

Regulation of Diacylglycerol Content in Olfactory Neurons Determines Forgetting or Retrieval of Olfactory Memory in *Caenorhabditis elegans*

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Proper management of memories by forgetting and retrieval is essential for animals to adapt their behavior to changing environments. To elucidate the mechanisms underlying forgetting, we use olfactory learning to an attractive odorant, diacetyl, in *Caenorhabditis elegans* hermaphrodites as a model. In this learning paradigm, the TIR-1/JNK-1 pathway in AWC sensory neurons accelerates forgetting of the olfactory memory, which is stored as a sensory memory trace in AWA sensory neurons. Our genetic screening revealed that increased neuronal diacylglycerol in the olfactory neuronal circuit, by mutations in diacylglycerol kinase-1, *egl-30* or *goa-1*, Gq and Go type G-proteins, suppresses the forgetting defect in the behavior of *tir-1* mutants, although the calcium imaging analyses of the olfactory neurons revealed that the sensory memory trace to the odorant was maintained. In contrast, the expression of a gain-of-function *goa-1* gene exclusively in AWC neurons caused a forgetting defect in behavior, although their sensory memory trace declined. Furthermore, the behavioral analysis of animals applied with diacylglycerol analog and measurement of diacylglycerol content by fluorescent imaging suggested that diacylglycerol content in AWC is important for the proper forgetting. These findings raise a possibility that diacylglycerol signaling plays a crucial role in determining whether to forget or to recall in olfactory learning.

Key words: *C. elegans*; Ca²⁺ imaging; diacylglycerol; forgetting; memory retrieval; memory trace

Significance Statement

Forgetting and retrieval are important processes for proper management of memories, although the mechanisms underlying these processes remain largely unclear. We found that, in *Caenorhabditis elegans*, diacylglycerol signaling works in a forgetting mechanism downstream of TIR-1/JNK-1 pathway. Mutations that change diacylglycerol content in the olfactory neurons affect behavioral forgetting, although they did not alter the sensory memory trace. This suggests that diacylglycerol in specific neurons may determine the occurrence of retrieving, rather than modifying, the memory traces. Consistent with this hypothesis, application of diacylglycerol analog to animals suggests that diacylglycerol content until memory acquisition decides whether to retrieve or to forget the memory.

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Introduction

The proper use of memory through forgetting is important for animals to choose advantageous behavior in constantly changing environments. Proper forgetting is important to prevent interference and confusion by accumulated dispensable memories (Davis and Zhong, 2017), and proper retrieval is a must to prevent the unavailability of memories or incorrect recall (Frankland et al., 2019). Disorders in these processes not only impede proper memory management but also disturb proper decision-making and behavioral changes to suit the current situation and consequently lead to a survival disadvantage (Kraemer and Golding, 1997).

Active forgetting is essential for regulating proper retention of memory (Davis and Zhong, 2017) because eliminating old memories that are not fit for the current conditions provides a

flexible behavior to adapt to noisy environments (Richards and Frankland, 2017). Recently, several mechanisms committed to active forgetting likely accompanied by erosion of memory traces have been revealed in *Drosophila melanogaster* (Shuai et al., 2010, 2015; Berry et al., 2012, 2018; Dong et al., 2016; Himmelreich et al., 2017), and in *Caenorhabditis elegans* (Inoue et al., 2013; Hadziselimovic et al., 2014; Kitazono et al., 2017). Similar forgetting mechanisms have also been proposed in mammals (Hayashi-Takagi et al., 2015; Liu et al., 2016); thus, general mechanisms for regulating active forgetting are conserved evolutionarily.

The abovementioned forgetting mechanisms generally entail biological degradation of memory engrams, whereas others disturb the retrieval of relatively intact memory engrams (Davis and Zhong, 2017). Despite having an available intact memory trace, absence of appropriate retrieval cues or recollection of other items can lead to inability to access the memory and recall, resulting in an apparent forgetting of the memory (Tulving and Pearlstone, 1966; Wimber et al., 2015; Davis and Zhong, 2017; Frankland et al., 2019; Sabandal et al., 2021). On the contrary, mouse models of early Alzheimer's disease show a defect in memory consolidation, but they can recall the memory by optogenetic stimulation of the engram cells; thus, the cause of amnesia in the disease is failure to recall (Roy et al., 2016). Consequently, failure to recall also leads to apparent forgetting (Davis and Zhong, 2017).

C. elegans has a simple neuronal circuit comprising only 302 neurons. Despite their simplicity, it can sense diverse stimuli and show behavioral plasticity to these (Hedgecock and Russell, 1975; Rankin et al., 1990; Bargmann et al., 1993; Colbert and Bargmann, 1995; Saeki et al., 2001). *C. elegans* show chemotactic behavior to diacetyl, an attractive odorant sensed via G-protein-coupled receptor ODR-10 in a pair of AWA sensory neurons in the case of low concentrations (Bargmann et al., 1993; Sengupta et al., 1996; Chou et al., 2001). One simple learning example is that olfactory adaptation by preexposure to diacetyl in the absence of food reduced the chemoattraction of worms to diacetyl within a few hours (Colbert and Bargmann, 1995). Recently, molecular and neuronal mechanisms of active forgetting of olfactory learning have been reported (Inoue et al., 2013; Hadziselimovic et al., 2014; Kitazono et al., 2017). We have reported that the TIR-1/JNK-1 pathway in AWC sensory neurons accelerates forgetting of olfactory memory for diacetyl in AWA sensory neurons (Inoue et al., 2013), which are major sensory neurons for diacetyl. Ca^{2+} imaging analysis showed that the sensory responses to diacetyl in AWA neurons alter in accordance with the behavioral changes during memory acquisition and forgetting processes; hence, the Ca^{2+} response can be considered as the sensory memory trace (Inoue et al., 2013). However, the molecular basis and neuronal circuits of forgetting remain largely unknown.

In this study, through unbiased genetic screening, we found that mutations that induce a high level of diacylglycerol (DAG) in AWC sensory neurons and AIA interneurons suppresses the forgetting defect in *tir-1* mutant in *C. elegans*. Moreover, we discovered that manipulating the DAG content changed behavioral forgetting but not the memory trace in AWA neurons. These results imply the function of the memory traces and neuronal circuits in proper forgetting.

Materials and Methods

Strains and culture

All experiments were conducted on well-fed young adult hermaphrodite worms. The strains used in this study were as follows: N2, IG685 *tir-1(tm3036)*, VC218 *dgk-3(gk110)*, CE1047 *egl-30(ep271 gf)*, MT2426 *goa-1*

(*n1134*), JN1715 *pels1715[*str-1p::mCasp-1+unc-122p::GFP*]* as AWB(–) (Yoshida et al., 2012), and PY7502 *oyls85[*ceh-36p::TU#813+ceh-36p::TU#814+srtx-1p::GFP+unc-122p::DsRed*]* as AWC(–) (Beverly et al., 2011), which were obtained from Caenorhabditis Genetics Center, QD203 *tir-1(tm3036);dgk-1(qj203)*, QD124 *tir-1(tm3036);dgk-1(qj203);qjEx24[dgk-1(10.8 kb)+myo-3p::gfp]*, QD143 *dgk-1(qj203)*, QD144 *tir-1(tm3036);dgk-1(nu62)*, QD145 *tir-1(tm3036);dgk-1(sy428)*, QD146 *tir-1(tm3036);dgk-3(gk110)*, QD147 *egl-30(ep271);tir-1(tm3036)*, QD148 *goa-1(n1134);tir-1(tm3036)*, QD125 *tir-1(tm3036);qjEx25[H20p::egl-30(Q205L gf)+myo-3p::gfp]*, QD126 *tir-1(tm3036);qjEx26[odr-1p::egl-30(Q205L gf)+myo-3p::gfp]*, QD127 *tir-1(tm3036);qjEx27[gcy-28.dp::egl-30(Q205L gf)+myo-3p::gfp]*, QD128 *goa-1(n1134);tir-1(tm3036);qjEx28[goa-1p::goa-1+myo-3p::gfp]*, QD129 *goa-1(n1134);tir-1(tm3036);qjEx29[H20p::goa-1+myo-3p::gfp]*, QD130 *goa-1(n1134);tir-1(tm3036);qjEx30[odr-1p::goa-1+myo-3p::gfp]*, QD131 *goa-1(n1134);tir-1(tm3036);qjEx31[gcy-28.dp::goa-1+myo-3p::gfp]*, QD138 *lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15];qjEx38[odr-1p::goa-1(gf)]*, QD132 *tir-1(tm3036);dgk-1(qj203);qjEx32[H20p::dgk-1a+myo-3p::gfp]*, QD133 *tir-1(tm3036);dgk-1(qj203);qjEx33[ins-1(long)p::dgk-1a+myo-3p::gfp]*, QD134 *tir-1(tm3036);dgk-1(qj203);qjEx34[odr-1p::dgk-1a+myo-3p::gfp]*, QD135 *tir-1(tm3036);dgk-1(qj203);qjEx35[gcy-28.dp::dgk-1a+myo-3p::gfp]*, QD136 *tir-1(tm3036);dgk-1(qj203);qjEx36[odr-1p::dgk-1a+gcy-28.dp::dgk-1a+myo-3p::gfp]*, and QD137 *goa-1(n1134);tir-1(tm3036);qjEx37[odr-1p::TeTx+myo-3p::gfp]* (Schiavo et al., 1992), which were made by this study, *Is[odr-1p::mCasp1]* as AWB/AWC(–), which was gifted by Dr. Wakabayashi, for behavioral assays. QD139 *lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]* (Inoue et al., 2013), QD140 *tir-1(tm3036);lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]* (Inoue et al., 2013), QD160 *ceh-36(ks86)/lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]*, QD141 *goa-1(n1134);lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]*, QD142 *goa-1(n1134);tir-1(tm3036);lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]*, QD159 *tir-1(tm3036);dgk-1(qj203)/lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15]*, and QD138 *lin-15(n765);qjEx39[odr-10p::YC3.60/lin-15];qjEx38Ex[odr-1p::goa-1(gf)]*, which were made in this study, for Ca^{2+} imaging. JN2513 *peEx2513[ceh-36p::DownwardDAG2(worm)+unc-122p::mCherry]*, which was obtained from Caenorhabditis Genetics Center, QD149 *tir-1(tm3036);peEx2513[ceh-36p::DownwardDAG2(worm)+unc-122p::mCherry]*, QD150 *goa-1(n1134);peEx2513[ceh-36p::DownwardDAG2(worm)+unc-122p::mCherry]*, QD151 *goa-1(n1134);tir-1(tm3036);peEx2513[ceh-36p::DownwardDAG2(worm)+unc-122p::mCherry]*, which were made in this study, for DAG imaging. QD122 *Ex[odr-10 tagged gfp/ttx-3p::rfp]*, which was made by Fujiwara and the construct was provided by Bargmann (Sengupta et al., 1996), QD161 *tir-1(tm3036);Ex[odr-10 tagged gfp/ttx-3p::rfp]*, QD162 *dgk-1(qj203);Ex[odr-10 tagged gfp/ttx-3p::rfp]*, and QD163 *tir-1(tm3036);dgk-1(qj203);Ex[odr-10 tagged gfp/ttx-3p::rfp]*, which were made in this study, for quantification of ODR-10 protein. All strains were cultured on Nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP-50 under standard conditions (at 20°C) (Brenner, 1974).

