm3028)*, *gcy-28(ky713) I; Pstr-2::t01a4.1d::gfp* (CX10586), *gcy-28(ky713) I; Pgcy-28.d::t01a4.1d::gfp* (UT1307), *nsy-1(ky542) II* (CX4731), *gcy-28(ky713) I; nsy-1(ky542) II* (CX6510), *inx-19(ky634) I* (CX6161), *gcy-28(ky713) I; inx-19(ky634) I* (CX6519), *cng-1(jh111) V* (KJ461), *cng-3(jh113) IV* (KJ462), and *cng-1(jh111) V; cng-3(jh113) IV* (KJ5560). We obtained CX10586, CX4731, CX6510, CX6161, and CX6519 from the laboratory of Dr. Cornelia I. Bargmann, *gcy-28(qj4)* from the laboratory of Dr. Takeshi Ishihara, and *gcy-28(tm2411)* and *gcy-28(tm3028)* from the National BioResource Project of Japan (Dr. Shohei Mitani). We created UT1307, and all other strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, Minnesota, USA).

2.2 | Culture

All strains were cultured in petri dishes containing Nematode Growth Medium (NGM) and were fed the *Escherichia coli* strain OP50 (Wood, 1988). All experiments used worms that were grown to young adults at 20°C (52 hr post-hatching).

2.3 | Experimental Design

Chemotaxis assays were performed essentially as per Tsui and van der Kooy (2008). Well-fed worms were placed on agar plates in the absence of *E. coli*. To condition the worms, 2 μL of either 100% benzaldehyde, 100% butanone, or 100% 2,3-pentandione was placed on the lid of the plate for 1 hr. To test whether preference for the odorant has changed, 1 μL of either 1% benzaldehyde, 0.1% butanone, or 0.01% 2,3-pentanedione (all of which were dissolved in 100% ethanol, the diluent) was placed on one side of a new agar plate without food, while a counterpoint of 1 μL of 100% ethanol was placed on the other as a control. Worms were distributed evenly on the middle of the new plate. Movement toward one

side or the other can therefore be attributed to the effects of the odorant placed on one side. Odor chemotaxis index (C.I.) was observed and calculated after 1 hr based on the following formula (worms that did not move from the initial location of placement were omitted from calculations):

$$\frac{\text{No. worms that approach the conditioning odorant} - \text{No. worms that approach ethanol}}{\text{total no. worms on the plate}}$$

In Figure 2a, worms were counted every 15 min following 1 hr of training until 90 min post-training. In Figure 2b, there was a 45-min delay between the end of training and the start of testing, and worms were again first counted at 15 min (which corresponds to the 60-min time point in Figure 2a). In Figures 4a and 4b, 2 μL of each of butanone and 2,3-pentanedione was used for training, and 1μL of each was used for testing. 2 μL of ethanol was used as the counterpoint. All odorants were used at the same concentration as they were used singly. In the cross-adaptation assay (Figure 6), trained N2 and *gcy-28(ky713)* mutant worms were treated with 2 μL of 100% benzaldehyde and tested with 1 μL of 0.01% 2,3-pentanedione. 1 μL of 100% ethanol was used as the counterpoint. All experiments were performed at 20°C.

2.4 | Statistical analysis

Multiple group comparisons were performed using two-way or three-way ANOVAs, followed by Bonferroni *t*-tests. The accepted level of statistical significance for all comparisons is $p < 0.05$. The data and error bars in all figures represent means ± standard errors of the means (SEMs), calculated from at least six chemotaxis test plates, each seeded with approximately 50 to 150 worms, per group. Graphs were generated using GraphPad Prism 5 and analyses were performed using SigmaPlot 12.5.

3 | RESULTS

3.1 | *gcy-28(ky713)* mutants have deficits in naïve attraction to benzaldehyde and in learning associatively that benzaldehyde predicts starvation

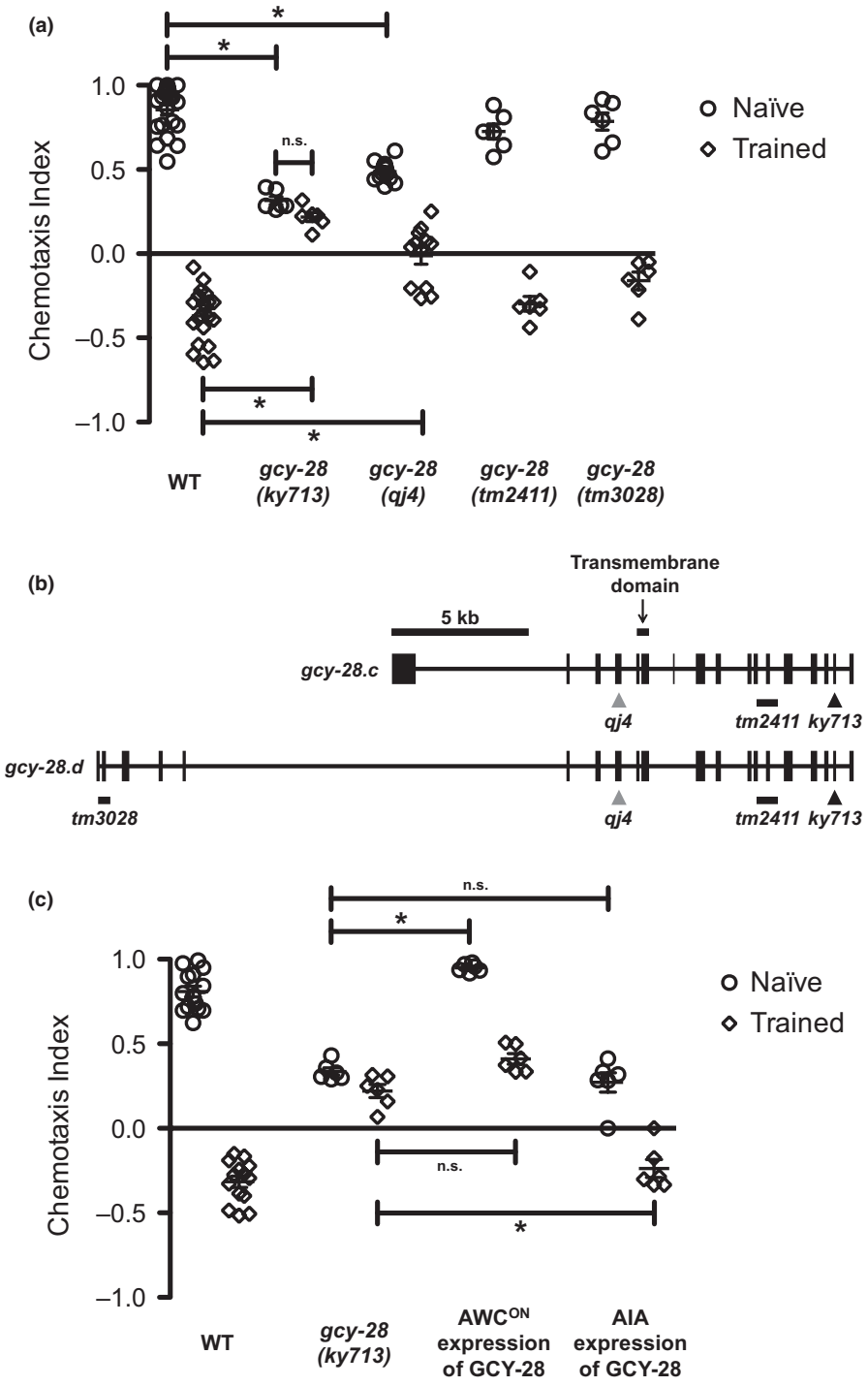
To investigate the behavioral processes that GCY-28 is involved in, we tested *gcy-28* mutants in the benzaldehyde/starvation learning assay, where the odor of benzaldehyde is paired with the absence of food (see Materials and Methods for details). Our results show that worms that have a loss-of-function mutation in *gcy-28(ky713)* exhibit deficits in two behaviors when compared to wildtype worms: naïve attraction to benzaldehyde ($F_{(4,86)} = 55.040$, $p < 0.001$, ANOVA) and association of benzaldehyde with starvation ($F_{(4,86)} = 55.040$, $p < 0.001$, ANOVA) (Figure 1a). Our data below suggest that these two deficits may be caused by two separate behavioral mechanisms, rather than a single pre-learning phenotype (Tsunozaki et al., 2008).

