Behavioral forgetting of olfactory learning is mediated by interneuron-regulated network plasticity and multiple signaling in Caenorhabditis elegans

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Behavioral forgetting of olfactory learning is mediated by interneuron-regulated network plasticity and multiple signaling in *Caenorhabditis elegans*

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Abstract

Forgetting is important for animals to manage acquired memories to enable adaptation to changing environments; however, the neural network in mechanisms of forgetting is not fully understood. To understand the mechanisms underlying forgetting, I examined olfactory adaptation, a form of associative learning, in *Caenorhabditis elegans (C. elegans). C. elegans* displays weak chemotaxis behavior toward diacetyl, one of the attractants, after they are exposed to it for a while but able to recover the chemotaxis behavior eventually. Such behavioral change is considered as forgetting. The forgetting of diacetyl olfactory adaptation in *C. elegans* is regulated by secreted signals from AWC sensory neurons via the TIR-1/JNK-1 pathway. These signals cause a decline of the sensory memory trace in AWA neurons where diacetyl is mainly sensed. Yet, the neuron network of this forgetting mechanism is not fully revealed. To further understand the neural network and mechanism that regulate this forgetting, I investigated the function of interneurons downstream of AWA and AWC neurons. I found that a pair of interneurons, AIA, is indispensable for the proper regulation of behavioral forgetting of diacetyl olfactory adaptation. Loss of or inactivation of AIA not only caused the impairment of the chemotaxis recovery after adaptation without causing severe chemotaxis defects in naïve animal, but also cause defect in forgetting behavior of two distinctive olfactory adaptation regulated by AWAs and AWCs respectively. Furthermore, based on the AWA and AIA Ca^{2+} imaging result in reported studies, even though AIAs Ca^{2+} response can be seen in naïve animal and after recovery, loss of AIAs cause behavioral defect without causing declination of sensory memory trace in AWAs. In addition, lack of both neuropeptide and glutamate cause prolonged retention of the olfactory adaptation, suggest that multiple chemicals signaling involved in regulating forgetting of olfactory adaptation. Here, I propose that 1) the functional neuronal circuit for attractive chemotaxis to diacetyl is changed temporally at the recovery phase so that AIA interneurons are required for chemotaxis,

although AIAs are dispensable for attractive chemotaxis to diacetyl in naïve animals and 2) forgetting mechanism might regulated via multiple signaling.

Introduction

Animals are able to learn and form memories depending on the experience from their surroundings; however, to adapt to changing environments, it is essential that dispensable information is discarded to manage accumulating memories. Recent studies reveal that memories can be actively forgotten by interference with other memories or by activating forgetting in neurons that are important for maintaining memories. (Davis and Zhong, 2017; Hardt et al., 2013). However, the molecular mechanisms and neural networks engaged in forgetting are not well understood.

The complexity of brain structure in higher organisms makes studies on active forgetting at molecular and cellular levels challenging; therefore, invertebrates with simple nervous systems, such as *Caenorhabditis elegans (C. elegans)*, have been used (Hadziselimovic et al., 2014; Inoue et al., 2013; Kitazono et al., 2017). Despite a simple neural network, *C. elegans* shows behavioral plasticity towards various stimuli, such as volatile and water-soluble chemicals (Bargmann et al., 1993; Colbert and Bargmann, 1995; Saeki et al., 2001). In the well-studied neural network of *C. elegans* (Cook et al., 2019; White et al., 1986), most attractive volatile odorants, such as diacetyl and isoamyl alcohol, are sensed by two pairs of amphid sensory neurons, AWA and AWC, respectively, and these neurons have distinct sensory mechanisms (Bargmann, 1993, 2006; Colbert et al., 1997; Colbert and Bargmann, 1995; L'Etoile and Bargmann, 2000; Sengupta et al., 1994, 1996). These amphid sensory neurons make synapses to first-layer interneurons, mainly AIA, AIB and AIY, which also regulate the plasticity of various behaviors, such as associative learning (Chalasani et al., 2010; Cho et al., 2016; Rankin et al., 1990; Tomioka et al., 2006), as well as integrate multiple sensory signals, including contradicting information (Dobosiewicz et al., 2019; Larsch et al., 2015; Shinkai et al., 2011; Wolfe et al., 2019), to generate appropriate cellular responses and animal behavior.

In invertebrates, despite their simple neural networks, several studies showed that forgetting is actively regulated. In *Drosophila*, dopamine neurons regulate both learning and active forgetting through distinctive dopamine receptors in mushroom body neurons (Berry et al., 2018, 2012). One of the dopamine receptors in mushroom body neurons, dDA1, leads to memory formation (Berry et al., 2012), while for forgetting, another receptor, DAMB, activates Scribble scaffold to initiate forgetting by actin cytoskeleton remodeling (Berry et al., 2018, 2015, 2012; Cervantes-Sandoval et al., 2016; Shuai et al., 2010). *C. elegans is* also used to study active forgetting through olfactory adaptation, a form of associative learning (Bargmann, 2006; Colbert and Bargmann, 1995). Forgetting of diacetyl olfactory adaptation, which is sensed by AWA olfactory neurons, is regulated by the TIR-1/JNK-1 pathway in another type of olfactory neuron, AWC. In wild-type and the mutants that are defective in TIR-1/JNK-1 pathway, the sensory Ca^{2+} response of AWA neurons to diacetyl is positively correlated with behavioral change through memory formation and forgetting, suggesting that the AWA Ca^{2+} responsiveness can be considered as the sensory memory trace and that the memory trace in AWAs is actively and non-cell-autonomously regulated by AWCs (Inoue et al., 2013; Kitazono et al., 2017). In addition, a membrane protein, MACO-1, and a tyrosine kinase pathway involving SCD-2 and HEN-1 regulate the forgetting (Kitazono et al., 2017). Another study showed that, similar to Rac1 in *Drosophila*, the Arp2/3 complex, which regulates the actin cytoskeleton in AVA interneurons, is important for forgetting downstream of RNA binding protein, *Musashi* (Hadziselimovic et al., 2014). Although these studies indicate that active forgetting is important, even in simple learning paradigms of model organisms, the corresponding neural network has not been fully revealed.

Types of signaling molecules which involved in forgetting, especially the chemical substances that are required for the neurotransmission during forgetting, have also been studied to unveiled the mechanisms of the forgetting. Besides the dopamine signaling mentioned above

in *Drosophila*, neurotransmitter such as glutamate, the most abundant excitatory signal in brain, and its receptor, AMPA receptor, also involved in memory related mechanism, such as memory retrieval, in mammals (Hardt et al., 2013; Pereyra and Medina, 2021; Medina, 2018; Sachser et al., 2017; Dong and Han, 2015). In *C. elegans*, neurotransmission from AWC sensory neurons is predicted to actively regulate forgetting of olfactory adaptation memory in AWA sensory neurons (Inoue et al., 2013; Kitazono et al., 2017). Since neuropeptide signaling is also important for regulating various behavioral plasticity of *C. elegans* (Chalasani et al., 2010; Choi et al., 2015; Jeong and Paik, 2017; Lee and Mylonakis, 2017; McDiarmid et al., 2015), the studies on the neurotransmission for forgetting is a promising target to be investigated. In addition to the chemical synaptic transmission, electric signals via gap junctions, which are formed in *C. elegans* by innexins instead of connexins (Hall, 2017a), are important for neuronal communication. The gap junctions, which are formed from various types of innexins subunits, can be found in sensory and interneurons to relay external stimuli and in motor neurons to modulate various muscular activity in *C. elegans* (Hall, 2017a, 2017b; Macosko et al., 2009; Simonsen et al., 2014; Walker and Schafer, 2020), suggesting their important roles in regulating behavioral plasticity. Despite studies in forgetting has progressed greatly, neural signaling which involve in forgetting is yet to be fully comprehended.

Here, I demonstrate that a pair of neurons in *C. elegans*, AIA interneurons, which are the first layer interneurons in olfactory circuits, is required to regulate forgetting processes of olfactory adaptation. Although absence or inactivation of functional AIA interneurons (AIA-) slightly affect attractive chemotaxis to diacetyl, it caused prolonged retention of the olfactory adaptation to diacetyl, suggesting that AIAs accelerate forgetting. Loss of AIA interneurons also cause retention of the olfactory adaptation to isoamyl alcohol, odorant sensed by different mechanism than those of diacetyl, suggesting that AIAs regulate forgetting of several different types of olfactory adaptation. In addition, this study also showed that lack of either

neuropeptides or glutamate cause prolonged retention of the olfactory adaptation, indicating that the involvement of multiple neuronal signaling in regulating forgetting. Moreover, calcium imaging analyses in reported studies showed that, even though the behavioral response in AIA-animals did not recover after cultivation for 4 hours, the calcium responses to diacetyl in AWAs were recovered. Together, these results suggest that AIAs are indispensable for the behavioral response of olfactory adaptation forgetting mechanism, probably because the functional neuronal circuit is changed temporally and AIAs are required for the chemotaxis after the recovery from adaptation.