Behavioral assays

Chemotaxis toward attractive odorants was performed as described previously (Bargmann et al., 1993), except the assay plates contained 50 mM NaCl (Inoue et al., 2013; Kitazono et al., 2017). We used 10^{-2} diluted diacetyl (0.11 M; Sigma-Aldrich catalog #B85307-100ML) as the “Odorant” for the chemotaxis assays, unless otherwise mentioned. Exceptionally, in Figure 1F, we used 10^{-3} diluted diacetyl (0.011 M) as the “Odorant.” The chemotaxis index was calculated as (“Odorant” – “Control”)/all worms on 90 mm agar plate, where “Odorant” was the number of worms within 15 mm of the diacetyl spot and “Control” was the number of worms within 15 mm of the solvent (ethanol) spot. Before the naive assays, adult worms were washed twice with S-basal buffer (100 mM NaCl, 50 mM K_2HPO_4 , pH 6, 0.02% gelatin) and once with distilled water. The worms for adaptation were preexposed to 0.02% (0.0022 M) diacetyl in S-basal buffer with rotation for 90 min. Next, the worms were washed once with distilled water and cultured on OP-50-seeded NGM plates for 4 h for recovery. In the chemotaxis assay with isoamyl alcohol, we used 10^{-2} diluted isoamyl alcohol (0.09 M; Nacalai Tesque catalog #02715-45) as the attractant and 0.01% isoamyl alcohol (0.0009 M) in S-basal buffer for adaptation. In the chemotaxis assay with pyrazine, we used 10 mg/ml

pyrazine (Nacalai Tesque catalog #29502-24) dissolved in ethanol as the attractant and 100 $\mu\text{g}/\text{ml}$ pyrazine in S-basal buffer for adaptation.

Forward genetic screen for the suppressor of the loss-of-function mutants of *tir-1* and positional cloning of *dgk-1* (qj203)

We mutagenized *tir-1*(*tm3036*) worms, a loss-of-function mutant of *tir-1*, by ethyl methanesulfonate and collected 5450 F1 worms. To search for mutants showing normal forgetting, even when animals were cultivated on food after conditioning, we performed a set of chemotaxis assays to diacetyl just after adaptation and after 4 h on food recovery. We then allowed the candidates, which showed normal adaptation and forgetting, to lay the next generations. After these screening assays were performed on F2-F8 worms individually, we chose worms that showed normal avoidance just after adaptation and normal forgetting after recovery despite of the *tir-1* mutation as candidate mutants that suppress the *tir-1* defect. Whole genome sequencing of these mutant lines was performed, followed by analysis with MAQ Gene (Mapping and Assembly with Qualities gene) software (Bigelow et al., 2009). Subsequently, genetic mappings were performed using single nucleotide polymorphisms between *tir-1*(*tm3036*) and the suppresser mutants.

DNA constructs and germline transformation

The transgenic strains for behavioral assays of rescue or overexpression experiments and calcium imaging were prepared by injection of each DNA fragment at a concentration of 5–90 ng/ μl into each worm. *goa-1*, *goa-1*(Q205L *gf*), and *egl-30*(R243Q *gf*) cDNAs were kindly provided by Y. Iino (Matsuki et al., 2006). *dgk-1* fragment (10.8 kb) for the rescue experiment (see Fig. 1B) was made by the following: forward: CAGCACATGAACATCCCCAACTACAA and reverse: CCCTAAGTTGCTGGACCTTCTGA primers from WT *dgk-1*. *dgk-1.a* cDNA ATGGCAGCT-TTTCTCTGA (2853 bp) was synthesized based on the sequence of DGK-1.a isoform in N2. *myo-3p::gfp* or *lin-44p::gfp* were used as injection markers for transgenic strains for behavioral assays.

Measurement of expression of the ODR-10 protein tagged with GFP

The expression levels of ODR-10 receptor were measured using ODR-10 protein tagged with GFP (Sengupta et al., 1996) in AWA cilia. Worms with ODR-10 tagged GFP were washed and anesthetized with 10 mM NaN_3 on 5% agar pads. Fluorescent images of the sensory cilium were acquired by Olympus BX53 microscope equipped with a 60 $\times$ objective and captured by ORCA-Flash4.0 (Hamamatsu). Fluorescent intensities of ODR-10::GFP were the average of ≥ 64 worms in each strain.

Calcium imaging

Calcium imaging of AWA neurons was performed as described previously (Inoue et al., 2013; Kitazono et al., 2017). In brief, we used 0.02% (0.0022 M) diacetyl for 90 min adaptation and 2×10^{-7} diluted diacetyl (0.0022 mM) in S-basal buffer for odor stimulation. Fluorescence images, which were acquired by an Olympus BX53-F microscope equipped with a 60 $\times$ objective and ORCA-D2 (Hamamatsu), were analyzed using AQUACOSMOS (Hamamatsu).

PMA administration assay

WT and *tir-1* mutant were cultured on the OP-50 spread NGM plates added dropwise of 10 μl of 0.05 mg/ml PMA (Abcam catalog #ab120297) in ethanol or 10 μl of ethanol (vehicle) at 20°C for 2 h before naive chemotaxis assay. Subsequently, worms were exposed to PMA (0.25 $\mu\text{g}/\text{ml}$) or solvent (ethanol) with 0.02% (0.0022 M) diacetyl in S-basal buffer with rotation for 90 min as adaptation, and then adapted animals were cultured on the food plates added dropwise of 10 μl of 0.05 mg/ml PMA or 10 μl of ethanol for 4 h until after recovery assay.

Measurement of DAG levels

DAG levels of AWC neurons were measured using DownwardDAG2 (Tewson et al., 2012, 2013; Ohno et al., 2017) in the cell body by using animals carrying *ceh::36p*-DownwardDAG2, which were provided from Caenorhabditis Genetic Center (Strains and Culture). We used 0.02% (0.0022 M) diacetyl for 90 min adaptation. Worms in naive, after

adaptation, and after 4 h cultivation on the food plates were washed and anesthetized with 10 mM NaN_3 on 5% agar pads. Fluorescent images were acquired by Olympus BX53 microscope equipped with a 60 $\times$ objective and captured by ORCA-Flash4.0 (Hamamatsu). Fluorescent intensity of DownwardDAG2 was the average of ≥ 44 worms in each strain.

DiI staining

To confirm that DownwardDAG2 expresses in AWC neurons, we stained amphid neurons (ADL, ASH, ASI, ASJ, ASK, AWB) used by 0.002 mg/ml DiI (1,1-diiododecyl-3,3',3'-tetramethylindolecarbocyanine perchlorate; Sigma-Aldrich catalog #42364) in dimethyl formamide and identified AWC (Garriga et al., 1993).

Quantification and statistical analysis

Behavioral analysis. To analyze chemotactic behaviors, we used ~ 100 hermaphrodite worms in each assay and repeated 4 or more times (the detailed sample sizes are noted in the figure legends). Behavioral assays were statistically analyzed using two-way ANOVA with Bonferroni multiple correction (Bellcurve for Excel). Statistical information including each power was presented in the figure legends (see Figs. 1–4, 6).

Analysis of expression levels of ODR-10 receptor. To analyze expression levels of ODR-10 receptor, we used 64 or more hermaphrodite worms for each strain (the detailed sample sizes are noted in the legend of Fig. 1F). ODR-10 receptor levels were statistically analyzed using one-way ANOVA with Tukey–Kramer *post hoc* test (Bellcurve for Excel). Statistical information including each power was presented in the legend of Figure 1F.

Calcium imaging analysis. We analyzed calcium responses of 10 or more hermaphrodite worms for each condition and strain (the detailed sample sizes are noted in the figure legends) and performed as described previously (Inoue et al., 2013; Kitazono et al., 2017). $(R_{\text{max}} - R_0)/R_0$ was calculated as the peak amplitude of the YFP/CFP ratio (R_{max}) after stimulation relative to the mean basal ratio (R_0) during the 5 s interval preceding the application of diacetyl. Peak values of the quantitated calcium responses were statistically analyzed using one-way ANOVA with Tukey–Kramer *post hoc* test (Bellcurve for Excel). Statistical information, including each power, is presented in the Figure 5H–N legend.

Analysis of measurement of DAG levels. To analyze DAG levels by *ceh-36p::* DownwardDAG2, we used 44 or more hermaphrodite worms for each condition and strain (the detailed sample sizes are noted in the figure legends). Measured fluorescent intensities of *ceh-36p::* DownwardDAG2 (see Fig. 6D) were statistically analyzed using one-way ANOVA with Tukey–Kramer *post hoc* test (Bellcurve for Excel). Statistical information, including each power, is presented in the Figure 6D legend.

Results

dgk-1 mutation suppressed the forgetting defect of the *tir-1* loss-of-function mutant

In *C. elegans*, WT animals show attractive chemotaxis to various odorants. It has been reported that low diacetyl concentrations are mainly sensed by AWA olfactory neurons, whereas high diacetyl concentrations are also sensed by AWC neurons (Bargmann et al., 1993; Sengupta et al., 1996; Chou et al., 2001). While naive animals were strongly attracted to diacetyl (Fig. 1A, Naive), after preexposure to 0.02% diacetyl for 90 min without food, animals showed olfactory adaptation in which they changed their behavior to show weaker chemotaxis to diacetyl (Bargmann et al., 1993; Colbert and Bargmann, 1995) (Fig. 1A, Adaptation). We studied this behavioral plasticity as a model of memory acquisition of diacetyl and starvation (Inoue et al., 2013; Hadziselimovic et al., 2014; Kitazono et al., 2017). After adaptation, WT animals restored the chemotaxis to diacetyl within 4 h to the level of naive animals, which is considered as “forgetting” (Inoue et al., 2013;