In addition to *gcy-28(ky713)*, we examined three other alleles of *gcy-28*: *gcy-28(qj4)*, *gcy-28(tm2411)*, and *gcy-28(tm3028)*. *gcy-28(tm2411)* and *gcy-28(tm3028)* mutants have essentially wildtype phenotypes in the benzaldehyde learning assay. On the other hand, *gcy-28(qj4)* shows a decrease in naïve attraction that is comparable with *gcy-28(ky713)*, as well as a partial deficit in learning (since there is a difference between naïve and trained scores), in contrast to the absence of learning that is observed in *gcy-28(ky713)* (Figure 1a). Naïve *gcy-28(ky713)* and *gcy-28(qj4)* worms are not significantly different in benzaldehyde attraction ($F_{(4,86)} = 55.040$, $p = 0.125$, ANOVA), but the chemotaxis scores of trained *gcy-28(ky713)* and *gcy-28(qj4)* are significant ($F_{(4,86)} = 55.040$, $p = 0.010$, ANOVA), suggesting that the naïve attraction and learning deficits may be separable, at least partially. No noticeable differences in movement patterns were observed between these two mutants.

The naïve attraction and associative learning phenotypes observed in *gcy-28(ky713)* and *gcy-28(qj4)* mutants are specific to these alleles, which harbor different mutations: *gcy-28(ky713)* contains a single nucleotide polymorphism causing a missense mutation that results in the substitution of an amino acid (phenylalanine to leucine) in the cytosolic cyclase domain of the protein (Tsunozaki et al., 2008), whereas *gcy-28(qj4)* contains a Mos1 transposon insertion in the fourth exon that results in the premature termination of GCY-28 in its extracellular domain close to the transmembrane domain (Shinkai et al., 2011). *gcy-28(tm2411)* and *gcy-28(tm3028)*, the alleles that do not show behavioral effects, contain deletions that occur in the cytosolic kinase homology domain and a more distal portion of the extracellular domain, respectively, and are predicted to cause frameshift mutations (Figure 1b). This suggests that the cyclase domain of GCY-28 may be most crucial for its functions in naïve benzaldehyde attraction and associative learning. As *gcy-28(ky713)* mutants have the strongest deficits in associative learning of benzaldehyde and starvation, these worms were used for subsequent experiments.

3.2 | Presence of GCY-28 in AWC^{ON} and AIA rescues naïve attraction and learning, respectively

The differential effects of the alleles *gcy-28(ky713)* and *gcy-28(qj4)* suggest a separation of the naïve and learning



phenotypes. Thus, we tested whether they could be dissociated by their actions in separate neuronal circuits. First, naïve attraction to benzaldehyde in *gcy-28(ky713)* mutants was rescued by expressing the wildtype allele in AWC^{ON}. Using the AWC^{ON}-selective *str-2* promoter to drive *gcy-28.d* cDNA in *gcy-28(ky713)* mutants (Tsunozaki et al., 2008), naïve attraction to benzaldehyde was rescued to wildtype levels, recapitulating previously published results with butanone (Tsunozaki et al., 2008), an odorant that is sensed by AWC^{ON} exclusively (compared to benzaldehyde, which is sensed by both AWC^{ON} and AWC^{OFF}). Three other isoforms of *gcy-28* exist that share the same intracellular domains but have different N-terminal extracellular domains. The *d* isoform, when expressed under the AWC^{ON}-selective *str-2* promoter, produces a more complete rescue of chemotaxis than the *c* isoform (Tsunozaki et al., 2008). However, we found that learning was not restored by expressing wildtype *gcy-28.d* in AWC^{ON}, as there is no significant difference between the chemotactic behaviors of trained wildtype worms and trained rescue worms (Figure 1c), and therefore investigated