Materials and Methods

Strains and Culture

Strain name	Genotype	Source
	N2	CGC
	tir-1(tm3036)	National Bioresource Project
RB1085	*tir-1(ok1052)*	CGC
NY7	*flp-1(yn2)*	CGC
NY16	*flp-1(yn4)*	CGC
AX1410	*flp-18(db99)X*	CGC
MT1241	*egl-21(n611)*	CGC
	ins-1(nr2091)	CGC
VC1218	*ins-18(ok1672)* I	CGC
RB2059	*ins-28(ok2722)* I	CGC
FX01922	*ins-29(tm1922)*	National Bioresource Project
VC2357	*nlp-38(ok2330)* I	CGC
CX13325	*inx-2(ok376)*	CGC
RB1834	*inx-4(che-7)(ok2373)*	CGC
RB1086	*inx-5(ok1053)*	CGC
RB1792	*inx-7(ok2319)*	CGC
VC116	*inx-8(gk42)*	CGC
CX12726	*inx-9(ok1502)*	CGC
RB2051	*inx-10(ok2714)*	CGC

RB2108	*inx-11(ok2783)*	CGC
AU98	*inx-14(ag17)*	CGC
VC40193	*inx-17[gk506358(W176stop)]*	CGC
RB1896	*inx-18(ok2454)*	CGC
CX6161	*inx-19(nsy-5)(ky634)*	CGC
CX12725	*inx-20(ok681)*	CGC
RB1929	*inx-21(ok2524)*	CGC
XM1011	*inx-22(tm1661)*	National Bioresource Project
DA464	*eat-5(ad464)*	CGC
MT6308	*eat-4(ky5)*	CGC
JN578	peIs578[npr-9p::casp1, npr-9p::venus, unc-122p::mCherry] (AIB-)	Satoh et al., 2014
JN579	peIs579[ttx-3p::casp1, ttx-3p::venus, lin-44p::gfp] (AIY-)	Satoh et al., 2014
JN580	peIs580[ins-1(short)p::casp1, ins-1(short)p::venus, unc-122p::gfp] (AIA-)	Satoh et al., 2014
QD155	qjEx3[*gcy-28.dp::mec-4(d), gcy-28::gfp, lin-44p::gfp*]	Shinkai et al., 2011
QD156	qjEx4[*gcy-28.dp::unc-103(gf), myo-3p::gfp*]	Shinkai et al., 2011
QD157	peIs578[*npr-9p::casp1, npr-9p*::venus, *unc-122p*::mCherry]; peIs579[*ttx-3p::casp1, ttx-3p*::venus, *lin-44p*::gfp] (AIB-; AIY-)	This thesis
QD165	*tir-1(ok1052)*; qjEx4[*gcy-28.dp::unc-103(gf), myo-3p::gfp*]	This thesis

QD164	tir-1(tm3036); qjEx3[*gcy-28.dp::mec-4(d), gcy-28::gfp, lin-44p::gfp]*	This thesis
QD166	*tir-1(tm3036); qj*Ex4[*gcy-28.dp::unc-103(gf), myo-3p::gfp*]	This thesis

Table 1: Strain list

All strains were cultured on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 (Brenner, 1974) and were grown at 20°C prior to experiments. In all experiments, I used young adult hermaphrodites that were prepared as described in each section.

Behavioral Assay

Chemotaxis toward attractive odorants was performed on assay plates (2% Bacto agar, 50 mM NaCl, 10 mM K_2HPO_4 [pH 6], 1 mM $MgSO_4$, 1 mM $CaCl_2$) with 1:100 dilutions of odorants (diacetyl and isoamyl alcohol) (Bargmann et al., 1993). During behavioral assays, animals were placed in the middle of the assay plate while the odorant and control solution (ethanol, the odorant diluent) were spotted on opposite sides of the plate. The chemotaxis index was calculated as (A-B)/N, where A refers to the number of animals within 1.5 cm of the odorant spot, B refers to the number of animals within 1.5 cm of the control spot, and N is the total number of animals. Chemotaxis index was normalized to the average naïve chemotaxis index respectively. In the forgetting assay (Inoue et al., 2013), adult animals were firstly washed three times with S-basal buffer (100 mM NaCl, 50 mM K_2HPO_4 [pH 6], 0.02% gelatin) (naïve) and pre-exposed to 1:5000 diluted diacetyl or isoamyl alcohol in S-basal buffer with slow rotation for 90 min at room temperature (adaptation). Next, the worms were washed once and allowed to recover on OP50-seeded NGM plates for 4 hours (recovery). In the extended forgetting assay,

animals were recovered on OP50-seeded NGM plates for 24 hours, and behavioral assays after recovery were conducted after 4, 8 and 24 hours of recovery.

Experimental design and statistical analyses

For all experiments, adult hermaphrodites were used. In the behavioral assays, the stage of animals was synchronized by removing adult animals from OP50-seeded NGM plates 16 to 20 hours after transfer (Ishihara et al., 2002). Injection markers, such as *myo-3p::*gfp, *lin-44p::*gfp, *unc-122*p::mCherry and *unc-122*p::gfp, were used to distinguish transgenic animals with extrachromosomal transgenes, and the values of animals with extrachromosomal transgenes were compared with those of animals without transgenes on the same plates as internal controls.

All values are presented in box plot. Data analyses were performed using Bell Curve for Excel (Social Survey Research Information Co., Ltd, version 3.22). Statistical significance between means was determined by Student's *t*-test or two-way ANOVA followed by a post-hoc *t*-test with Bonferroni's correction. Sample sizes and statistical values are noted in the figure legends.

Results

AIA interneurons are required to regulate forgetting in AWA olfactory adaptation

C. elegans shows strong attractive chemotaxis to diacetyl, which is mainly sensed by AWA sensory neurons. After animals are exposed to diacetyl without food for 90 minutes, they show significantly weaker responses to diacetyl (olfactory adaptation) (Colbert and Bargmann, 1995). The conditioned animals are able to recover the attractive chemotaxis toward diacetyl to a level similar to that of naïve animals after cultivation with food for 4 hours (recovery), and such recovery is considered as forgetting (Inoue et al., 2013). Consistent with the behavioral change, the Ca^{2+} responses to diacetyl in AWA neurons are decreased after conditioning and recover with cultivation. This correlation between behavior and sensory responses is also observed in mutants defective in TIR-1/JNK-1 pathway, which function in AWC sensory neurons. In *tir-1 (tm3036)* null mutant, naïve animals show the sensory response to diacetyl and, after conditioning, the Ca^{2+} responses decrease to levels similar to those of wild-type animals. However, in *tir-1* null animals, similar to the behavioral changes, the Ca^{2+} responses in AWAs to diacetyl do not recover with cultivation. Therefore, forgetting of diacetyl olfactory adaptation in AWA neurons is regulated by AWC sensory neurons via the TIR-1/JNK-1 pathway (Inoue et al., 2013). AWC neurons do not make direct connections to AWAs; therefore, other neurons may be involved in this regulation.

Figure 1A shows the olfactory circuit including olfactory sensory neurons, AWAs and AWCs, and their downstream interneurons (Chalasani et al., 2010, 2007; Cook et al., 2019; Dobosiewicz et al., 2019; White et al., 1986). As shown in Figure 1A, AWCs and AWAs mainly relay signals to the first layer interneurons, AIA, AIB and AIY. Among these interneurons, AIB and AIY interneurons, the main synaptic target of AWCs, were first to be examined to investigate whether they are involved in forgetting by using animals with

genetically ablated AIB and AIY, in which cell-specific cell death is promoted by expressing mouse Caspase 1 (*Casp1)* (Satoh et al., 2014). However, in animals without AIBs and/or AIYs, we detected no significant differences in changes of chemotaxis to diacetyl among naïve, conditioned and recovered animals (Figure 1B, C). This indicated that AIB and AIY interneurons are dispensable for the regulation of forgetting in this olfactory adaptation.