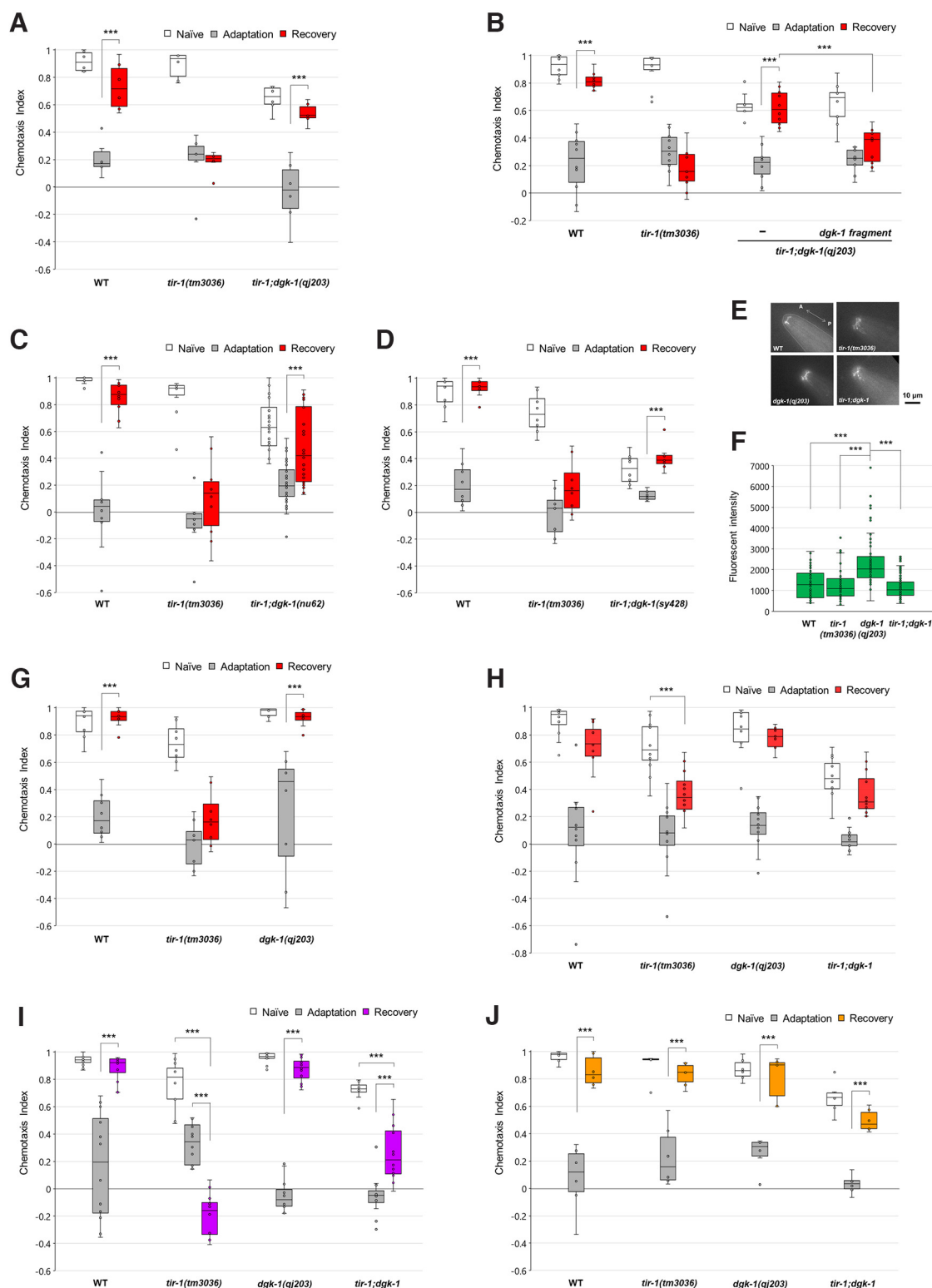


Figure 1. *dgk-1* mutation suppressed the forgetting defect of the *tir-1* loss-of-function mutant. **A**, Suppression of the forgetting defect of the *tir-1* mutant by *dgk-1* mutation. Chemotaxis of naïve, adapted, and 4 h recovered animals was analyzed. Asterisks indicate significant differences between after adaptation and after 4 h recovery. WT: $p = 3.35 \times 10^{-7}$; *tir-1;dgk-1(qj203)*: $p = 4.29 \times 10^{-8}$ ($n = 6$, $F_{(4,45)} = 9.98$, $p = 7.18 \times 10^{-6}$). **B**, Transgenic rescue of *tir-1(tm3036);dgk-1(qj203)* animals expressing WT *dgk-1* genomic fragments. Asterisks indicate significant differences between after adaptation and after 4 h recovery or between recovery with or without *dgk-1* fragment. WT: $p = 2.84 \times 10^{-17}$; *tir-1;dgk-1(qj203)* without *dgk-1* fragment: $p = 5.23 \times 10^{-10}$; recovery with or without *dgk-1* fragment: $p = 4.08 \times 10^{-5}$ ($n = 10$, $F_{(6,108)} = 22.22$, $p = 6.72 \times 10^{-17}$). **C**, *dgk-1(nu62)* and *dgk-1(sy428)* mutations also suppressed *tir-1*'s forgetting defect. Asterisks indicate significant differences between after adaptation and after 4 h recovery. **C**, WT: $p = 2.32 \times 10^{-15}$; *tir-1;dgk-1(nu62)*: $p = 9.38 \times 10^{-6}$ ($n \geq 10$, $F_{(4,135)} = 17.42$, $p = 1.50 \times 10^{-11}$). **D**, WT: $p = 1.53 \times 10^{-15}$; *tir-1;dgk-1(sy428)*: $p = 0.000247$ ($n = 8$, $F_{(4,63)} = 22.51$, $p = 1.40 \times 10^{-11}$). **E**, Representative images of the GFP-tagged ODR-10 protein at cilia of AWA in WT and mutants. Scale bar, 10 μ m. **F**, Quantitative analyses of fluorescent intensities of expression of the GFP-tagged ODR-10 protein at cilia. Statistics: one-way ANOVA with Tukey–Kramer multiple test. Asterisks indicate significant differences between *dgk-1(qj203)* and others. *dgk-1(qj203)* versus WT: $p = 9.14 \times 10^{-13}$; versus *tir-1(tm3036)*: $p = 9.37 \times 10^{-13}$; versus *tir-1;dgk-1*: $p = 9.52 \times 10^{-13}$ ($n = 72, 77, 64, 134$, $F_{(3,343)} = 44.42$, $p = 2.82 \times 10^{-24}$). **G**, Phenotype of the *dgk-1*

Hadziselimovic et al., 2014) (Fig. 1A, Recovery in WT). We have previously found that a *tir-1* loss-of-function (*lf*) mutant (*tm3036*) shows prolonged retention of the memory (Fig. 1A) and the TIR-1/JNK-1 pathway, which functions in the AWC olfactory neurons, accelerates forgetting of the memory of diacetyl in AWA neurons (Inoue et al., 2013). However, the molecular and neuronal mechanisms by which the TIR-1/JNK-1 pathway in AWC neurons regulates forgetting the memory in the AWA neurons is still unknown.

In this study, to explore new components downstream of the TIR-1/JNK-1 pathway, we first performed an unbiased suppressor screening of the *tir-1* (*tm3036 null*) forgetting defect phenotype. In this screening, we identified several suppressor mutations that restored the forgetting phenotype. Using whole genome sequencing and single nucleotide polymorphism analyses, we found that, among the identified mutants, *qj203* mutation in *dgk-1*, which encodes DAG kinase-1, suppressed the defective forgetting phenotype in the *tir-1* mutant (Fig. 1A). This suppression could be rescued by the WT *dgk-1* gene, indicating this *dgk-1* mutation (*qj203*) is responsible for the suppression (Fig. 1B). This mutation causes a premature stop codon (W646) to stop, which is the same as the *n892* allele in the DAG kinase catalytic domain (Nurrish et al., 1999; Jose and Koelle, 2005). We also showed that the other *dgk-1* mutants, *dgk-1(nu62)* and *dgk-1(sy428)*, both of which are regarded as null mutants (Hajdu-Cronin et al., 1999; Nurrish et al., 1999; Jose and Koelle, 2005), suppressed the forgetting defect of the *tir-1* mutant, like *dgk-1(qj203)* (Fig. 1C,D). Although the double mutants *tir-1;dgk-1* showed slightly lower chemotaxis to diacetyl (Fig. 1A–D), we could not detect significant differences in expression of a GFP-tagged ODR-10 protein, a receptor for diacetyl, between WT and *tir-1;dgk-1(qj203)* (Fig. 1E,F), suggesting that the expression of ODR-10 receptor does not affect the naive chemotaxis. Although the single mutant *dgk-1(qj203)* showed significantly higher intensity than other strains (Fig. 1F), the single mutation did not affect chemotaxis in naive, adaptation, and forgetting (Fig. 1G). These results suggest that the function of *dgk-1* itself is not required for chemotaxis, adaptation to diacetyl, and its forgetting, but it is required for the regulation of forgetting downstream of *tir-1*.

Confirming that the effect of the *dgk-1* mutations is involved in the forgetting process in AWA neurons, we found that *dgk-1*

suppressed the forgetting defect of *tir-1*, even when attracted to lower concentrations (10^{-3}) of diacetyl of which sensing is thought to be more restricted to AWA neurons (Sengupta et al., 1996; Chou et al., 2001) (Fig. 1H). Next, to examine whether forgetting of the memory for odorants other than diacetyl was regulated by the same pathway, we analyzed the behavioral forgetting of the memory for isoamyl alcohol, which is predominantly sensed by AWC neurons, and pyrazine, which is predominantly sensed by AWA neurons (Bargmann et al., 1993). We found that the forgetting defect of the *tir-1* mutant in the memory of isoamyl alcohol was only modestly suppressed by the *dgk-1* mutation (Fig. 1I), while, for pyrazine, even in *tir-1* mutant, the forgetting defect was not observed (Fig. 1J), suggesting that the forgetting of the olfactory adaptation is differently regulated depending on the type of odorants.

DGK-1, a human DGK θ homolog, depletes DAG by changing it into phosphatidic acid (PA) via phosphorylation (Houssa et al., 1997; Nurrish et al., 1999; Van Blitterswijk and Houssa, 2000). DAG is an important second messenger in various biological processes, including facilitation of synaptic release (Hajdu-Cronin et al., 1999; Lackner et al., 1999; Nurrish et al., 1999; Miller et al., 2000; Van Blitterswijk and Houssa, 2000) (Fig. 2A). In *C. elegans*, DAG controls various types of behavior, such as locomotion (Nurrish et al., 1999; Miller et al., 2000), egg-laying (Hajdu-Cronin et al., 1999), thermotaxis (Biron et al., 2006; Nakano et al., 2020), odor chemotaxis (Tsunozaki et al., 2008), and learning of salt and food (Adachi et al., 2010; Kunitomo et al., 2013; Ohno et al., 2017).

To examine whether the regulation of DAG content is important for the regulation of forgetting, we analyzed the *tir-1*-suppressive activity of the DAG kinase *dgk-3*, which controls thermotaxis as a thermal memory molecule (Biron et al., 2006) and adaptation to AWC-sensed odorants (Matsuki et al., 2006). We found that a *dgk-3* (*lf*) mutation suppressed the forgetting defect of the *tir-1* mutant (Fig. 2B), suggesting that multiple DAG kinases coordinately regulate forgetting by controlling the DAG content downstream of the TIR-1/JNK-1 pathway.

Two types of G-proteins are involved in the regulation of forgetting

In *C. elegans*, the G-proteins, EGL-30 (Gq α), and GOA-1 (Goa α), function antagonistically in the regulation of the DAG content (Mendel et al., 1995; Segalat et al., 1995; Brundage et al., 1996; Hajdu-Cronin et al., 1999; Lackner et al., 1999) (Fig. 2A): EGL-30 upregulates DAG content, whereas GOA-1 downregulates it in motor neurons (Lackner et al., 1999; Nurrish et al., 1999; Miller et al., 2000) (Fig. 2A). Furthermore, EGL-30 and GOA-1 in AWC neurons regulate the adaptation to AWC-sensed odorants, but not to AWA-sensed odorants (Matsuki et al., 2006). Additionally, activation of Gq α signaling in AWC neurons enhances memory consolidation of the associative learning of butanone, an AWC-sensed odorant, with food (Arey et al., 2018). Therefore, we examined whether these G-proteins regulate forgetting of the olfactory memory by analyzing the behavioral phenotypes of an *egl-30* gain-of-function (*gf*) mutant and a *goa-1* (*lf*) mutant, which are considered to have higher DAG content, like the *dgk-1* (*lf*) mutant.