FIGURE 1 Mutations in *gcy-28* cause naïve attraction and associative learning deficits that can be rescued by expressing the wildtype allele in AWC^{ON} and AIA, respectively. (a) *gcy-28(ky713)* mutants show deficits in naïve attraction to benzaldehyde ($p < 0.001$) as well as associative learning of benzaldehyde and starvation ($p < 0.001$), compared to wildtype (WT) worms, at 60 min post-training. A two-way ANOVA revealed a significant interaction between genotype and conditioning (naïve vs. trained) ($F_{(4,86)} = 55.040$; $p < 0.001$). There is no significant difference between naïve and trained *gcy-28(ky713)* ($p = 0.210$). *gcy-28(tm2411)* and *gcy-28(tm3028)* behave similar to WT worms, although *gcy-28(tm3028)* has a slight learning deficit (naïve WT vs. *gcy-28(tm2411)*: $p = 0.889$; trained WT vs. *gcy-28(tm2411)*: $p = 1.000$; naïve WT vs. *gcy-28(tm3028)*: $p = 1.000$; trained WT vs. *gcy-28(tm3028)*: $p = 0.028$). The attraction and learning deficits of *gcy-28(qj4)* are less drastic than those of *gcy-28(ky713)*, but still significant (naïve WT vs. *gcy-28(qj4)*: $p < 0.001$; trained WT vs. *gcy-28(qj4)*: $p < 0.001$). Naïve *gcy-28(ky713)* and *gcy-28(qj4)* worms are not significantly different ($p = 0.125$), but the comparison between the trained worms of these genotypes is significant ($p = 0.010$). Data are represented as means ± SEMs. *$p < 0.001$; n.s., not significant; Bonferroni tests. (b) Rescuing *gcy-28* in AWC^{ON} restores naïve attraction to benzaldehyde compared to WT ($p = 0.654$), while rescuing it in AIA restores learning compared to WT ($p = 1.000$). A two-way ANOVA revealed a significant interaction between genotype and conditioning ($F_{(3,48)} = 60.207$; $p < 0.001$). There is a significant difference in naïve attraction between *gcy-28(ky713)* worms and their AWC^{ON}-specific rescue ($p < 0.001$), but no significant difference between *gcy-28(ky713)* worms and their AIA-specific rescue ($p = 1.000$). For trained worms, *gcy-28(ky713)* is not significantly different from their AWC^{ON}-specific rescue ($p = 0.263$), but is significantly different from their AIA-specific rescue ($p < 0.001$). A double dissociation exists between the roles of GCY-28 in AWC^{ON} (naïve odor attraction) and in AIA (odor/starvation associative learning). (c) Locations of mutations of *gcy-28*. The *gcy-28.c* and *gcy-28.d* isoforms have different extracellular domains. Black rectangles represent deletions, black triangles represent missense mutations, and gray triangles represent Mos1 insertions. The *gcy-28(ky713)* missense mutation occurs in the cyclase domain. *gcy-28(tm2411)* and *gcy-28(tm3028)* cause frameshifts, and *gcy-28(qj4)* results in the premature termination of the protein

the other potential neuronal sites of action of GCY-28 in benzaldehyde/starvation learning.

Given that *gcy-28* is also expressed in the AIA interneurons, and that the pair of AWC neurons directly project to AIA and vice versa, we hypothesized that the presence of *gcy-28* in AIA is necessary for learning. INS-1, a neuropeptide orthologous to human insulin and present in AIA, as well as its receptor DAF-2 in AWC are necessary for benzaldehyde/starvation associative plasticity (Lin et al., 2010). Under its own promoter, *gcy-28.d* was reintroduced specifically in AIA in *gcy-28(ky713)* mutants, which were tested in the benzaldehyde learning assay to see if the wildtype learning phenotype would be recovered. The *pgcy-28.d::gcy-28.d::gfp* fusion gene has been shown to be expressed specifically in the AIA interneurons, whereas *pgcy-28.c::gcy-28.c::gfp* is expressed in many neurons (Ortiz et al., 2006; Shinkai et al., 2011). Indeed, expression of *gcy-28* in AIA is able to restore benzaldehyde/starvation learning ($F_{(3,48)} = 60.207$, $p < 0.001$, ANOVA) (Figure 1c). Notably, this rescue does not restore naïve attraction to benzaldehyde ($F_{(3,48)} = 60.207$, $p = 1.000$, ANOVA). Therefore, although *gcy-28* may be expressed in multiple neurons according to the localization data of *gcy-28.c*, a full rescue of learning can be achieved with *gcy-28.d*, suggesting that *gcy-28* can function cell-autonomously in AIA. These results suggest that there is a double dissociation between the naïve attraction and associative learning deficits that *gcy-28(ky713)* mutants demonstrate.

3.3 | *gcy-28(ky713)* mutants have impaired memory retention

Given that a deficit in benzaldehyde/starvation learning may result from a failure of any one of memory acquisition, memory storage, or memory retrieval, the nature of the learning deficit in *gcy-28(ky713)* mutants was explored. A time course assay in which the movement of worms was tracked over a 90-min period following training (during which benzaldehyde is presented with starvation for 1 hr) revealed that naïve *gcy-28(ky713)* mutants reached their maximum attraction to benzaldehyde at the 15-min time point during testing, while naïve wildtype worms continued to increase their approach until 45 min post-training (Figure 2a). Trained *gcy-28(ky713)* worms behaved similar to trained wildtype worms at 15 min post-training, but travelled away from the counterpoint to eventually reach the chemotaxis index of naïve *gcy-28(ky713)* over the 90-min time course (Figure 2a). This suggests that *gcy-28(ky713)* mutants are able to initially learn the association.

Worms in this associative benzaldehyde training paradigm are exposed to benzaldehyde during both training and testing sessions. To rule out the possibility that the behaviors of *gcy-28(ky713)* naïve and trained mutants were merely the result of a rapid adaptation phenomenon (shown by Nuttley et al. (2001) to be actually habituation due to the absence of receptor down-regulation characteristic of adaptation) due to exposure to the odorant during chemotaxis testing, we performed a delay experiment in which we waited 45 min after training to test the animals, and first counted them at 15 min after plating. The phenotypes of the naïve and trained *gcy-28(ky713)* mutants at 15 min are similar to those at 60 min in the time course experiment (Figure 2b), which suggests that exposure to benzaldehyde during testing does not account for the results.

3.4 | Mutations eliminating AWC asymmetry suppress the benzaldehyde/starvation learning phenotype of *gcy-28(ky713)* mutants

To further understand the mechanisms by which GCY-28 functions in two different neurons (AWC^{ON} and AIA), we examined