Next, AIAs were examined to using several AIAs-malfunctioned strains (AIA-). By using an AIA specific *gcy-28.d* promoter (Shinkai et al., 2011) and an *ins-1*(short) promoter (Satoh et al., 2014), we expressed 1) a hyperactive form of the DEG/ENaC sodium channel, MEC-4 [MEC-4(d)] to cause neural toxicity (Harbinder et al., 1997; Shinkai et al., 2011), 2) a constitutively active form of the ERG-like potassium channel, UNC-103[gain of function (gf)] to hyperpolarize and consequently inactivate neurons (Shinkai et al., 2011) and 3) *Casp1* for genetic ablation (Satoh et al., 2014). In naïve animals, chemotaxis to diacetyl in AIA- strains was weakly defective (Figure 1D and E), probably because AIA interneurons are involved in diacetyl perception (Larsch et al., 2015). In spite of this weak naïve chemotactic defect, more prominent decreases in chemotaxis to diacetyl after recovery from adaptation was detected in AIA- animals (Figure 1D), indicating that AIA interneurons are required for forgetting diacetyl olfactory adaptation.

AIA interneurons accelerate forgetting of olfactory adaptation

Next, I examined whether AIA- animals completely lost the ability to forget, or decelerated the forgetting progress, as in *tir-1(tm3036)* null animal (Figure 2; Inoue et al., 2013). To test this, I analyzed the time course of memory retention for up to 24 hours of recovery (4, 8 and 24 hours after conditioning) (Figure 2). At the first 4 and 8 hours of recovery, although wild-type animals showed full recovery of chemotaxis, AIA- and *tir-1 (tm3036*) null animals showed very weak recovery. After 24 hours, AIA- animals, similar to *tir-1(tm3036)* null mutants,

showed almost full recovery to diacetyl, suggesting that, even without AIAs, animals can slowly forget the memory. AIA interneurons, therefore, accelerate forgetting of olfactory adaptation.

AIA interneurons regulate forgetting downstream of the TIR-1 pathway

As mentioned later in the discussion section, AWA Ca^{2+} imaging analyses, performed by my collaborator, showed that the ablation of AIA interneuron did not affect the AWA Ca^{2+} responses at all the phases (naïve, adaptation, and after recovery) (Fig. 7), although the mutants defective in TIR-1/JNK-1 pathway showed the prolonged retention of the sensory memory trace in AWA. Therefore, I suspected that AIA interneurons might regulate forgetting independently of the TIR-1/JNK-1 pathway. I examined the genetic relationship between AIA interneurons and the TIR-1/JNK-1 pathway in the forgetting mechanism, by analyzing genetic epistasis using *tir-1(ok1052 gf) animals*, which show weak adaptation after conditioning probably because of hyper-forgetting (Chuang and Bargmann, 2005; Inoue et al., 2013). Consistent with previous studies, *tir-1(ok1052 gf)* animals showed weak adaptation after conditioning (Figure 3; Inoue et al., 2013). I prepared *tir-1 (ok1052 gf)* animals without AIA interneurons and found that the animals showed normal adaptation, and also showed prolonged retention of the adaptation (Figure 3). These phenotypes cannot be distinguished from those of AIA- animals, suggesting that AIA interneurons regulate a forgetting process downstream of the TIR-1/JNK-1 pathway.

AIA interneurons regulate forgetting of AWC-sensed isoamyl alcohol olfactory adaptation

As mentioned in earlier section, in *C. elegans*, odorants are sensed by AWAs and AWCs via different mechanisms, including olfactory adaptation. However, Inoue et al. (2013) and Kitazono et al. (2017) reported that parts of forgetting mechanism are somehow shared among

distinctive odorants. AIAs are reported to be downstream target of not only AWAs but also AWCs(Cook et al., 2019; White et al., 1986); thus, I suspect that loss of AIAs might affect the chemotaxis of animal after recovery of AWC-sensed olfactory adaptation. To investigate this hypothesis, I observed the behavior of two AIA- transgenic animals in forgetting assay using AWC-sensed isoamyl alcohol. Both AIA- animals showed retention of olfactory adaptation toward isoamyl alcohol (Figure 4), suggesting that AIAs are required not only for AWA-sensed olfactory forgetting but also for AWC-sensed olfactory forgetting mechanism.

Multiple types of signals may involve in regulating olfactory forgetting behavior.

AIAs were reported to be the hub of multiple behavior changes where both chemical and electric signal were involved (Chalasani et al., 2010; Cho et al., 2016; Ji et al., 2021; Larsch et al., 2015; Shinkai et al., 2011; Tomioka et al., 2006; Wolfe et al., 2019), suggesting that AIAs connect to other neurons via multiple signals. Thus, I also investigated the signaling molecules involved in AIAs function that regulate such behavioral changes.

Previous studies suggested that forgetting behavior might be regulated by AWC sensory neurons via neurotransmission (Inoue et al., 2013; Kitazono et al., 2017). To test if AIAs regulate the behavioral changes through chemical signals, I listed out neuropeptide encoding genes (insulin-like peptides INS, FMRFamide related peptides FLP, neuropeptide-like proteins NLP; Li, 2008) , which are expressed in AIA via CeNGEN database (Table 3; Hammarlund et al., 2018; Taylor et al., 2020; https://cengen.shinyapps.io/CengenApp/) and examined the behavior of mutants of several genes among them (Fig. 5). *ins-28* showed a forgetting defect phenotype (Figure 5C), although the phenotype was much weaker than the AIA-f strain. I also found that *egl-21*, a mutant of carboxypeptidase E involved in all neuropeptide processing (Li, 2008), showed severe forgetting defects phenotypes, similar to those of *tir-1(tm3036)* null mutants (Figure 5A). These results suggest that some neuropeptide may be involved in regulating diacetyl olfactory forgetting.

Next, I examined the behavior of animals lacking a neurotransmitter, glutamate, which, along with their receptors, are reported to be involved in learning and memory, including memory retention, memory retrieval and forgetting in mammals and invertebrates (Davis and Zhong, 2017; Hardt et al., 2014, 2013; Kano et al., 2008; Pereyra and Medina et al., 2021; Migues et al., 2019, 2016; Sachser et al., 2017). A mutant of *eat-4* gene that encodes vesicular glutamate transporter of *C. elegans* (Lee et al., 1998) showed defect in chemotaxis after recovery from adaptation (Figure 5C), indicating that glutamate might also be involved in regulating forgetting of olfactory adaptation in *C. elegans*.

AIAs receive signals from AWA for diacetyl chemosensory behavior via gap junctions (Cook et al., 2019; Dobosiewicz et al., 2019; Liu et al., 2018; White et al., 1986). I examined the behavior of mutants of 16 innexin genes which encode subunits of gap junctions of *C. elegans* (Altun et al., 2009) and found that, besides *inx-10* and *inx-20* showed weaker chemotaxis after recovery, almost all gap junction subunit mutants were able to show recovered behavior (Figure 6) as wild-type animals. Thus, defects in single gap junction subunit caused no defect in forgetting.

		Data Structure	Type of test	Power (α=0.05)
a	Fig. 1B, C	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
b	Fig. 1D	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
c	Fig. 1E	Normal Distribution	Student's T-test	$1:10^{-2}$: 0.0201 $1:10^{-3}$: 0.5159 $1:10^{-4}$: 0.2595 $1:10^{-5}$: 0.466
d	Fig. 2	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
e	Fig. 3	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
f	Fig. 4	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
g	Fig. 5	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
h	Fig. 6	Normal Distribution	Two-way ANOVA	Strains: <0.0001 Conditions: <0.0001
i	Fig. 7B	Normal Distribution	Two-way ANOVA	Strains: 0.0271 Conditions: <0.0001
j	Fig. 8B	Normal Distribution	Student's T-test	Naïve: 0.032 Adaptation: 0.13 Recovery: 0.0149

Table 2: Statistical table

Discussion

Forgetting is important for animals to manage information to properly respond to changing environments. Yet, the neuronal mechanisms for forgetting are not fully understood. In this study, I discovered that a pair of interneurons, AIA, is required to regulate behavioral forgetting of olfactory adaptation.

Interneurons accelerate forgetting of olfactory adaptation

In this study, I found that AIA interneurons are required to accelerate forgetting of olfactory adaptation (Figure 2). Without functional AIAs, conditioned animals were unable to regain chemoattraction toward diacetyl after cultivation with food for 4 hours. However, after cultivation with food for 24 hours, chemoattraction was recovered in AIA- animals, suggesting that, even in the absence of the functional AIA interneurons, animals can slowly forget. Therefore, AIAs are important to accelerate forgetting of olfactory adaptation.