The single mutants *egl-30(ep271 gf)* and *goa-1(n1134 lf)* did not exhibit significant differences in naive chemotaxis, adaptation, and forgetting, like the WT and *dgk-1* single mutant (Fig. 2C,D). The double mutants with *tir-1* revealed that both *egl-30(gf)* and *goa-1* suppressed the forgetting defect of the *tir-1*

(*qj203*) single mutant. Chemotaxis of naive, adapted, and 4 h recovered animals was analyzed. Asterisks indicate significant differences between after adaptation and after 4 h recovery. WT: $p = 1.44 \times 10^{-9}$; *dgk-1(qj203)*: $p = 5.09 \times 10^{-9}$ ($n = 8$, $F_{(4,63)} = 6.67$, $p = 0.000153$). **H**, *dgk-1* also suppresses the forgetting defect of the *tir-1* mutant, even, when attracted by an even lower concentration (1:1000) of diacetyl. Asterisks indicate significant difference between naive and after recovery. *tir-1(tm3036)*: $p = 8.91 \times 10^{-5}$ ($n = 12$, $F_{(6,132)} = 4.06$, $p = 0.000907$). **I**, *dgk-1* only modestly, but significantly, suppresses the forgetting defect in *tir-1* in the case of the memory of isoamyl alcohol. Asterisks indicate significant differences between after adaptation and after recovery or naive and after recovery. Adaptation versus recovery in WT: $p = 2.54 \times 10^{-18}$; *tir-1(tm3036)*: $p = 9.96 \times 10^{-12}$; *dgk-1(qj203)*: $p = 7.76 \times 10^{-26}$; *tir-1;dgk-1*: $p = 3.03 \times 10^{-5}$. Naive versus recovery in *tir-1*: $p = 1.17 \times 10^{-27}$; *tir-1;dgk-1*: $p = 2.11 \times 10^{-9}$ ($n = 12$, $F_{(6,132)} = 45.34$, $p = 8.98 \times 10^{-30}$). **J**, *tir-1* does not show a forgetting defect in the memory of pyrazine. Asterisks indicate significant differences between after adaptation and after recovery. WT: $p = 3.73 \times 10^{-14}$; *tir-1(tm3036)*: $p = 3.36 \times 10^{-10}$; *dgk-1(qj203)*: $p = 3.36 \times 10^{-9}$; *tir-1;dgk-1*: $p = 3.35 \times 10^{-7}$ ($n = 6$, $F_{(6,60)} = 2.04$, $p = 0.0744$). Box plots: center line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA with Bonferroni correction (except **F**).

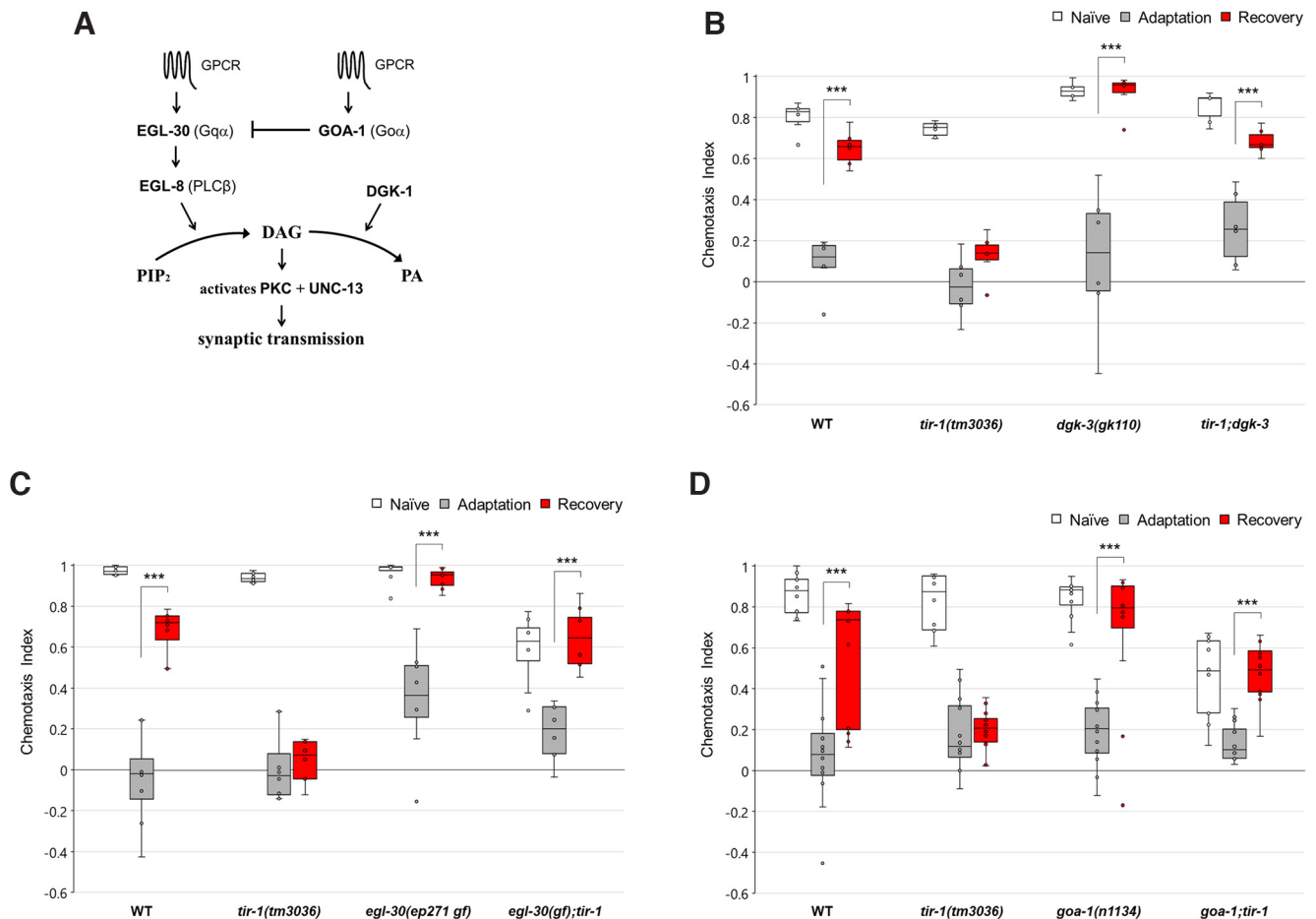


Figure 2. DGK-3, EGL-30 (Gqα), and GOA-1 (Goα) are also involved in the regulation of forgetting downstream of the TIR-1/JNK-1 pathway. **A**, A model of the DAG signaling pathway based on Nurrisch et al. (1999); Lackner et al. (1999); and Miller et al. (2000). DAG is produced by EGL-8 (PLCβ) downstream of EGL-30, which is inhibited by GOA-1, activates protein kinase C and UNC-13, and results in enhancing synaptic transmission. DGK-1 degrades DAG to PA. **B**, Suppression of the forgetting defect of the *tir-1* mutant by the DAG kinase *dgk-3*. Chemotaxis of naive, adapted, and 4 h recovered animals was analyzed. Asterisks indicate significant differences between after adaptation and after 4 h recovery. WT: $p = 8.87 \times 10^{-9}$; *dgk-3(gk110)*: $p = 5.76 \times 10^{-14}$; *tir-1; dgk-3*: $p = 8.10 \times 10^{-6}$ ($n = 6$, $F_{(6,60)} = 7.90$, $p = 2.73 \times 10^{-6}$). **C**, Suppression of the forgetting defect of the *tir-1* mutant by *egl-30* gain-of-function mutation (*ep271*). WT: $p = 2.51 \times 10^{-15}$; *egl-30(ep271 gf)*: $p = 7.33 \times 10^{-12}$; *egl-30(gf);tir-1*: $p = 2.96 \times 10^{-8}$ ($n = 8$, $F_{(6,84)} = 22.48$, $p = 1.25 \times 10^{-15}$). **D**, Suppression of the forgetting defect of the *tir-1* mutant by *goa-1* loss-of-function mutation (*n1134*). WT: $p = 5.77 \times 10^{-8}$; *goa-1*: $p = 1.87 \times 10^{-8}$; *goa-1;tir-1*: $p = 0.000104$ ($n = 12$, $F_{(6,132)} = 8.50$, $p = 8.22 \times 10^{-8}$). Box plots: center line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. *** $p < 0.001$ (two-way ANOVA with Bonferroni correction).

mutant, like *tir-1;dgk-1* (Fig. 2C,D), suggesting that these G-proteins also regulate the forgetting process, probably through the DAG content. Our data suggest that this forgetting regulation by DAG signaling is also necessary for proper forgetting in WT animals (see below).

DAG regulates forgetting of the adaptation in the olfactory circuit

To determine where the DAG content-modulating molecules regulate forgetting, we performed cell-specific overexpression and rescue experiments. First, we tested whether the expression of *egl-30(gf)*, a constitutively active form of *egl-30*, under a pan-neuronal, AWC, or AIA promoter suppresses the forgetting defect of the *tir-1* mutant (Fig. 3A–C). Pan-neuronal expression of *egl-30(gf)* in the *tir-1* mutant restored the chemotaxis after a 4 h recovery to the same extent as that of the naive nematode (Fig. 3A), although these animals showed lower chemotaxis in naive conditions. The expression of *odr-1 promoter::egl-30(gf)*, which gives an expression predominantly in AWC sensory neurons (L'Etoile and Bargmann, 2000), suppressed the forgetting defect of the *tir-1* mutant, without affecting its naive chemotaxis (Fig.

3B). On the other hand, the expression of *gcy-28.d promoter::egl-30(gf)*, which gives an expression specifically in AIA interneurons that are downstream of AWA and AWC neurons (Tsunozaki et al., 2008; Chalasani et al., 2010), did not show significant suppression of the forgetting defect (Fig. 3C). These results suggest that activating EGL-30, which presumably resulted in the upregulation of the DAG content, accelerates forgetting, even in the absence of *tir-1* function; thus, for proper forgetting, the activity of EGL-30 is downregulated in AWC neurons downstream of the TIR-1/JNK-1 pathway. Additionally, in naive condition, relatively weak chemotaxis in the double mutants of *tir-1* with mutations, which elevate DAG (Figs. 1A, 2C,D), did not occur in the overexpression of *egl-30* in AWC neurons (Fig. 3B), suggesting that their weak chemotaxis in naive is a side effect unrelated to the suppression of *tir-1*'s forgetting defect.