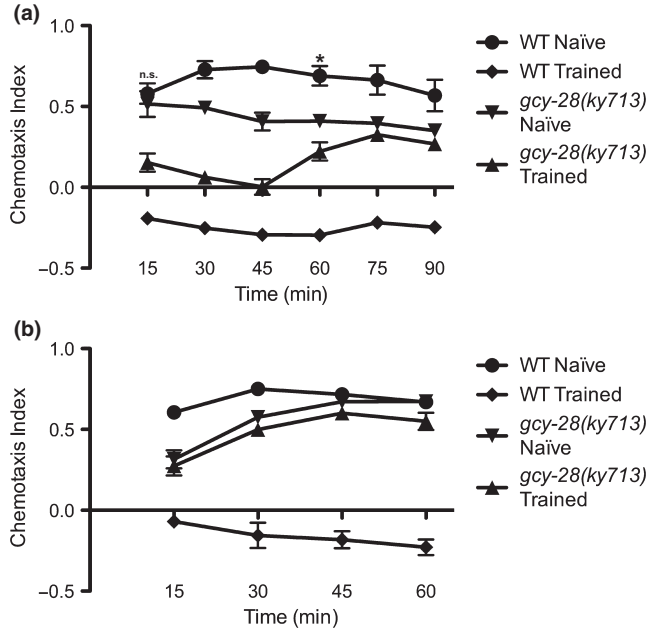


FIGURE 2 The nature of the learning deficit of *gcy-28(ky713)* mutants is not one of memory acquisition or storage. (a) *gcy-28(ky713)* mutants can initially learn about the association of benzaldehyde with starvation, but demonstrate early forgetting of this memory and/or a failure to form or retain longer term memories. A three-way ANOVA revealed a significant interaction between genotype, conditioning, and time ($F_{(5,120)} = 8.014$; $p < 0.001$). Instead of scoring the worms only at 60 min post-training, they were scored every 15 min (up to 90 min post-training). Naïve WT and *gcy-28(ky713)* mutant worms exhibit maximum attraction to benzaldehyde 15 min and 45 min post-training, respectively. Trained *gcy-28(ky713)* animals approach the ethanol counterpoint at 15 min post-training, but move away from it to reach eventually the chemotaxis index of naïve *gcy-28(ky713)* mutants. There is no significant difference between naïve WT and *gcy-28(ky713)* at 15 min (n.s.), nor between trained WT and *gcy-28(ky713)* at 15 min (n.s.). At 60 min, there is a significant difference between naïve WT and *gcy-28(ky713)* ($p < 0.001$, indicated by the asterisk), as well as between trained WT and *gcy-28(ky713)* ($p < 0.001$, indicated by the asterisk). (b) The phenotypes of naïve and trained *gcy-28(ky713)* mutants are not caused by rapid habituation to benzaldehyde during chemotaxis testing. In this delay experiment, there was a 45-min gap between the end of training and the start of testing. The behaviors of naïve and trained *gcy-28(ky713)* mutants at 15 min post-training mimic those at 60 min post-training in Figure 2a ($p = 0.120$ and $p = 0.548$)

the effect of the *gcy-28(ky713)* allele in animals carrying mutations affecting AWC cell specification. *gcy-28(ky713);nsy-1(ky542)* and *gcy-28(ky713);inx-19(ky634)* double mutants, as well as *nsy-1(ky542)* and *inx-19(ky634)* single mutants as controls, were tested for deficits in naïve attraction to benzaldehyde and learning of the benzaldehyde/starvation association. *nsy-1* encodes a MAP kinase kinase kinase (MAP3K) required for asymmetric AWC neuron fates, the absence of which results in the development of two AWC^{ON} neurons (Sagasti et al., 2001). In contrast, *inx-19* encodes an innexin, proteins that form gap junction channels. Together with NSY-4, INX-19 functions to specify the ON fate of AWC neurons. Without INX-19, worms develop two AWC^{OFF} neurons (Chuang, VanHoven, Fetter, Verselis, & Bargmann, 2007).

gcy-28(ky713);nsy-1(ky542) double mutants show a naïve aversion toward benzaldehyde, a phenotype that was first reported by Tsunozaki et al. (2008) and is consistent with their conclusion that *gcy-28* is needed in AWC^{ON} to mediate attraction (Figure 3). However, *gcy-28(ky713);inx-19(ky634)* animals not only have wildtype naïve attraction to benzaldehyde but also can learn associatively (Figure 3). The naïve behavior of these animals is expected, as *gcy-28* is not expressed in AWC^{OFF} and therefore does not play a role in mediating attraction in this neuron (Tsunozaki et al., 2008). Other chemosensory neurons may have a minor role in the detection of benzaldehyde, as worms that lack both AWC neurons (by laser ablation) exhibit a slight approach toward benzaldehyde (Bargmann et al., 1993). The restoration of the ability to perform benzaldehyde/starvation learning in *gcy-28(ky713);inx-19(ky634)* animals, however, was unexpected. The phenotype of the *inx-19(ky634)* single mutant confirms previous reports that AWC^{OFF} alone is sufficient for complete benzaldehyde attraction (Tsunozaki et al., 2008). The observation that *gcy-28(ky713);inx-19(ky634)* worms can learn suggests that there may be an integrative process occurring between signals from AWC^{ON} and AWC^{OFF}, and that *gcy-28* is only required in AIA to mediate learning if the benzaldehyde conditioned stimulus signals come from both AWC^{ON} and AWC^{OFF}. This mechanism is consistent with the known role of GCY-28 in sensory integration (Shinkai et al., 2011).

3.5 | Worms with an AWC^{ON}-specific rescue of *gcy-28* can learn about butanone and 2,3-pentanedione separately but not their combination

To further test the hypothesis that GCY-28 in AIA is important in the integration of sensory cues from the two separate AWC neurons, we performed associative learning assays with odorants sensed by only one of the two AWC neurons. We chose to use butanone (sensed only by AWC^{ON}), 2,3-pentanedione (sensed only by AWC^{OFF}), as well as the combination of these two odorants to mimic the effects of benzaldehyde in stimulating both AWC^{ON} and AWC^{OFF} (Troemel, Sagasti, & Bargmann, 1999; Wes & Bargmann, 2001). Wildtype animals are able to associate both single cues, as well as their combination, with starvation (Figure 4a). The baseline learning with butanone and 2,3-pentanedione is not as dramatic as with benzaldehyde, which is a phenomenon that we see consistently. Conversely, worms in which *gcy-28* is rescued exclusively in AWC^{ON} are capable of learning about butanone-only/starvation and about 2,3-pentanedione-only/starvation, but show a learning deficit when the odorants are combined and paired with starvation (Figure 4b). This confirms that GCY-28 is not required for learning if only one of the two AWC cells is activated; however,

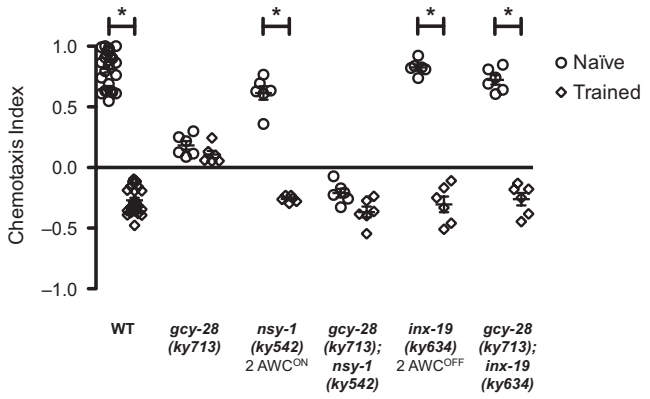


FIGURE 3 The activation of a single AWC neuron can overcome the block of learning caused by the absence of GCY-28 in AIA. A two-way ANOVA revealed a significant interaction between genotype and conditioning ($F_{(5,76)} = 36.153$; $p < 0.001$). *gcy-28(ky713)* and *nsy-1(ky542)* double mutants have a naïve aversion to benzaldehyde that is not significantly different from trained WT animals ($p = 0.205$). There is a significant difference between naïve and trained *gcy-28(ky713);nsy-1(ky542)* ($p = 0.033$). *gcy-28(ky713)* and *inx-19(ky634)* double mutants have a wildtype phenotype in benzaldehyde/starvation learning (naïve WT vs. *gcy-28(ky713);inx-19(ky634)*: $p = 1.000$; trained WT vs. *gcy-28(ky713);inx-19(ky634)*: $p = 1.000$). *nsy-1(ky542)* and *inx-19(ky634)* single mutants behave essentially like WT animals and serve as controls (naïve WT vs. *nsy-1(ky542)*: $p = 0.022$; trained WT vs. *nsy-1(ky542)*: $p = 1.000$; naïve WT vs. *inx-19(ky634)*: $p = 1.000$; trained WT vs. *inx-19(ky634)*: $p = 1.000$). *$p < 0.001$