Interneurons regulate forgetting of distinct types of olfactory adaptation

Besides showing retention of AWAs-sensed diacetyl olfactory adaptation, such behavior was also observed in isoamyl alcohol olfactory adaptation, odorant that is sensed by AWC sensory neurons, in animal without functional AIA interneurons (Figure 4). These observations suggest that AIA interneurons are involved in both AWA-sensed and AWC-sensed olfactory forgetting. Due to the simplicity of neural network, it is not uncommon to have the same neuron, or pathway, to regulate multiple behavior plasticity in *C. elegans*, especially the first layer interneurons which received signals from multiple sensory neurons (Bargmann, 2012; Dobosiewicz et al., 2019; Ghosh et al., 2017; Hobert, 2003; Macosko et al., 2009; Summers et al., 2015). For instance, AIAs themselves are involved in detecting the change in odorant concentration (Larsch et al., 2015) and also in integration of signals from multiple sensory neurons for behavioral output (Sreekanth H. Chalasani et al., 2010; Shinkai et al., 2011; Wolfe

et al., 2019). Furthermore, in previous studies, TIR-1/JNK-1 pathway and MACO-1 are reported to regulate olfactory forgetting of both AWA-sensed and AWC-sensed odorant (Inoue et al., 2013; Kitazono et al., 2017). Observations in this study suggest that, even for different odorants, at least, a part of forgetting mechanisms may be shared, and hence further investigation is necessary to clarify this assumption.

AIA interneurons are indispensable for chemotactic behavior to diacetyl only after recovery and, thereby, for behavioral forgetting.

AIA interneurons are part of the olfactory sensory circuit and Ca^{2+} response of AIA interneurons was observed during chemotaxis to diacetyl (Dobosiewicz et al., 2019; Larsch et al., 2015). A minor defect in chemoattraction of naïve AIA- animals to diacetyl was observed (Figure 1D and E), indicating that the neuronal circuit for chemotaxis can function in naïve animals in the absence of AIA interneurons. However, after recovery for 4 hours, AIA- animals still showed a lowered chemotaxis to diacetyl (Figure 1D), observed as a defect in behavioral forgetting, suggesting that AIAs activity is more critical for chemotaxis after recovery than those in naïve.

Diacetyl-evoked Ca^{2+} response in AWAs was reported to show correlation with behavioral change in naïve, conditioned and recovered animals (Inoue et al., 2013; Kitazono et al., 2017). Therefore, the weakened Ca^{2+} response in AWAs after conditioning is considered as a sensory memory trace. In the forgetting defect mutant, such as *tir-1 (tm3036)* null mutants and *maco-1*, the sensory memory traces are retained inappropriately after 4 hours of recovery, in consistent with their behavioral responses (Inoue et al., 2013; Kitazono et al., 2017). On the other hand, in AIA- animals, the sensory memory trace in AWA was reported to decline normally after 4 hours of recovery (Figure 7; Teo et al., 2022). Furthermore, Ca^{2+} imaging analyses of AIAs showed that AIAs respond to diacetyl stimuli at naïve and after recovery, but

not at just after conditioned (Figure 8; Teo et al., 2022). Thus, after recovery from adaptation, even if AWA restores the response to diacetyl, it may not be able to induce chemotaxis to diacetyl without AIA activity.

With the Ca^{2+} response observed in AWA and AIA during forgetting assay, two possible circuit plasticity are suggested. One is that although redundant neuronal circuits can regulate chemotaxis to diacetyl in naïve animals, after conditioning, the circuit that does not include AIAs becomes non-functional so that the AIAs become indispensable for the chemotaxis (Figure 9A). Another one is that, only after conditioning the neuronal circuit for chemotaxis to diacetyl requires the activity of AIAs, which is distinct from the naïve circuit (Figure 9B). In these hypotheses, the functional neuronal circuit that does not include AIAs may recover slowly so that chemotaxis to diacetyl recovers after conditioning for 24 hours. Based on the Ca^{2+} imaging analyses of AIA (Figure 8; Teo et al. 2022), the model for the redundant neuronal circuits in naïve is highly supported as AIAs responses are similar to those after the recovery. These kinds of circuit plasticity based on internal states are important for behavioral plasticity in higher organisms (Herry et al., 2008; Kim et al., 2017; Kuchibhotla et al., 2017; Ramaswami, 2014). Furthermore, such circuit plasticity involving AIA interneurons might also be used by other olfactory adaptation mechanisms because AIA- animals displayed a defective forgetting phenotype toward AWC-sensed isoamyl alcohol without causing a severe chemotactic defect (Figure 4). In order to clarify the precise role of AIA interneurons in both circuit and behavior plasticity, further experiments including optogenetic inactivation or activation of the olfactory circuits in naïve and conditioned animals are required.

Genetic epistasis experiment indicates that AIAs function downstream of the TIR-1 pathway in the regulation of forgetting (Figure 3). TIR-1 is required to accelerate the forgetting of olfactory adaptation of diacetyl; therefore, the adaptation defect to diacetyl in the *tir-1(gf)* mutant might be caused by forced chemotactic recovery from adaptation during conditioning

(Inoue et al., 2013). If this is the case, suppression of the adaptation defect by AIA- is consistent with the role of AIAs in chemotaxis during the recovery phase.

Behavioral change after recovery might be regulated by multiple signaling

Our screening of types of the neurotransmittion shown in Figure 5 suggests that forgetting behavior might regulated via multiple types of signaling. I found that *egl-21*, a mutant of carboxypeptidase E involved in most neuropeptide processing (Li, 2008), showed a severe forgetting defective phenotype similar to those in AIA- (Figure 5A), AWC- and *tir-1(tm3036)* null mutant (Inoue et al., 2013). This suggests that neuropeptide(s) regulate olfactory forgetting in *C. elegans*, as mentioned in the papers by Inoue et al (2013) and Kitazono et al (2017). Identification of neuropeptide(s) involved in forgetting mechanism as well as the neuron(s) which release the neuropeptide(s) would reveal the neuronal circuit and signal(s) for olfactory forgetting in *C. elegans*. In addition to neuropeptide, lack of functional vesicular glutamate transporter EAT-4/VGLUT in *C. elegans* caused the forgetting defect (Figure 5C), suggesting that glutamatergic signaling might also be involved in regulating forgetting of olfactory adaptation. Glutamate, as the major excitatory neurotransmitter in brain, was reported to not only modulate learning and memory but also involved in forgetting mechanisms across species (Hardt et al., 2013; Kano et al., 2008; Pereyra and Medina et al., 2021; Migues et al., 2019, 2016; Sachser et al., 2017). However, in addition to memory related mechanism, in *C. elegans*, glutamatergic signal regulates numerous behavior plasticity such as locomotion(Choi et al., 2015), foraging behavior (Hills et al., 2004), as well as maintaining tonic neural activity (Dobosiewicz et al., 2019), making it difficult to simply explain the role of glutamate in such behavior changes without further investigation.

Overall, this study shows that AIA interneurons in *C. elegans* are required to regulate behavioral forgetting of olfactory adaptations. This indicates that intact neural circuits are

important for simple forgetting regardless of the simplicity of the neural system. Studies that reveal learning, memory formation and forgetting pathways in invertebrates might be conserved across species (Costa et al., 2020; Lipina et al., 2016; Rahmani and Chew, 2021; Stein and Murphy, 2014; Vorster and Born, 2015); therefore, I believe that studies in invertebrates are important to elucidate the mechanisms of forgetting in higher organisms.