Second, we analyzed where *goa-1* functions in the forgetting process (Fig. 3D–G). We found that, in *goa-1;tir-1* double mutant in which the adaptation is recovered within a few hours, the expression of *goa-1* cDNA under its own promoter or a pan-neuronal promoter caused the forgetting defect (Fig. 3D, E). The expression of *goa-1* in AWC neurons or AIA neurons

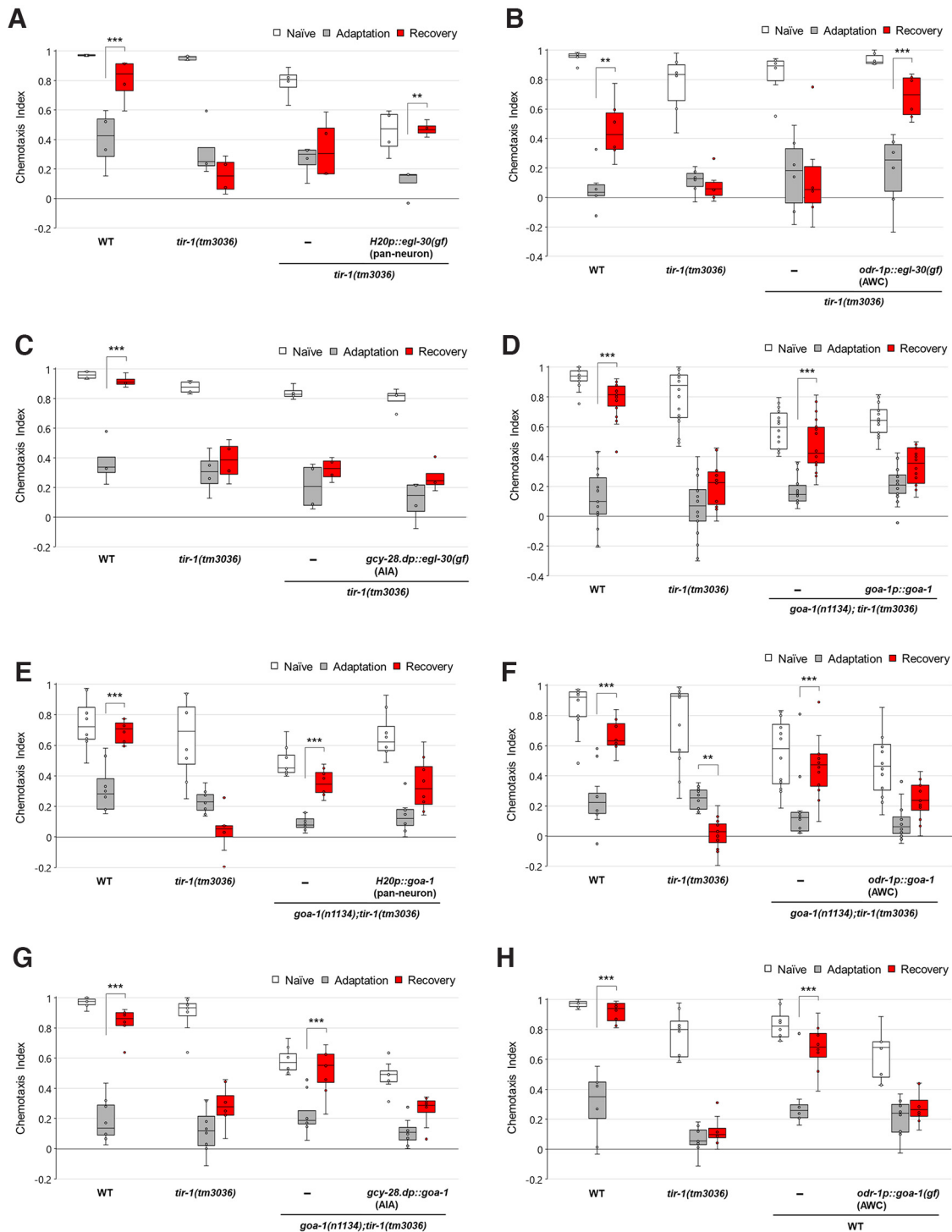


Figure 3. The DAG content needs to be reduced in AWC and AIA neurons during forgetting. **A–C**, Transgenic rescue of *tir-1(tm3036)* mutants by expressing a constitutively active form of the *egl-30* gene under a pan-neuronal (*H20*) (**A**), AWC (*odr-1*) (**B**), or AIA (*gcy-28.d*) promoter (**C**). Chemotaxis of naïve, adapted, and 4 h recovered animals was analyzed. Asterisks indicate significant differences between after adaptation and after 4 h recovery. **A**, WT: $p = 0.000465$; *tir-1;Ex[H20p::egl-30(gf)]*: $p = 0.00166$ ($n = 4$, $F_{(6,36)} = 7.81$, $p = 2.00 \times 10^{-5}$). **B**, WT: $p = 0.00136$; *tir-1;Ex[odr-1p::egl-30(gf)]*: $p = 4.40 \times 10^{-5}$ ($n = 6$, $F_{(6,60)} = 3.81$, $p = 0.00280$). **C**, WT: $p = 3.15 \times 10^{-8}$ ($n = 4$, $F_{(6,36)} = 6.40$, $p = 0.000118$). **D–G**, Transgenic rescue of the *goa-1(n1134);tir-1(tm3036)* mutant's phenotype by expressing the WT *goa-1* gene under its own (*goa-1*) (**D**), pan-neuronal (*H20*) (**E**), AWC (*odr-1*) (**F**), or AIA (*gcy-28.d*) promoter (**G**). **D**, WT: $p = 8.07 \times 10^{-31}$, *goa-1;tir-1* (Ex-): $p = 4.06 \times 10^{-9}$ ($n = 18$, $F_{(6,204)} = 22.73$, $p = 1.87 \times 10^{-20}$). **E**, WT: $p = 2.79 \times 10^{-6}$; *goa-1;tir-1* (Ex-): $p = 0.000961$ ($n = 8$, $F_{(6,84)} = 8.60$, $p = 2.71 \times 10^{-7}$). **F**, WT: $p = 1.09 \times 10^{-7}$; *tir-1* (*tm3036*): $p = 0.00618$; *goa-1;tir-1* (Ex-): $p = 0.000388$ ($n = 12$, $F_{(6,132)} = 11.30$, $p = 3.67 \times 10^{-10}$). **G**, WT: $p = 3.51 \times 10^{-18}$, *goa-1;tir-1* (Ex-): $p = 8.36 \times 10^{-6}$ ($n = 8$, $F_{(6,84)} = 17.22$, $p = 6.99 \times 10^{-13}$). **H**, Overexpression of *goa-1* in AWC neurons prevents WT animals from forgetting. WT: $p = 1.91 \times 10^{-13}$; WT (Ex-): $p = 2.62 \times 10^{-6}$ ($n = 8$, $F_{(6,84)} = 11.00$, $p = 5.16 \times 10^{-9}$). Box plots: center line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA with Bonferroni correction.

also caused the forgetting defect after a 4 h recovery (Fig. 3F,G). These data suggest that GOA-1, which presumably downregulates the DAG content, works not only in AWC but also AIA neurons to prevent forgetting.

As mentioned above, mutations that were expected to upregulate the DAG content may induce forgetting despite the *tir-1* defect (Figs. 2, 3A–G). Consistently, downregulation of the DAG content was inferred to prevent forgetting. As expected, animals expressing *goa-1(gf)* (Q205L) in AWC neurons in a WT background, in which the DAG content in AWC neurons may decrease, still showed weak chemotaxis to diacetyl after the 4 h recovery, suggesting that they have a forgetting defect like the *tir-1* mutant (Fig. 3H). These results indicate that the regulation of forgetting by changing the DAG content is not restricted to the absence of *tir-1*.

Finally, we examined where DGK-1 functions. The suppression of the forgetting defect in the *tir-1* mutant by the lack of *dgk-1* was rescued by *dgk-1a* cDNA expressed under a pan-neuronal promoter or *ins-1(long)* promoter, which expresses in multiple neurons (Kodama et al., 2006), including AWC and AIA (Hammarlund et al., 2018) (Fig. 4A,B). However, *dgk-1a* expression in AWC neurons can slightly, but significantly, rescue the behavioral phenotype of *tir-1;dgk-1* double mutant (Fig. 4C), although *dgk-1a* expression in AIA neurons did not show the significant effect of the rescue (Fig. 4D). In contrast, the combined expression of both promoters was effective to rescue the suppression of *tir-1*'s forgetting defect by *dgk-1* (Fig. 4E). These findings suggest that DGK-1 functions simultaneously in AWC and AIA neurons in the regulation of forgetting. Together, changes in the DAG content in AWC sensory neurons and AIA interneurons probably regulate forgetting.

Regulation of the DAG content controls behavioral forgetting via synaptic transmission from AWC

In *C. elegans*, the DAG content is important for synaptic transmission (Nurrish et al., 1999; Miller et al., 2000) (Fig. 2A). We examined whether synaptic transmission is involved in the DAG-regulated forgetting process, using animals expressing Tetanus Toxin light chain (TeTx) to inhibit synaptic transmission (Schiavo et al., 1992). Inhibition of synaptic transmission from AWC neurons overrode the suppression of the forgetting defect of the *tir-1* mutant by the *goa-1* mutation in the *goa-1;tir-1* double mutant (Fig. 4F).

Additionally, we examined whether AWB neurons are also required for proper forgetting because the *odr-1* promoter used as an AWC promoter in this study has also been reported to be expressed in AWB sensory neurons (Yu et al., 1997). AWB-ablated animals did not show the forgetting defect, whereas AWC-ablated animals showed the forgetting defect (Fig. 4G). This result is consistent with our previous report on the *ceh-36* mutant (Inoue et al., 2013); thus, synaptic transmission from AWC neurons, but not AWB neurons, are necessary for the suppression of *tir-1* effect by *dgk-1* and its upstream factor *goa-1*, and GOA-1 disturbs synaptic transmission from AWC neurons by modulating the DAG content to regulate forgetting (Fig. 2A). Accordingly, these results suggest that the rescues by expression by *odr-1* promoters (Figs. 3B,F, 4C,E) are caused by expression in AWC neurons.

Behavioral forgetting can occur even without elimination of the memory traces in AWA sensory neurons

Our previous report revealed that the Ca^{2+} responses in AWA neurons change in parallel with the behavioral change in WT

and *tir-1* animals (Inoue et al., 2013). Because AWA is the primary sensory neuron for diacetyl, the diminished Ca^{2+} response after adaptation can be considered as a sensory memory trace for this plasticity. To examine whether the G-proteins and DAG are important for the maintenance or elimination of the memory trace, we analyzed the Ca^{2+} responses to diacetyl in AWA neurons of these strains. First, we confirmed the previously reported finding (Inoue et al., 2013; Kitazono et al., 2017) that in WT animals, Ca^{2+} responses to diacetyl in AWA neurons are evident under naive conditions, become weak after adaptation, and are recovered after a 4 h cultivation after adaptation (Fig. 5A,H). On the other hand, the Ca^{2+} responses in the *tir-1* mutant were very similar to those in WT in the naive state and after adaptation, although after a 4 h recovery, the Ca^{2+} responses were still very weak (Fig. 5B,I), indicating that we reproduced the previous results and that the Ca^{2+} response can be considered as a sensory memory trace (Inoue et al., 2013). Additionally, *ceh-36* mutants, which lack functional AWC neurons (Lanjuin et al., 2003), also had retained the memory trace after 4 h recovery in accord with the behavior similarly to *tir-1* (Fig. 5C,J; Inoue et al., 2013; Fig. 4G, “AWC (–)”). Therefore, the forgetting defect in loss of *tir-1* or AWCs is probably caused by inability to degrade the memory trace.