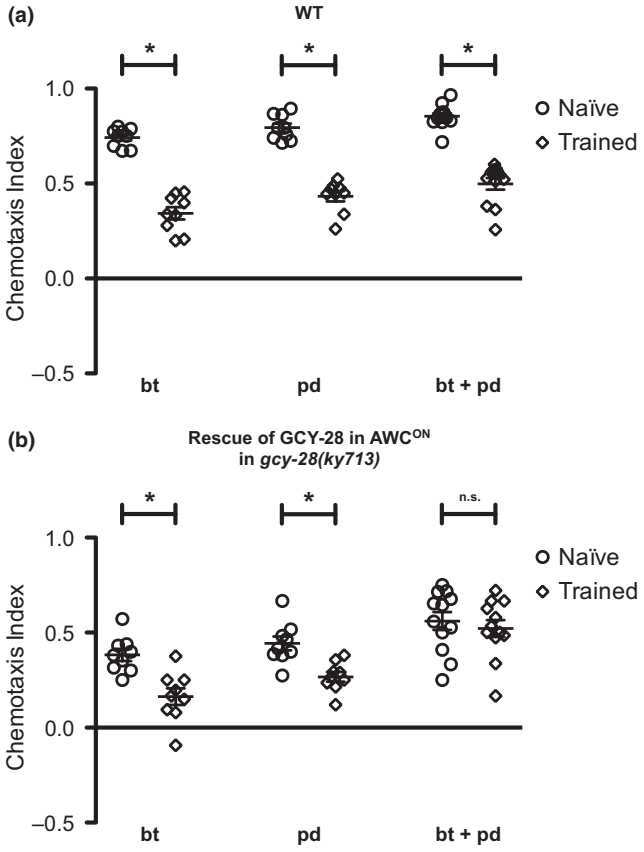


FIGURE 4 *gcy-28(ky713)* mutants can learn about butanone/starvation and 2,3-pentanedione/starvation, but not their combination with starvation. (a) WT worms can learn to associate butanone (bt), 2,3-pentanedione (pd), and their combination (bt + pd) with starvation (naïve vs. trained: $p < 0.001$, for all three groups). A two-way ANOVA revealed no interaction between odorant and conditioning ($F_{(2,54)} = 0.00274$; $p = 0.657$); however, there is a significant main effect of conditioning ($F_{(1,54)} = 315.891$; $p < 0.001$), showing that all genotypes learned. (b) Worms with an AWC^{ON}-specific rescue of *gcy-28* can associate butanone and 2,3-pentanedione with starvation separately ($p < 0.001$ and $p = 0.006$, respectively, when naïve and trained worms within these groups are compared), but cannot do the same for their combination, resulting in the absence of learning (naïve vs. trained: $p = 0.455$). The interaction between odorant and conditioning is not significant ($p = 0.069$). There is a significant main effect of odorant ($F_{(2,54)} = 23.990$; $p < 0.001$), as well as a significant main effect of conditioning ($F_{(1,54)} = 18.485$; $p < 0.001$). *$p < 0.001$

if cues are received from both cells, GCY-28 becomes essential in AIA to mediate their integration, which allows learning to be demonstrated in response to these stimuli (see Discussion for details).

3.6 | CNG-1 and CNG-3 are downstream effectors of GCY-28 in AIA

CNG-1 and CNG-3 are cGMP-gated ion channels, expressed in AIA but not in AWC^{ON}, and have previously been implicated as the downstream effectors of GCY-28 in sensory integration (Cho, Cho, Song, & Park, 2005; He, Altshuler-Keylin, Daniel, L'Etoile, & O'Halloran, 2016; Shinkai et al., 2011). To determine whether they also function downstream of GCY-28 in the benzaldehyde/starvation associative learning pathway, we observed the behaviors of *cng-1* and *cng-3* single mutants, as well as their double mutant. Single mutants can learn about benzaldehyde/starvation, whereas the double mutant has a learning deficit ($F_{(4,38)} = 17.853$, $p < 0.001$, ANOVA) (Figure 5). This suggests that CNG-1 and CNG-3 act redundantly in AIA to receive and process the cGMP signal generated by GCY-28. Worms that contain a deletion mutation in the *cng-2* gene are homozygous lethal and therefore could not be studied. However, given that CNG-2 is not known to be expressed in AIA, it is unlikely to be a downstream effector of GCY-28 in this neuron.

3.7 | *gcy-28(ky713)* mutants can cross-adapt to 2,3-pentanedione after learning about benzaldehyde

In *C. elegans*, learning about olfactory cues is generally specific to the odorant used during training. However, experiments performed previously by Colbert and Bargmann (1995) have shown that wildtype worms can cross-adapt between learning about benzaldehyde and isoamyl alcohol, such that they exhibit trained responses to both odorants after being trained with just one. This suggests that certain memories

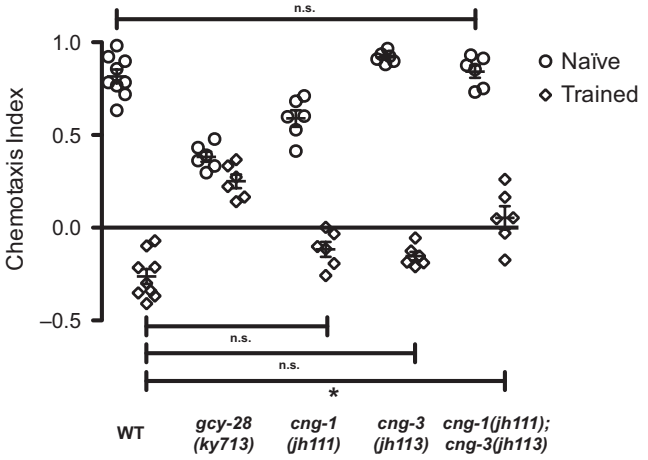


FIGURE 5 CNG-1 and CNG-3 likely act redundantly as the downstream effectors of GCY-28 in AIA. Trained *cng-1(jh111)* and *cng-3(jh113)* single mutants show similar benzaldehyde/starvation learning compared to trained WT ($p = 0.249$ and $p = 1.000$, respectively), but their double mutant shows a small learning deficit compared to WT ($p < 0.001$), suggesting that CNG-1 and CNG-3 may work in part redundantly downstream of GCY-28 in AIA. Naïve WT and *cng-1(jh111);cng-3(jh113)* are not significantly different ($p = 1.000$). A two-way ANOVA revealed a significant interaction between genotype and conditioning ($F_{(4,38)} = 17.853$; $p < 0.001$). *$p < 0.001$