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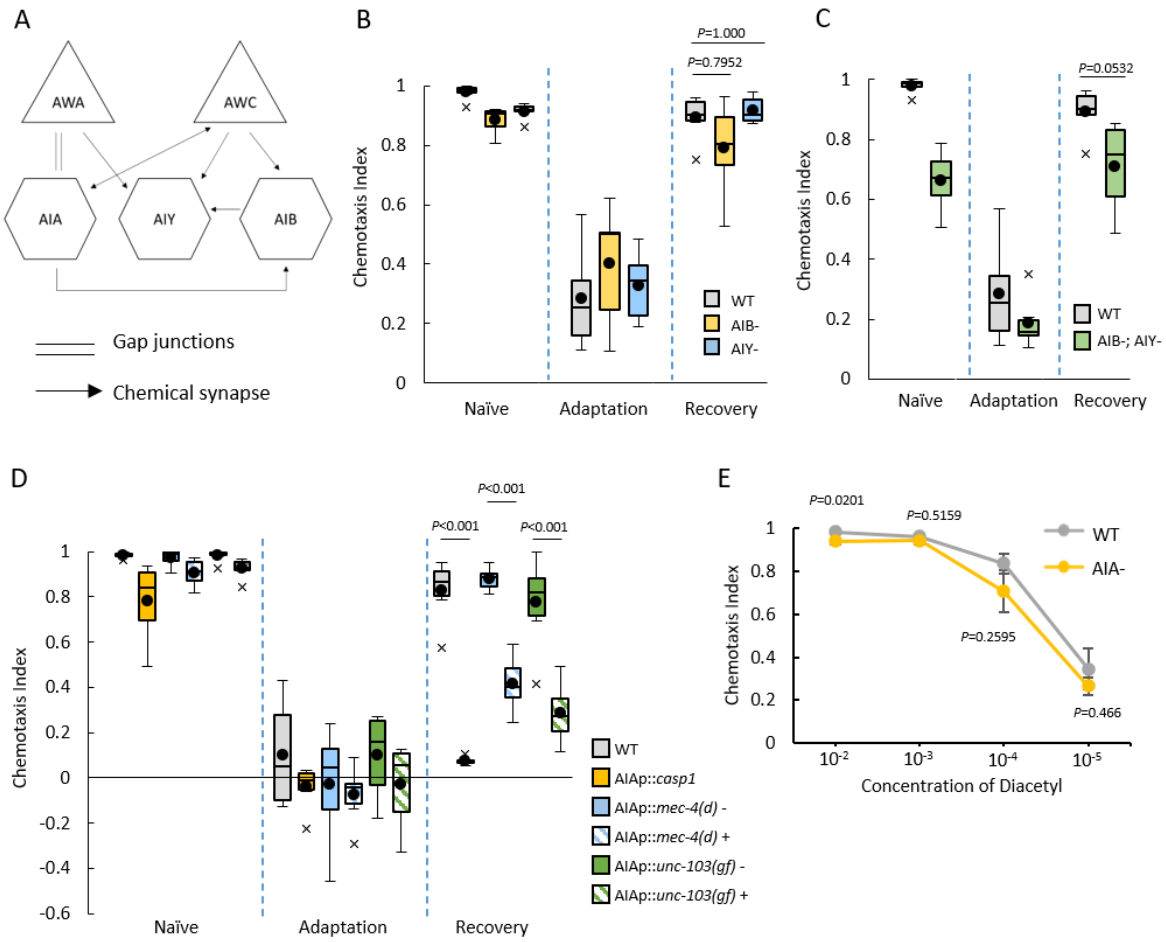

Figure 1. AIA interneurons are required to regulate forgetting of diacetyl olfactory adaptation.

A, A simplified neural network for olfactory sensing in *C. elegans* (Chalasani et al., 2010; Dobosiewicz et al., 2019; Larsch et al., 2015; White et al., 1986). **B–D**, Behavioral assays of animals with ablation of AIA, AIB, and AIY. Chemotaxis of naïve, adapted and 4 hour recovered animals was analyzed. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (AIB-, AIY- and AIB-; AIY-: n=6, 2-way ANOVA, $F_{strain(3,60)}$=12.11, p=2.66e^{-6}, η^2=0.3772; AIA-: n=6, 2-way ANOVA, $F_{strain(5,90)}$=21.07, p=6.86 e^{-14}, η^2=0.5393). **E**, Dose dependency of chemotaxis to diacetyl in AIA- animals. (1:10^{-2} diacetyl: n=6, t(5)=3.3608, p=0.0201; 1:10^{-3}

diacetyl: $t(5)=0.6986$, $p=0.5159$; 1:10^{-4} diacetyl: $t(5)=1.2714$, $p=0.2595$; 1:10^{-5} diacetyl: $t(5)=0.7888$, $p=0.466$; mean± SEM) **B-D** *Post hoc t*-test with Bonferroni's correction or **E** Student's *t*-test. Error bars represent SEM.

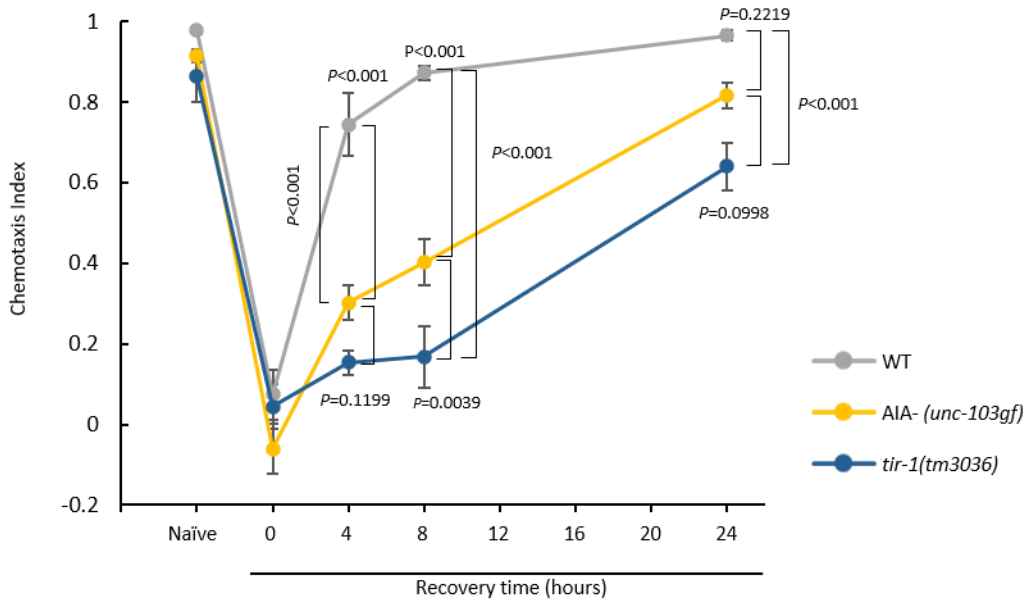

Figure 2. Time course of olfactory adaptation recovery.

Time course of chemotaxis recovery after adaptation in *tir-1(tm3036)* and AIA- *(unc-103 gf)* animals. Chemotaxis at naive, adaptation (0 hour), and 4, 8 and 24-hour recovery were analyzed ($n \geq 6$, 2-way ANOVA, $F_{strain(2, 99)}=61.26$, $p=4.84e^{-18}$, $\eta^2=0.5531$; mean ± SEM). *Post hoc t*-test with Bonferroni's correction. Error bars represent SEM.

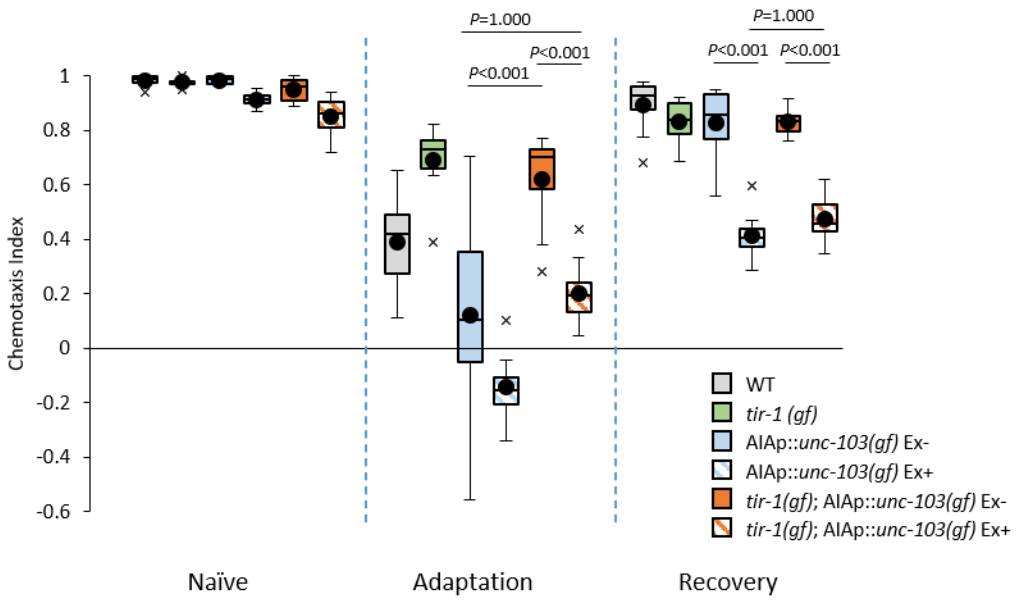

Figure 3. Genetic epistasis between TIR-1 and AIA interneurons.