Next, we analyzed the change in the sensory response in the *goa-1* single mutant, which showed similar behavioral phenotypes to WT in the naive and adaptation states, and after a 4 h recovery from the adaptation. We found that the Ca^{2+} responses in the *goa-1* mutant were also very similar to those in the WT, which is consistent with its behavior (Fig. 5D,K) and in agreement with our hypothesis. Subsequently, we examined whether the Ca^{2+} responses in the *goa-1;tir-1* double mutant are similar to those in the *goa-1* mutant. We found that, in the *goa-1;tir-1* double mutant, even after a 4 h recovery, the Ca^{2+} responses to diacetyl in the AWA neurons did not recover, which is similar to the response in the *tir-1* mutant (Fig. 5E,L). These findings suggest that, in the *goa-1;tir-1* double mutant, the sensory memory trace of adaptation is maintained even after a 4 h recovery. Furthermore, the Ca^{2+} responses in *tir-1;dgk-1* double mutant also did not recover after 4 h cultivation (Fig. 5F,M) differently from the behavioral recovery (Fig. 1A). Together, in the *goa-1;tir-1* and *tir-1;dgk-1* mutants, although the sensory responses had been diminished even after a 4 h recovery from adaptation (Fig. 5E,F,L,M), the chemotaxis behaviors were recovered, and these animals behaved as if their memory traces were lost (Figs. 1A, 2D), suggesting that the DAG content can control the forgetting process in behavior without affecting the memory trace in the sensory response.

Overexpression of *goa-1* in AWC neurons sustains the behavioral memory, even in the absence of a memory trace in AWA

We also analyzed the Ca^{2+} responses in AWA neurons in animals expressing *goa-1(gf)* in AWC neurons, which showed a behavioral forgetting defect (Fig. 3H). We found that the Ca^{2+} response to diacetyl recovered from adaptation after a 4 h cultivation similar to the WT animals (Fig. 5G,N). These results suggest that expressing *goa-1(gf)* in AWC neurons caused loss of the sensory memory trace in AWA neurons after a 4 h recovery; thus, the AWA neurons showed the Ca^{2+} responses to diacetyl like naive animals. Therefore, after a 4 h recovery from adaptation, despite the strong sensory response to diacetyl in the AWA neurons, the animals expressing *goa-1(gf)* in the AWC neurons exhibited weak chemotaxis as if they retained the memory traces.

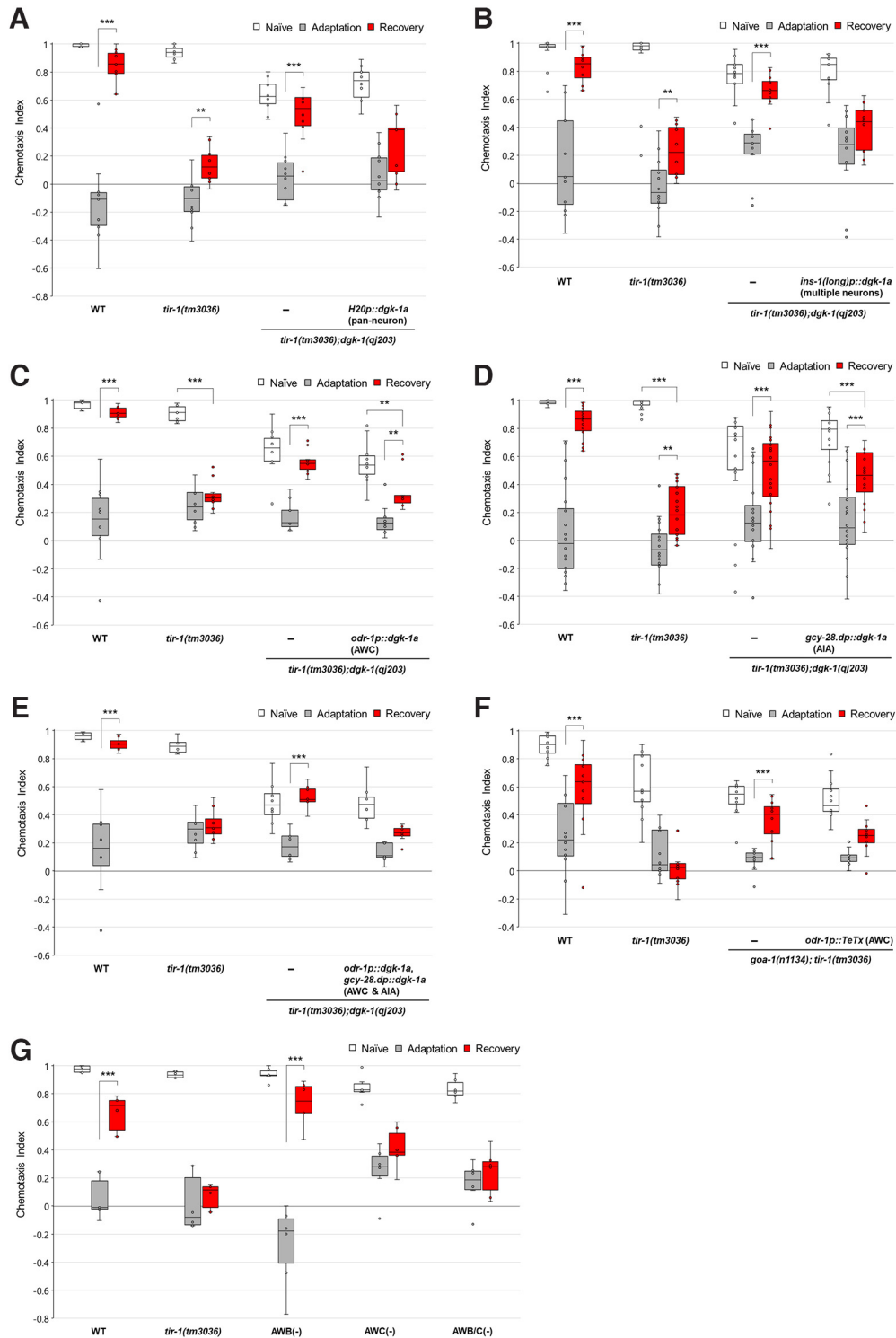


Figure 4. DGK-1 works in several neurons, including AWC and AIA in the regulation of forgetting. **A–E**, Transgenic rescue of the *tir-1(tm3036);dgk-1(q203)* mutant's phenotype by expressing the WT *dgk-1* gene isoform under its pan-neuronal (*H20*) (**A**), multiple neuronal (*ins-1(long)*) (**B**), AWC (*odr-1*) (**C**), AIA (*gcy-28.d*) (**D**) promoter, or AWC (*odr-1*) and AIA (*gcy-28.d*) promoter combined (**E**). Chemotaxis of naive, adapted, and 4 h recovered animals was analyzed. Asterisks indicate significant differences between after adaptation and after 4 h recovery or between naive and after 4 h recovery. **A**, WT: $p = 2.60 \times 10^{-23}$, *tir-1(tm3036)*: $p = 0.00248$; *tir-1;dgk-1* (Ex): $p = 1.03 \times 10^{-7}$ ($n = 10$, $F_{(6,108)} = 17.52$, $p = 4.47 \times 10^{-14}$). **B**, WT: $p = 7.15 \times 10^{-13}$, *tir-1(tm3036)*: $p = 0.00568$; *tir-1;dgk-1* (Ex): $p = 9.15 \times 10^{-6}$ ($n = 12$, $F_{(6,132)} = 6.73$, $p = 3.10 \times 10^{-6}$). **C**, Adaptation versus recovery in WT: $p = 1.58 \times 10^{-23}$, *tir-1;dgk-1* (Ex): $p = 6.65 \times 10^{-9}$; *tir-1;dgk-1;Ex(odr-1p::dgk-1a)*: $p = 0.00232$; and Naive versus recovery in *tir-1(tm3036)*: $p = 3.71 \times 10^{-16}$, *tir-1;dgk-1;Ex(odr-1p::dgk-1a)*: $p = 0.00191$ ($n = 10$, $F_{(6,108)} = 17.88$, $p = 2.63 \times 10^{-14}$). **D**, Adaptation versus recovery in WT: $p = 1.97 \times 10^{-21}$, *tir-1;dgk-1* (Ex): $p = 2.51 \times 10^{-5}$, *tir-1;dgk-1;Ex(gcy-28.dp::dgk-1a)*: $p = 0.000298$; and Naive versus recovery in *tir-1(tm3036)*: $p = 5.37 \times 10^{-21}$, *tir-1;dgk-1;Ex(odr-1p::dgk-1a)*: $p = 0.000316$ ($n = 20$, $F_{(6,228)} = 15.11$, $p = 1.51 \times 10^{-14}$). **E**, WT: $p = 2.30 \times 10^{-19}$, *tir-1;dgk-1* (Ex): $p = 1.47 \times 10^{-6}$ ($n = 8$, $F_{(6,84)} = 18.72$, $p = 1.05 \times 10^{-13}$). **F**, Effect of inhibiting synaptic transmission from AWC sensory neurons by expressing TeTx in *goa-1;tir-1* mutants. Asterisks indicate significant differences between after adaptation and after 4 h recovery. WT: $p = 1.84 \times 10^{-5}$; *goa-1;tir-1* (Ex): $p = 0.000260$ ($n = 12$, $F_{(6,132)} = 6.00$, $p = 1.42 \times 10^{-5}$). **G**, Analyzing forgetting defect of olfactory adaptation in AWC-ablated (*str-1p::mCasp-1*), AWC-ablated (*ceh-36p::caspase-3(p12)::n2 + ceh-36p::caspase-3(p17)*), and AWB/C-ablated (*odr-1p::mCasp1*) animals. WT: $p = 2.07 \times 10^{-9}$, AWB (–): $p = 2.48 \times 10^{-18}$ ($n = 6$, $F_{(8,75)} = 13.62$, $p = 5.51 \times 10^{-12}$). Box plots: center line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA with Bonferroni correction.