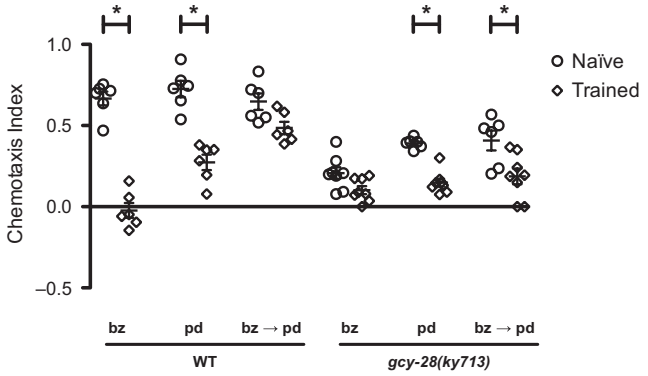


FIGURE 6 *gcy-28(ky713)* Worms can retrieve benzaldehyde/starvation memories when presented with a different context, 2,3-pentanedione, during testing. WT and *gcy-28(ky713)* worms were either trained and tested with benzaldehyde (bz), trained and tested with 2,3-pentanedione (pd), or trained with benzaldehyde and tested with 2,3-pentanedione (bz → pd). *gcy-28(ky713)* can cross-adapt from benzaldehyde to 2,3-pentanedione ($p = 0.002$), suggesting that the inability of *gcy-28(ky713)* to associate benzaldehyde with starvation is a form of retrieval deficit and not one of memory acquisition, storage, or retention. The difference between naïve and trained *gcy-28(ky713)* worms when they are trained and tested with benzaldehyde is significant ($p = 0.040$). The difference between naïve and trained WT cross-adapted (bz → pd) worms also is significant ($p = 0.027$). A three-way ANOVA revealed a significant interaction between genotype, conditioning, and odorant ($F_{(2,56)} = 12.450$; $p < 0.001$). A two-way ANOVA on WT and *gcy-28(ky713)* (naïve and trained) in the cross-adaption group shows no significant interaction between genotype and conditioning ($F_{(1,16)} = 0.233$; $p = 0.636$), but does show significant main effects of genotype ($F_{(1,16)} = 19.769$; $p < 0.001$) and of conditioning ($F_{(1,16)} = 9.904$; $p = 0.006$). *$p < 0.001$

formed during training are transferrable and can be retrieved within a different context.

To further investigate the part of the learning process (e.g., acquisition, storage, or retrieval) during which GCY-28 plays a crucial role, we performed a cross-adaptation assay using benzaldehyde and 2,3-pentanedione. We trained the worms to associate benzaldehyde with starvation and subsequently tested their attraction to 2,3-pentanedione. *gcy-28(ky713)* mutants were able to cross-adapt from benzaldehyde to 2,3-pentanedione ($F_{(2,56)} = 12.450$, $p = 0.002$, ANOVA), even though they showed weak learning when trained and tested with benzaldehyde ($F_{(2,56)} = 12.450$, $p = 0.040$, ANOVA) (Figure 6). These results indicate that in *gcy-28(ky713)* mutants, the memory is formed during the 1 hr training phase and is retrievable using 2,3-pentanedione during the 1 hr testing period. However, the memory is unable to be retrieved if the worms are re-exposed to benzaldehyde during training and subsequently tested with the same odorant for 1 hr (confirming the results shown in Figure 2a).

4 | DISCUSSION

4.1 | GCY-28 is necessary in AWC^{ON} for naïve attraction and in AIA for associative learning

The present data reveal a double dissociation between the naïve chemotaxis and associative learning deficits of *gcy-28(ky713)* mutants. GCY-28 functions in two distinct, reciprocally connected neurons: AWC^{ON} and AIA. Although expression of *gcy-28* has been found to be broad across neurons, selective GCY-28 re-expression in AWC^{ON} rescues naïve attraction and selective GCY-28 re-expression in AIA rescues associative learning (Figure 1c).

The naïve behavior of *gcy-28(ky713)* mutants toward benzaldehyde, producing a chemotaxis index of approximately half of that of naïve wildtype worms, is consistent with a previous finding reporting a similar decrease in naïve attraction. Tsunozaki et al. (2008) report that mutations in *gcy-28* switch the odor preference of butanone (sensed by AWC^{ON}) from attractive to aversive. As benzaldehyde is sensed by both AWC^{ON} and AWC^{OFF}, and a mutation in *gcy-28* only affects the former (Tsunozaki et al., 2008), AWC^{OFF} still functions normally in detecting benzaldehyde as an attractive odorant. Therefore, intermediate naïve attraction to benzaldehyde, generated by a balance between the aversive AWC^{ON} and attractive AWC^{OFF} signaling, can be seen in *gcy-28(ky713)* mutants. However, it also is possible that the absence of GCY-28 in AWC^{ON} reveals a slightly aversive

benzaldehyde chemoreceptor pathway in this neuron once the attractive pathway is hindered by *gcy-28* mutations in AWC^{ON}. This explanation also may apply to the observed naïve aversion to benzaldehyde in *gcy-28(ky713);nsy-1(ky542)* double mutants, as the expression of this potentially aversive naïve receptor would be heightened in these animals compared to in *gcy-28(ky713)*.

Although the pre-learning explanation for *gcy-28* deficits put forth by Tsunozaki et al. (2008) is sufficient to describe naïve behaviors, it cannot be used to explain the observed absence of learning in *gcy-28* mutants. Tsunozaki et al. (2008) suggest that the AWC^{ON} neuron is systematically switched to avoid odors that it senses in *gcy-28* mutants, thus causing naïve worms to behave as if they had been trained previously to avoid these chemosensory cues. Given that the absence of *gcy-28* alters the preference of AWC^{ON}-sensed odors, transforming AWC^{ON} into a genetically odor-aversive state (Tsunozaki et al., 2008), we would expect *gcy-28(ky713)* mutants to demonstrate normal benzaldehyde/starvation learning scores. As the switch in AWC^{ON} generates a pre-learning phenotype and AWC^{OFF} is presumably unaffected by mutations in *gcy-28*, mutant animals should be averse to benzaldehyde following its pairing with starvation, a finding that was not observed (Figure 1a). Our data suggest instead that GCY-28 also functions in AIA, independently from its role in AWC^{ON}, as its absence in AIA produces an associative learning deficit in memory retrieval rather than an already-learned phenotype. Thus, effects on two separate behavioral processes are required to explain the actions of GCY-28 in the AWC^{ON} versus AIA neurons.