Chemotaxis to diacetyl was analyzed in *tir-1(gf)*, AIA-, and *tir-1(gf)* animals with no functional AIA in naïve, adaptation, and recovery phases. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (n=8, 2-way ANOVA, $F_{strain(5,126)}$=38.4268, p=8.41e^{-24}, η^2=0.6039). *Post hoc t*-test with Bonferroni's correction.

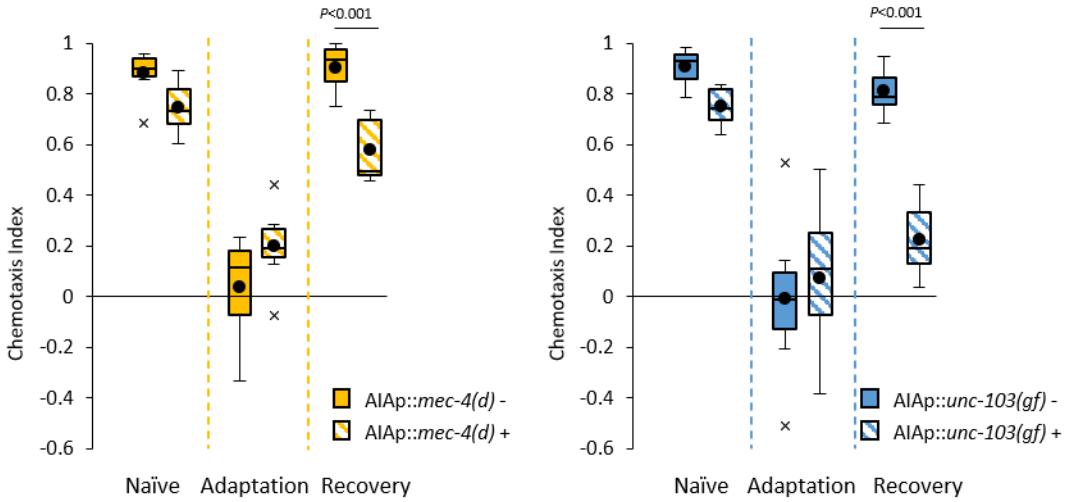

Figure 4. Chemotaxis to AWC-sensed isoamyl alcohol in AIA- animals.

Chemotaxis to isoamyl alcohol was analyzed in two AIA- transgenic animals, AIAp::*mec-4(d)* and AIAp::*unc-103 (gf)*, in naïve, adapted, and 4 hour recovery phases. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (n=8, 2-way ANOVA, $F_{strain(3,82)}$=11.4853, p=2.31e^{-6}, η^2=0.2959). *post hoc t*-test with Bonferroni's correction.

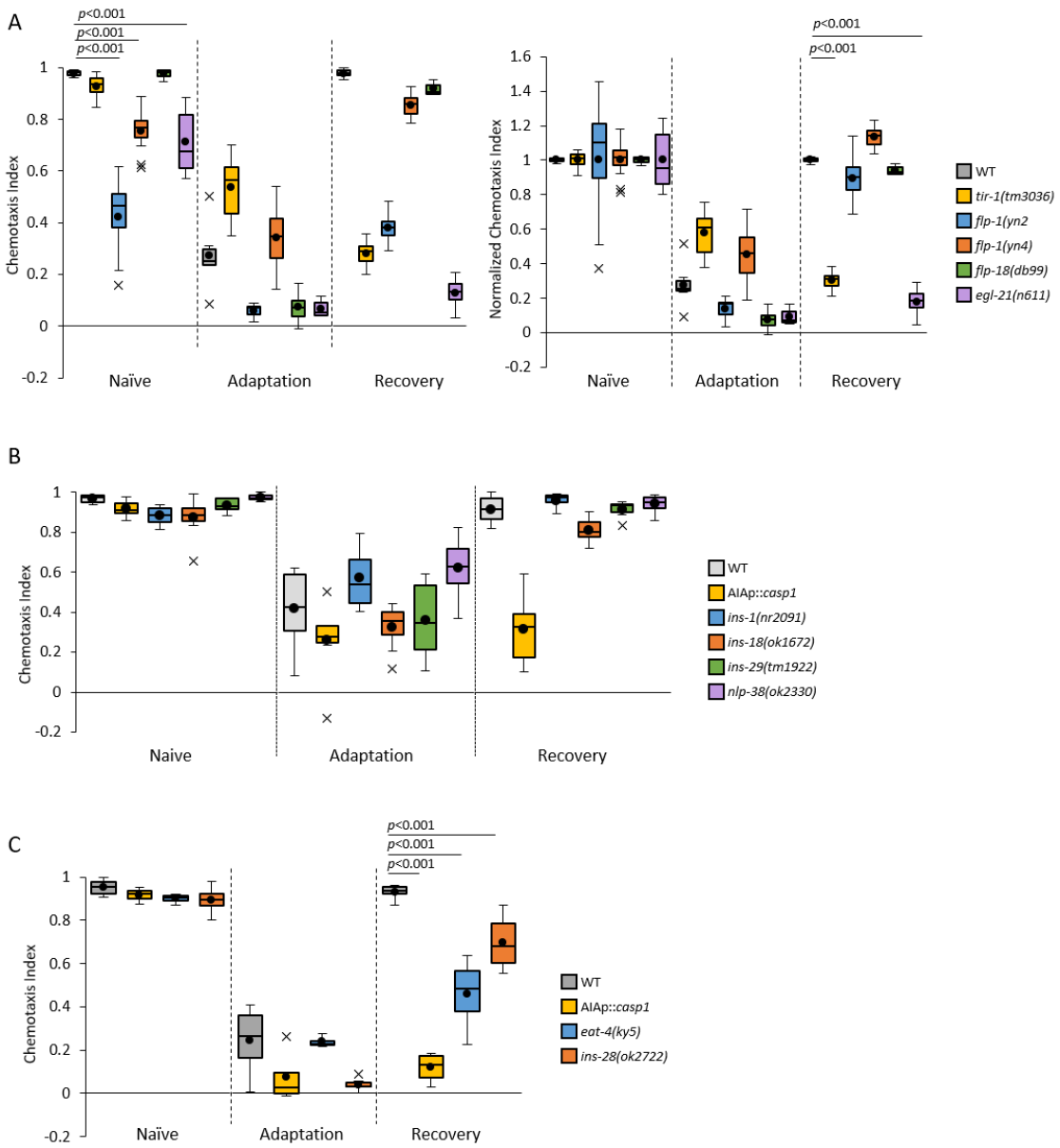

Figure 5. Chemotaxis of neuropeptides and glutamate transporter mutants.

A-C Behavioral assays of neuropeptides and glutamate transporters mutant animals toward diacetyl. Chemotaxis of naïve, adapted and 4 hour recovered animals was analyzed. A. Chemotaxis index in naïve, adaptation and recovery were normalized to naïve average C.I. (left panel). Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (**A:** left panel, n=8, 2-way ANOVA, $F_{strain(5,125)}$=140.7806, p=1.3452 e^{-49}, η^2=0.8492; right panel, n=8, 2-way ANOVA,

$F_{strain(5,125)}$=31.5061, p=1.1910e^{-20}, η^2=0.5576; **B:** n=8, 2-way ANOVA, $F_{strain(5,117)}$=28.3895, p=9.4233e^{-19}, η^2=0.5482; **C:** n=6, 2-way ANOVA, $F_{strain(3,53)}$=, p=2.154e^{-14}, η^2=0.7130). *Post hoc t*-test with Bonferroni's correction.