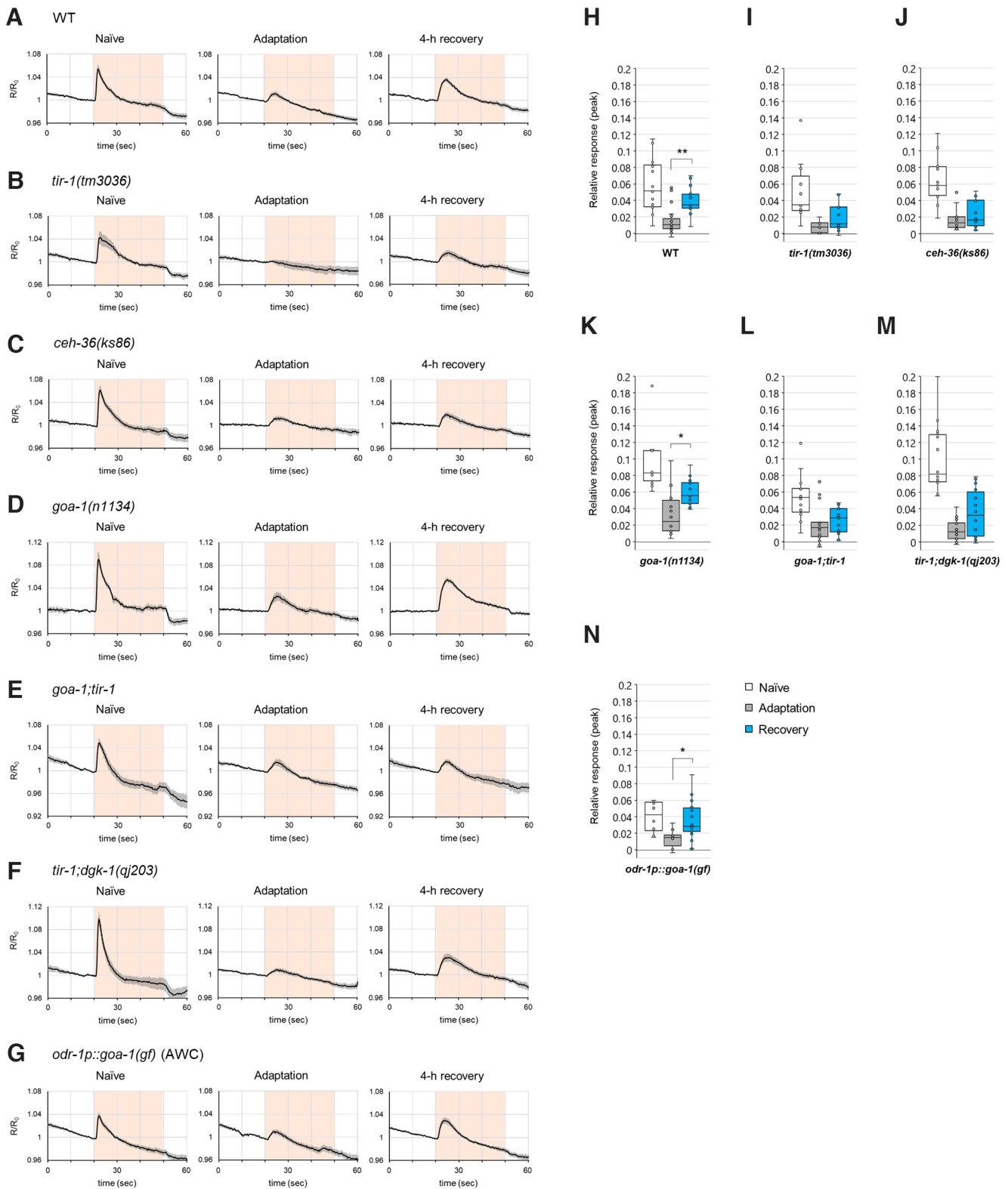


Figure 5. The Ca^{2+} response in AWA olfactory neurons is inconsistent with the behavior after a 4 h recovery of the *goa-1*; *tir-1* and *tir-1*; *dgl-1* double mutants, and the *goa-1(gf)*-expressing mutant. **A–G**, Quantification of the Ca^{2+} response of naïve, adapted, and 4 h recovered animals. Averaged traces of the Ca^{2+} response in WT animals ($n \geq 18$) (**A**), *tir-1(tm3036)* mutants ($n \geq 10$) (**B**), *ceh-36(ks86)* mutants ($n \geq 12$) (**C**), *goa-1(n1134)* mutants ($n \geq 10$) (**D**), *goa-1(n1134);tir-1(tm3036)* double mutants ($n \geq 15$) (**E**), *tir-1(tm3036);dgl-1(qj203)* double mutants ($n \geq 14$) (**F**), and WT animals with overexpression of *goa-1(gf)* in AWC neurons ($n \geq 10$) (**G**). Pink-colored background represents the timing of the diacetyl stimulation (from 20 to 50 s). R represents the YFP/CFP ratio, and R_0 represents the averaged ratio of 5 s before diacetyl stimulation. **H–N**, Peak values of the quantified Ca^{2+} responses (**A–G**) in WT (**H**), *tir-1(tm3036)* (**I**), *ceh-36(ks86)* (**J**), *goa-1(n1134)* (**K**), *goa-1(n1134);tir-1(tm3036)* (**L**), *tir-1(tm3036);dgl-1(qj203)* (**M**) animals, and WT animals with overexpression of *goa-1(gf)* in AWC neurons (**N**). Asterisks indicate significant differences between after adaptation and after 4 h recovery. **H**, WT: $p = 0.00182$ ($n = 18, 19, 23$, $F_{(2,57)} = 17.23$, $p = 1.40 \times 10^{-6}$); **I**, *tir-1*: ($n = 11, 10, 12$, $F_{(2,30)} = 9.75$, $p = 0.000548$); **J**, *ceh-36*: ($n = 13, 12, 14$, $F_{(2,36)} = 20.05$, $p = 1.41 \times 10^{-6}$); **K**, *goa-1*: $p = 0.0285$ ($n = 10, 14, 16$, $F_{(2,37)} = 16.97$, $p = 5.89 \times 10^{-6}$); **L**, *goa-1*; *tir-1*: ($n = 15, 15, 17$, $F_{(2,44)} = 9.92$, $p = 0.000278$); **M**, *tir-1*; *dgl-1*: ($n = 14, 15, 18$, $F_{(2,44)} = 32.68$, $p = 2.00 \times 10^{-9}$); **N**, *odr-1p::goa-1(gf)*: $p = 0.0120$ ($n = 12, 10, 18$, $F_{(2,37)} = 5.40$, $p = 0.00877$). Box plots: center

Together, the change in the DAG content in AWC neurons controls the forgetting process in behavior, but not the forgetting process in the sensory memory trace.

High DAG content until acquisition of memory and low DAG content after the acquisition lead to promote forgetting

To examine whether, as suggested by the genetic and imaging analyses, an increase of DAG really induces forgetting in *tir-1* mutant, we administered PMA, an analog of DAG, to WT and *tir-1* worms, during cultivation, conditioning, and/or recovery. In WT animals, the addition of PMA did not cause significant behavioral changes in all conditions (Fig. 6A). On the other hand, *tir-1* mutant incubated with PMA during cultivation or during adaptation caused to show the attractive chemotaxis after 4 h recovery (Fig. 6B), suggesting that PMA until acquisition of memory leads to induce forgetting. However, *tir-1* mutant exposed PMA during 4 h recovery, even if they exposed PMA during adaptation, showed forgetting defect (Fig. 6B). These results suggest that high DAG content before or during conditioning results in forgetting, even in *tir-1* loss-of-function mutant, and that the higher DAG content after the conditioning cancelled this effect. Additionally, by cultivation with PMA, the naive chemotaxis to diacetyl of *tir-1* mutant became weaker than that by cultivation without PMA (Fig. 6B). This result is consistent with the low chemotaxis of double mutants of *tir-1* with mutation elevating DAG like *dgk-1* (Figs. 1, 2). Moreover, the cultivation with PMA before conditioning, but not during adaptation, made the adaptation to diacetyl weaker in both of WT and *tir-1* lines (Fig. 6A,B), suggesting that, similar to *tir-1(gf)* animals (Inoue et al., 2013), high DAG content before memory acquisition prevents acquisition.

Furthermore, to examine whether DAG content in AWC neurons was distinct among strains and conditions, we monitored the DAG levels of AWC neurons at naive, after adaptation, and after recovery, in WT and the mutants by using a DAG indicator DownwardDAG2 (Tewson et al., 2012, 2013; Ohno et al., 2017) (Fig. 6C), of which lower DAG content increases fluorescent intensity. The fluorescent intensity of DownwardDAG2 in AWC neurons in *tir-1* at naive exhibited higher than WT (Fig. 6D, left), suggesting that the DAG content in AWC neurons of *tir-1* mutant is usually lower than that of WT. After adaptation, the fluorescent intensity of DownwardDAG2 in *tir-1* remained higher than that in WT, and that in *goa-1;tir-1* also became higher than in WT (Fig. 6D, middle). After 4 h recovery, *tir-1* kept higher fluorescent intensity than WT (Fig. 6D, right). These results suggest that, although the DAG content in AWC neurons of *tir-1* is constantly lower than that of WT, *goa-1* loss of function brings about higher DAG content to *tir-1* at least before memory acquisition (in naive) and leads to behavioral forgetting. Together, although the results of DownwardDAG2 expressed in AWC did not completely match with that of PMA administered assay (Fig. 6A,B), probably because administration of PMA affects DAG content of the whole body, a decrease in DAG content during until and after memory acquisition presumably gives rise to the behavioral forgetting.

Discussion

Recently, several lines of evidence in *C. elegans* demonstrated that components of the DAG signaling pathway are important for memory acquisition and consolidation in various learning procedures, including salt chemotaxis learning, adaptation, and associative learning of AWC-sensed odorants, and thermotaxis (Matsuki et al., 2006; Adachi et al., 2010; Kunitomo et al., 2013; Ohno et al., 2017; Arey et al., 2018; Nakano et al., 2020). In this study, we demonstrated that the DAG signaling pathway also regulates active forgetting of memory. Furthermore, the Ca^{2+} imaging of AWA neurons in the *goa-1;tir-1* double mutant and *goa-1*-overexpressing animals revealed that probably through a DAG signal, the sensory memory trace in the AWA neurons does not always induce behavioral memory retrieval, and hence the behavioral forgetting can be observed, even if a sensory memory trace is retained. Our results suggest that a DAG signal followed by synaptic transmission from AWC neurons and their downstream AIA neurons leads to proper behavioral forgetting consistent with the memory trace in AWA. Additionally, measurement and application of DAG raise the possibility that DAG content until acquisition of memory determines whether to recall or to forget the memory, although high DAG during recovery cancelled the DAG effect.

Based on these results, we propose that WT animals, in which DAG content in AWC is properly regulated, can forget the memory depending on the sensory memory trace in AWA (Fig. 7A). However, animals with higher DAG, including *goa-1*, *dgk-1*, *egl-30(gf)* animals, even if the memory trace in AWA exists, could not recall the memory and thereby they appear to forget the memory (Davis and Zhong, 2017) (Fig. 7A). On the other hand, animals with constantly lower DAG, including animals expressing *goa-1(gf)*, could not forget the memory because of too intense recall, although the memory trace in the AWA neurons was mostly eroded to the extent that the Ca^{2+} responses similar to naive animals could be observed. This model is consistent with a recent study on *Drosophila*, which revealed that transient forgetting evoked by distractors is caused by disruption of memory retrieval through a pathway different from that of permanent forgetting (Sabandal et al., 2021), suggesting that the mechanism for forgetting is conserved in various species.

In addition, as a second possibility, the DAG signal may also degrade another memory trace stored in other neurons but not in AWA neurons (Fig. 7B). In this hypothesis, although changing the DAG content did not affect the memory trace in the AWA neurons, the change could have potential to alter the state of another memory trace in other neurons. For example, a reduction in the DAG signal would induce the memory degradation in neurons other than AWA (as the candidates, AIA and AIZ interneurons, ADF, ASE, and ASK sensory neurons, which have direct connection with AWA and AWC or AIA, etc.). Therefore, the remaining memory in the olfactory neuronal circuit, regarded as the engram, would become insufficient and unresponsive to retrieval. Additionally, because the two possibilities, the regulation of memory retrieval by DAG (Fig. 7A) and the existence of the other memory trace (Fig. 7B), are not mutually exclusive, the DAG signal can regulate both erasing memory trace and retrieving simultaneously.