4.2 | GCY-28 is only required in AIA for learning when AWC^{ON} and AWC^{OFF} are both activated by odor stimuli

Upon further investigation of the role of GCY-28 in learning, we observed that *gcy-28(ky713);inx-19(ky634)* double mutants (with two AWC^{OFF} neurons) are able to learn about the benzaldehyde/starvation pairing (Figure 3). A similar normal associative learning phenotype is present in the AWC^{ON} rescue of *gcy-28(ky713)* when trained and tested with butanone alone (detected by AWC^{ON} exclusively) or 2,3-pentanedione alone (detected by AWC^{OFF} exclusively), but not when trained and tested with the combination of butanone and 2,3-pentanedione (Figure 4b). Taken together, we propose that GCY-28 is required in the AIA interneurons to mediate learning only if the odorant is sensed by both AWC^{ON} and AWC^{OFF}. *gcy-28(ky713)* mutants (and their AWC^{ON}-specific rescue) cannot learn to associate benzaldehyde (and its molecularly equivalent combination of butanone and 2,3-pentanedione) with starvation, as AWC^{ON} and AWC^{OFF} become activated simultaneously in these cases. AWC^{ON} is not present in *gcy-28(ky713);inx-19(ky634)* double mutants; therefore, only AWC^{OFF} senses benzaldehyde during the learning assay, explaining the ability of these worms to learn. Likewise, AWC^{ON}-specific rescues of *gcy-28* do not have a learning deficit with either butanone or 2,3-pentanedione because only one of the two AWC neurons is stimulated by each odor. These results suggest that there exists a sensory integrative process occurring in AIA that is mediated by GCY-28 only when olfactory signals are processed by both AWC^{ON} and AWC^{OFF}.

This explanation is consistent with the previously reported role of GCY-28 in AIA in sensory integration (Shinkai et al., 2011). In the case of benzaldehyde, two different signals coming from the distinct AWC neurons become integrated in AIA, and GCY-28 could be regulating this in a variety of ways. AWC neurons use the neurotransmitter glutamate as well as neuropeptides (Chalasani et al., 2007; Nathoo, Moeller, Westlund, & Hart, 2001). Therefore, the difference between AWC^{ON} and AWC^{OFF} could involve the identity of the neurotransmitter signals, or perhaps different neuronal output activation patterns. Thus, an integration strategy would be required to resolve the information from these signals, just as if signals were coming from a copper-sensing neuron and a diacetyl-sensing neuron, which is the canonical example of sensory integration (Shinkai et al., 2011). The synaptic connectivity and signaling properties of symmetrical AWC neurons have not been extensively studied. These circuit changes could be present at the sensory or interneuron level. However, these possibilities cannot be teased apart by behavioral consequences.

4.3 | *gcy-28(ky713)* mutants are unable to retrieve memories but can initially acquire and store them

The absence of associative learning in *gcy-28(ky713)* mutants could be caused by a deficit in the acquisition, storage, or retrieval of memories. By tracking the movement of these worms following training to benzaldehyde and starvation, we observed that they show avoidance of benzaldehyde initially for 15 min (but not longer) after training, which indicates that they are capable of memory acquisition (Figure 2a). This behavior can be explained by an early forgetting phenomenon of a single memory that leads to a deficit in long-term memory retention. Conversely, it is possible that there exist completely separate short-term and long-term memories, and that only the latter cannot be retrieved in the context of benzaldehyde.

The results from our cross-adaptation experiment provide further support for GCY-28 playing a more important role in the retrieval of memories than in their acquisition, storage, or erasure. *gcy-28(ky713)* learns to avoid 2,3-pentanedione after associating benzaldehyde with starvation (Figure 6) and can retain this memory for at least 1 hr following training. This suggests that mutations in *gcy-28* also do not affect longer