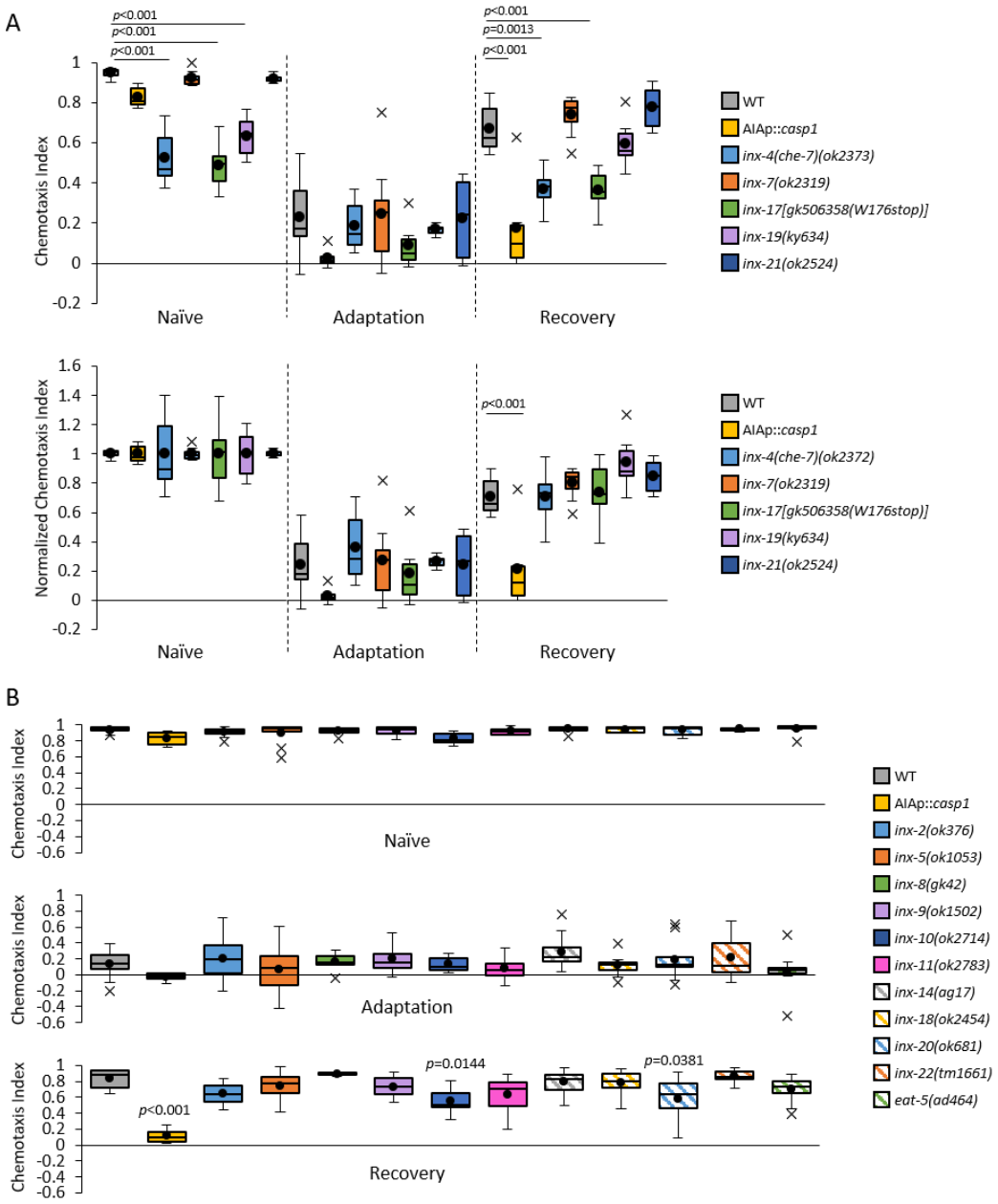

Figure 6. Behavioral assay of gap junction subunit mutant.

A-B Behavioral assays of gap junction subunit mutants. Chemotaxis of naïve, adapted and 4 hour recovered animals was analyzed. (A lower panel) Chemotaxis index was normalized to average chemotaxis index respectively. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and

Q1). (**A** upper panel: $n \geq 6$, 2-way ANOVA, $F_{strain(6,117)}=23.8445$, $p=2.8476e^{-18}$, $\eta^2=0.5501$; A lower panel: $n \geq 6$, 2-way ANOVA, $F_{strain(6,117)}=6.1808$, $p=1.1721e^{-5}$, $\eta^2=0.2407$; **B**: $n \geq 4$, 2-way ANOVA, $F_{strain(12,317)}=10.6651$, $p=8.829e^{-18}$, $\eta^2=0.2876$:). *Post hoc t*-test with Bonferroni's correction.

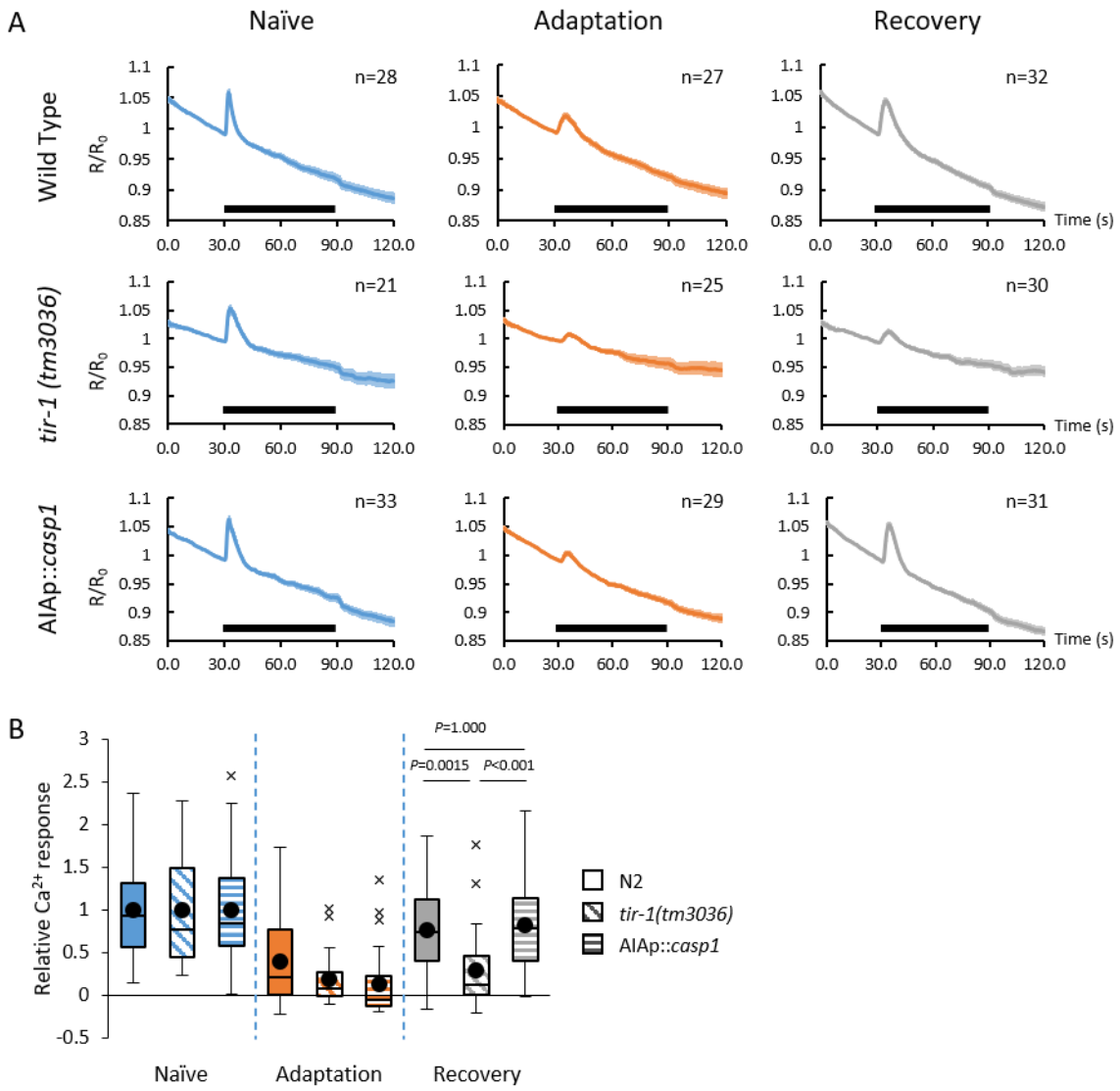

Figure 7. The Ca^{2+} responses to diacetyl of AWA neurons

A, Ca^{2+} responses of AWAs in wild-type, *tir-1(tm3036)* and AIA- animals in naïve, adaptation and recovery phases (n≥21; mean± SEM). Black line represents application of odor stimulation (1:10^{-7} dilution of diacetyl). **B,** Relative Ca^{2+} responses of AWAs in wild-type, *tir-1(tm3036)* and AIA- animals. Values are normalized to the average naïve value in respective animals. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (n≥21, 2-way ANOVA, $F_{strain(2,247)}$=3.6626, p=0.0271, η^2=0.0288). *Post hoc t*-test with Bonferroni's correction. A Error

bars represent SEM. This data was part of Teo et al. (2022) paper and conducted by Itsuki Kurokawa.