In the nervous system, DGKs regulate signal transduction by phosphorylating DAG, which is released as a second messenger and activates PKC and UNC-13/Munc13, and this regulatory system is conserved in various species (Lackner et al., 1999; Nurrish et al., 1999; Miller et al., 2000; Van Blitterswijk and Houssa, 2000). In *C. elegans*, DGK-1 suppresses acetylcholine

←

line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. * $p < 0.05$; ** $p < 0.01$; one-way ANOVA with Tukey–Kramer multiple test.

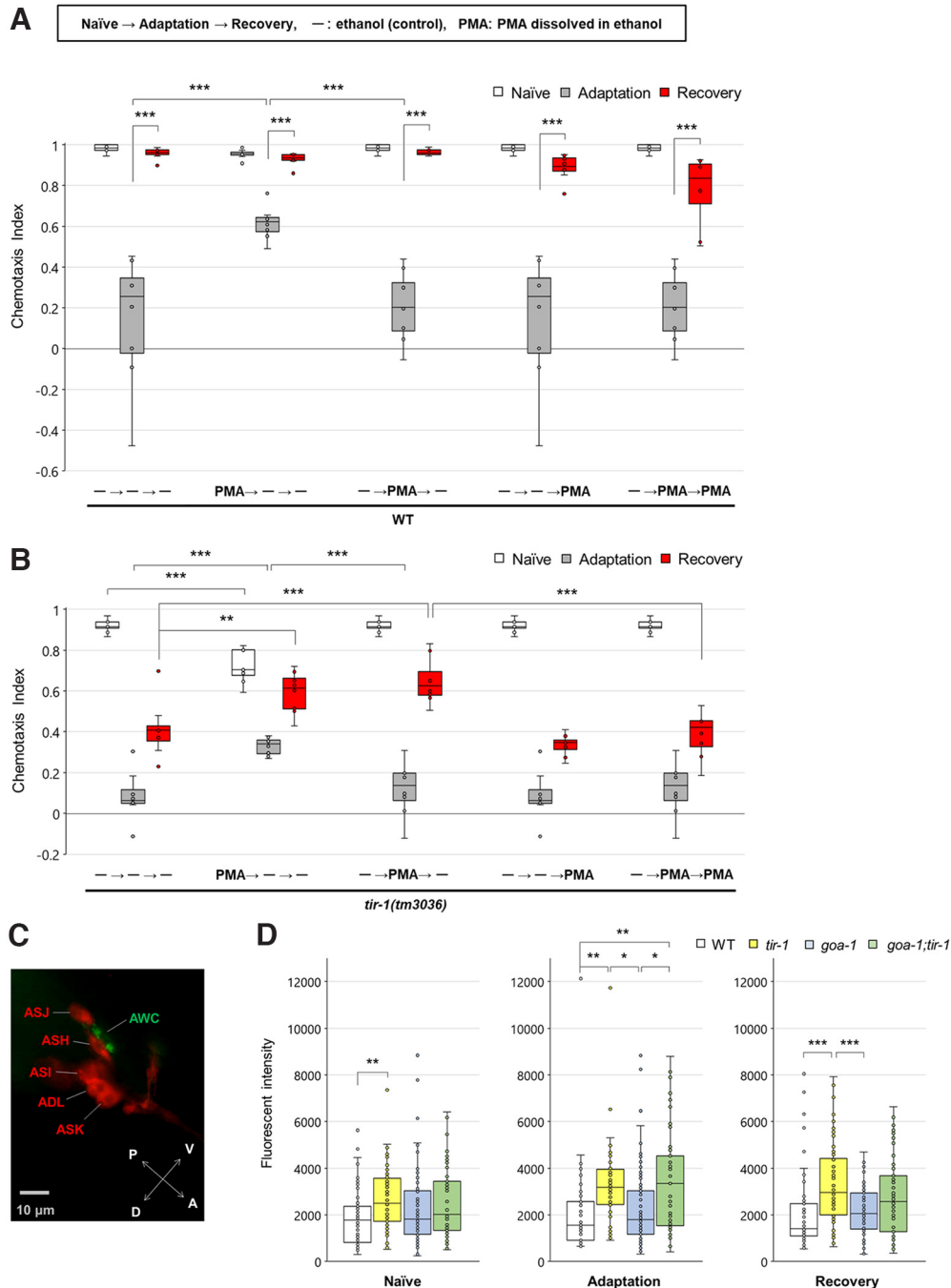
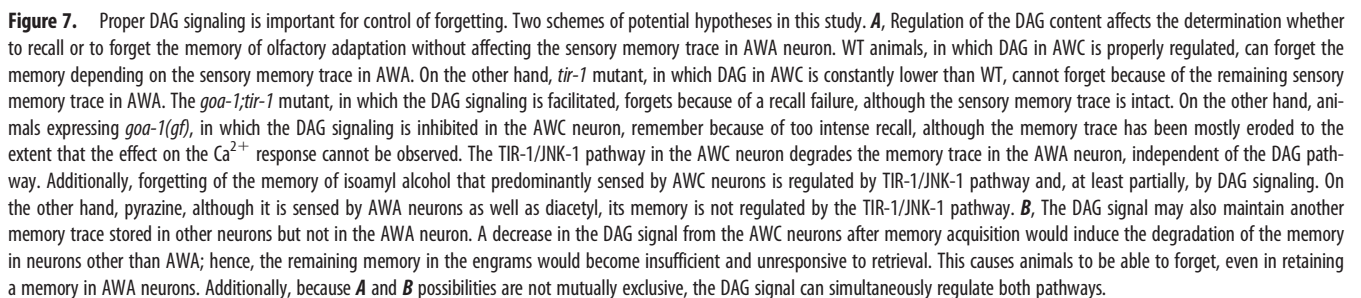


Figure 6. High DAG content until memory acquisition and low DAG content after the acquisition leads to promote forgetting. **A, B**, The chemotaxis of naïve, adapted, and 4 h recovery of PMA administered WT animals (**A**) and *tir-1* mutant (**B**) was analyzed. —, exposed only solvent (ethanol as control); PMA, exposed PMA dissolved in ethanol. For example, PMA → PMA → —, exposed to PMA dissolved in ethanol during cultivation and adaptation, and to ethanol during 4 h recovery. **A**, The chemotaxis of WT animals after recovery was not affected by PMA exposure during cultivation, during adaptation, and/or during recovery, individually. In addition, PMA exposure during cultivation made WT difficult to adapt to diacytl. Asterisks indicate significant differences between after adaptation and after 4 h recovery or between after adaptation of two conditions. Adaptation versus recovery in — → — → —: $p = 9.94 \times 10^{-20}$; PMA → — → —: $p = 7.40 \times 10^{-5}$; — → PMA → —: $p = 5.44 \times 10^{-18}$; — → — → PMA: $p = 1.56 \times 10^{-17}$; — → PMA → PMA: $p = 3.52 \times 10^{-12}$; and adaptation in — → — → — versus PMA → — → —: $p = 1.43 \times 10^{-8}$; PMA → — → — versus — → PMA → —: $p = 7.13 \times 10^{-7}$ ($n = 8$, $F_{(8,105)} = 6.13$, $p = 1.81 \times 10^{-6}$). **B**, *tir-1* mutant exposed PMA during cultivation or during adaptation rose the chemotaxis after recovery. However, PMA exposure during recovery canceled the effect. PMA exposure during cultivation reduced the naïve chemotaxis of *tir-1* and made it difficult to adapt to diacytl. Asterisks indicate significant differences between naïve, after adaptation, or after 4 h recovery of two conditions. Recovery in — → — → — versus PMA → — → —: $p = 0.00281$; — → — → — versus — → PMA → —: $p = 2.77 \times 10^{-5}$; — → — → PMA versus — → PMA: $p = 2.18 \times 10^{-6}$; naïve in — → — → — versus PMA → — → —: $p = 0.000633$; adaptation in — → — → — versus PMA → — → —: $p = 1.30 \times 10^{-5}$; PMA → — → — versus — → PMA → —: $p = 0.000217$ ($n = 8$, $F_{(8,105)} = 13.10$, $p = 5.91 \times 10^{-13}$). **C**, Representative expression of DownwardDAG2 (Green) with Dil (Red) staining pattern in WT. DownwardDAG2 expressed in AWC neurons. Scale bar, 10 μm. **D**, Fluorescent intensity of DownwardDAG2 was measured in AWC sensory neurons of WT and mutants at naïve, after adaptation, and after recovery. Asterisks indicate significant differences between strains in naïve, after adaptation, or after 4 h recovery. In naïve, WT versus *tir-1*: $p = 0.00711$ ($n = 66, 63, 80, 65$, $F_{(3,270)} = 4.03$, $p = 0.00787$). In adaptation, WT versus *tir-1*: $p = 0.00946$; WT versus *goa-1;tir-1*: $p = 0.00360$; *tir-1* versus *goa-1*: $p = 0.0315$; *goa-1* versus *goa-1;tir-1*: $p = 0.0129$ ($n = 49, 44, 68, 53$, $F_{(3,210)} = 6.55$, $p = 0.000297$). In recovery, WT versus *tir-1*: $p = 2.68 \times 10^{-5}$; *tir-1* versus *goa-1*: $p = 4.38 \times 10^{-5}$ ($n = 64, 56, 74, 45$, $F_{(3,235)} = 9.82$, $p = 3.97 \times 10^{-6}$). Box plots: center line indicates median. Box range, 25th–75th percentiles. Whiskers represent minimum–maximum values. Dots represent the data points. External dots from whisker represent outliers. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA with Bonferroni correction (**A, B**) or one-way ANOVA with Tukey–Kramer multiple test (**D**).



In this study, we also showed the specificity for odorants in regulation of the forgetting. *dgc-1* mutation only had a little effect on suppressing the forgetting defect of *tir-1* in isoamyl alcohol memory (Fig. 1I). Previously, we demonstrated that the expression of PKC-1(gf) in AWC neurons can restore the forgetting of isoamyl alcohol memory, suggesting that the secretion from AWC is important for forgetting of the memory like that of diacetyl (Inoue et al., 2013). Additionally, downstream of TIR-1/JNK-1, although HEN-1/SCD-2 pathway regulates forgetting of olfactory adaptation specific to diacetyl, MACO-1 regulates that of diacetyl and to isoamyl alcohol (Kitazono et al., 2017). Since isoamyl alcohol and diacetyl are sensed in the different sensory neurons, the regulatory mechanisms of forgetting can be, at least partly, shared among sensory neurons. On the other hand, pyrazine memory was forgotten, even in *tir-1* animals (Fig. 1J).

Since diacetyl and pyrazine are mainly sensed in the same AWA neurons, the mechanisms of forgetting are distinct, at least in some cases, dependent on the memories of odorants, even in the same sensory neurons. These results suggest that, even in the simple nervous system, the forgetting mechanisms, molecules, and circuits are highly variable for stimuli. Therefore, studies on forgetting of the various memories may give us important aspects of regulatory mechanisms of memory retentions.

Our study provides insights into the mechanisms that regulate forgetting which lead to specific behavior. To adapt to variable and noisy environments, it is important to provide adequate duration of each memory for flexible behavior and to determine whether forgetting or retrieval because memories that are unsuitable for the current circumstance or are unwellcome should be forgotten to prevent interference and exhaustion of energy. The molecular basis and the neuronal circuits for active forgetting and its regulatory mechanisms require further elucidation, but our findings using *C. elegans* will help uncover the big picture of mechanisms of the regulation of forgetting.

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