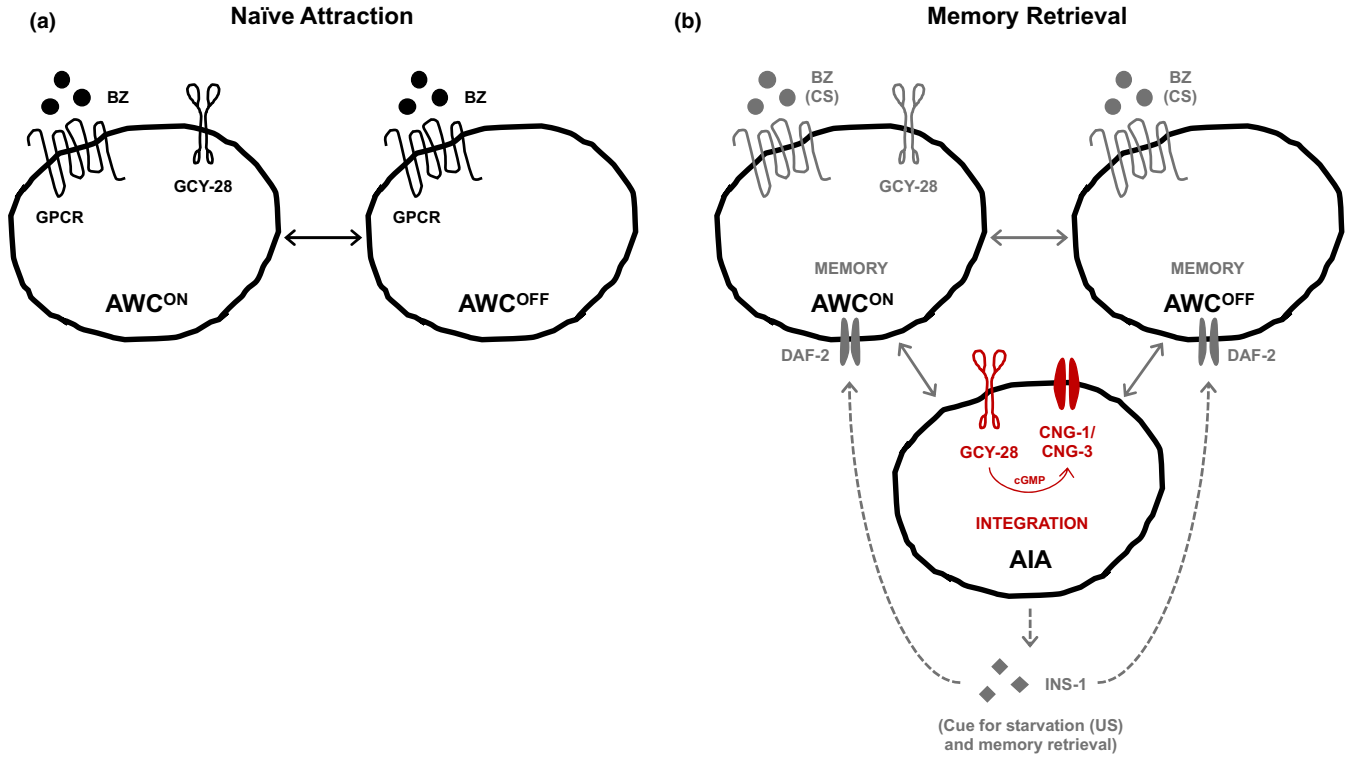


FIGURE 7 Schematic of the differential effects of GCY-28 in AWC^{ON} and AIA. (a) AWC^{ON} senses benzaldehyde (bz) using GPCRs and generates an attractive response toward it when GCY-28 is present. AWC^{OFF}, like AWC^{ON}, also detects benzaldehyde but does not require GCY-28 for attraction. (b) When both AWC^{ON} and AWC^{OFF} are activated by chemical stimuli in the presence of starvation, GCY-28 is required in AIA to resolve these different signals. When GCY-28 does not function in AIA, worms are unable to retrieve this associative memory (in the presence of benzaldehyde) from the AWC neurons. AWC^{ON}, AWC^{OFF}, and AIA are all reciprocally connected by chemical synapses, as shown by the solid arrows. INS-1, the starvation signal, is released from AIA and binds to its receptor DAF-2 on AWC^{ON} and AWC^{OFF}, as shown by the dashed arrows (which represent another chemical synapse). We hypothesize that GCY-28 acts through this pathway to mediate memory retrieval. CNG-1 and CNG-3 are downstream of GCY-28 in AIA and process the cGMP signal that is generated by GCY-28, resulting in sensory integration. Elements shown in gray represent past findings in literature and those that were confirmed by us in this paper. Elements shown in red represent novel findings. CS, conditioned stimulus; US, unconditioned stimulus

term memories, as benzaldehyde/starvation memories can be recalled when the worms are presented with a different context (2,3-pentanedione, in this case) than the one in which they were trained.

It is not clear whether the phenomenon described above is actually an example of cross-adaptation or rather an example of generalization of associative learning (from one stimulus to another). These processes, however, are distinguishable. Adaptation is thought to occur at the receptor level or just downstream of it in primary sensory neurons. Associative learning involves another layer of complexity; that is, it depends on the presence or absence of food. We have shown previously that what is often termed adaptation is really a version of associative learning (Lin et al., 2010; Nuttley et al., 2001). If the phenomenon described above depends on the presence or absence of food, it would be an example of associative learning rather than adaptation, and therefore should be referred to as generalization. However, given that this phenomenon happens in primary olfactory sensory neurons in worms, it also has properties of cross-adaptation.

Given that GCY-28 underlies separate behavioral processes in AWC^{ON} (naïve attraction to odors) and in AIA (learning about the pairing of odors and starvation), are the signaling pathways downstream of GCY-28 similar in these neurons? Downstream of the detection of chemosensory cues by GPCRs, guanylate cyclases convert GTP to cGMP, which signals through cGMP-gated or TRPV channels. The cyclic nucleotide-gated channel TAX-4/TAX-2 is expressed in AWC and is believed to regulate naïve attraction toward AWC-sensed odors (Coburn & Bargmann, 1996; Komatsu, Mori, Rhee, Akaike, & Ohshima, 1996). Based on our results, the cyclic nucleotide-gated channels CNG-1 and CNG-3 are likely downstream targets of GCY-28 that act redundantly in AIA, as the elimination of both causes a partial learning deficit.

The components further downstream of this molecular pathway remain unclear. There is evidence suggesting that benzaldehyde/starvation memories are stored in AWC (Pereira & van der Kooy, 2012), and INS-1 (insulin-like peptide), a neuropeptide orthologous to human insulin, is released from AIA and binds to its receptor DAF-2 on AWC (Lin et al.,

2010). *daf-2* mutants, similar to *ins-1* mutants, show an associative learning deficit after benzaldehyde/starvation training; however, if *daf-2* is reintroduced in constitutive mutants only in AWC, normal learning is restored (Lin et al., 2010). We hypothesize that GCY-28 functions upstream of INS-1 release from AIA to AWC, and that in the absence of GCY-28 in AIA, INS-1 transmission does not occur, explaining the learning deficit of *gcy-28(ky713)* mutants. In addition, previous research using temperature-sensitive *daf-2* mutants has revealed that insulin signaling is partially necessary for memory acquisition but entirely necessary for memory retrieval (Lin et al., 2010). These data are consistent with GCY-28 being upstream of INS-1 release in the memory retrieval pathway. Figure 7 is a graphical depiction of the results and model described above.

Our results offer new insights about how information is processed in the neural network of *C. elegans* during associative learning. A novel role of GCY-28 in benzaldehyde/starvation associative learning is revealed whereby GCY-28 functions in AIA to resolve the signals coming from AWC^{ON} and AWC^{OFF} during longer term memory retrieval when the training stimulus is used. Downstream of GCY-28 in AIA, the cGMP signal is likely received by CNG-1 and CNG-3, cyclic nucleotide-gated channels. Furthermore, *gcy-28(ky713)* mutants are unable to retrieve memories as opposed to being unable to acquire and store them, which results in the observed absence of learning. In contrast to the previous explanation put forth by Tsunozaki et al. (2008) in which *gcy-28* mutants were thought to be genetically averse to AWC^{ON}-sensed odorants and thus exhibit a pre-learning phenotype, we propose that their naïve odor attraction and associative memory retrieval deficits identify behavioral substrates that are differentially regulated and dissociable at the neuronal level. Understanding the components of associative learning pathways provides useful insights into the broader neural mechanisms involved in memory formation, retention, and retrieval.

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CONFLICT OF INTEREST

The authors declare no competing financial and non-financial interests.

DATA ACCESSIBILITY

Data will be shared with the research community upon request. Please contact the corresponding author.

AUTHOR CONTRIBUTIONS

N. L. and D. v. d. K. designed the experiments. N. L. performed the experiments. N. L. and D. v. d. K. analyzed the data and wrote the paper.

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