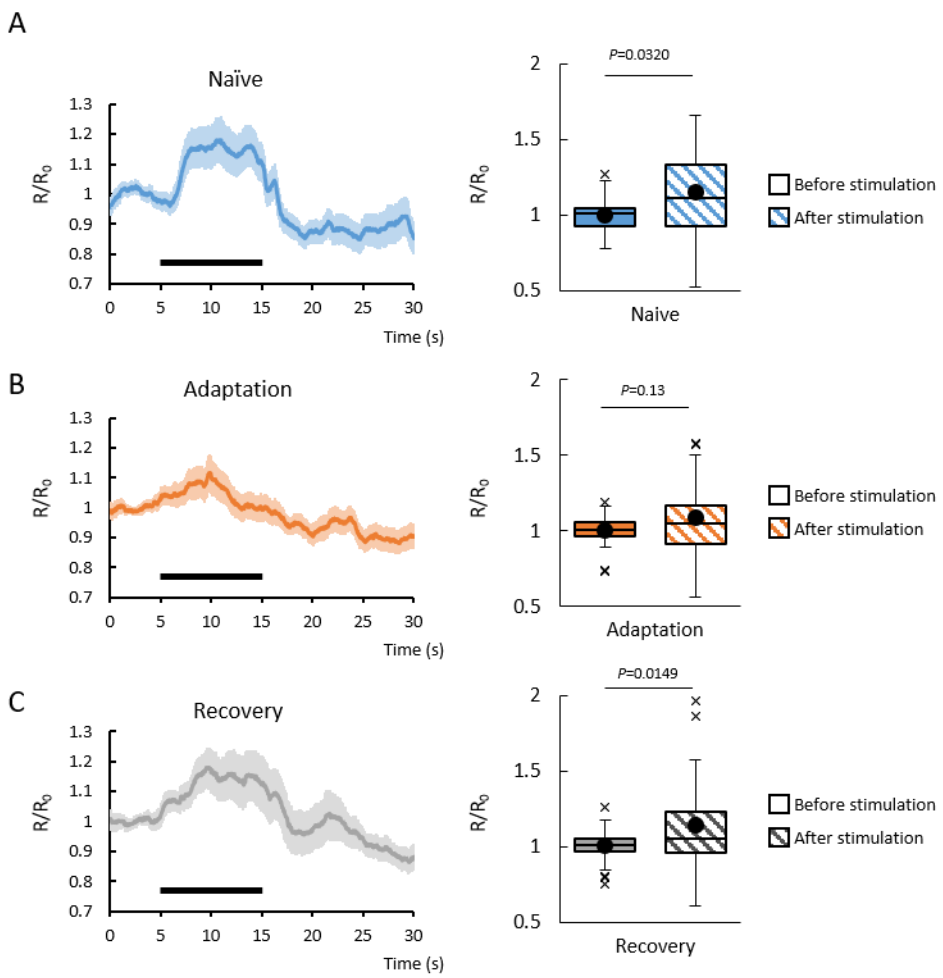

Figure 8. The Ca^{2+} responses to diacetyl of AIA interneurons

A-C, (Right Panel) Ca^{2+} responses of AIAs in wild-type animals in naïve, adaptation and recovery phases (n=36; mean± SEM). Black line represents application of odor stimulation (1:10^{-7} dilution of diacetyl). (Left Panel) Ca^{2+} responses of AIAs before (color; 2.5th s to 5th s) and after (pattern; 7.5th s to 10th s) stimulation in wild type animals in naïve, adaptation and recovery. Boxes: first to third quartiles (Q1 25th to Q3 75th percentile) of each dataset; black line in the boxes: medians; black dots: mean; whiskers: minimum and maximum, excluding outliers (beyond 1.5-fold interquartile range from Q3 and Q1). (Naive: n=36, t(35)=2.2336, p=0.032; Adaptation: t(35)=1.5505, p=0.13; Recovery: t(35)=2.5616, p=0.0149) Student's t-

test. Error bars represent SEM. This data was part of Teo et al. (2022) paper and conducted by Satoh Noriko.

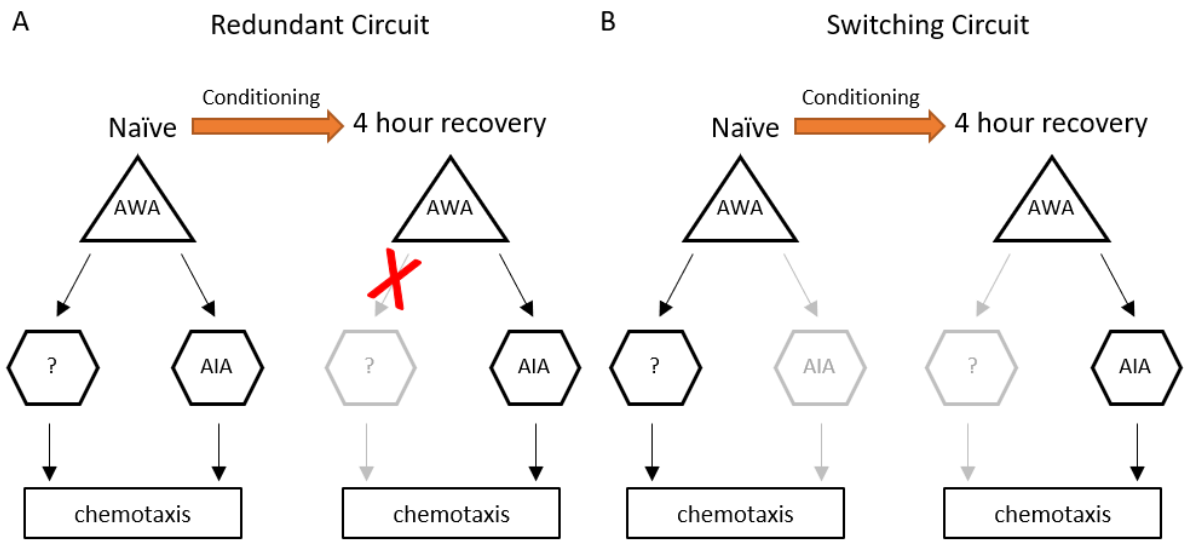

Figure 9. AIAs may regulate forgetting of diacetyl olfactory adaptation via circuit plasticity

A, B According to the AWA and AIA imaging data from Teo et al. (Figure 3 and 4, 2022), two hypothetical neural circuits of AIA-dependent behavioral plasticity in the forgetting of olfactory adaptation to diacetyl. In the two models, naïve chemotactic behavior might be regulated along with (A) or independently from (B) an AIA-dependent functional neural circuit. In both models, after conditioning, the AIA-dependent functional neural circuit is required to regulate the corresponding behavioral output after the animal recovers from adaptation.

	gene_name	gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj	cluster
1	*flp-2*	WBGene00001445	0.00E+00	5.37	0.976	0.054	0.00E+00	AIA
2	*flp-19*	WBGene00001462	0.00E+00	5.16	0.992	0.092	0.00E+00	AIA
3	*ins-28*	WBGene00002111	0.00E+00	4.68	0.96	0.026	0.00E+00	AIA
4	*ins-29*	WBGene00002112	0.00E+00	3.78	0.876	0.038	0.00E+00	AIA
5	*ins-18*	WBGene00002101	0.00E+00	3.55	0.9	0.077	0.00E+00	AIA
6	*nlp-38*	WBGene00007219	0.00E+00	3.27	0.892	0.084	0.00E+00	AIA
7	F09E5.16	WBGene00017292	0.00E+00	2.44	0.773	0.057	0.00E+00	AIA
8	*ins-25*	WBGene00002108	0.00E+00	2.3	0.661	0.026	0.00E+00	AIA
9	*pdf-2*	WBGene00018590	0.00E+00	2.18	0.705	0.024	0.00E+00	AIA
10	*ins-1*	WBGene00002084	0.00E+00	1.92	0.665	0.071	0.00E+00	AIA
11	*nlp-35*	WBGene00007881	0.00E+00	1.79	0.689	0.051	0.00E+00	AIA
12	H23L24.1	WBGene00019228	0.00E+00	1.51	0.526	0.009	0.00E+00	AIA
13	*ins-10*	WBGene00002093	0.00E+00	1.41	0.39	0.018	0.00E+00	AIA
14	*unc-39*	WBGene00006775	0.00E+00	1.01	0.418	0.005	0.00E+00	AIA
15	C08B6.4	WBGene00007425	0.00E+00	0.996	0.283	0.003	0.00E+00	AIA
16	F26A1.19	WBGene00270319	0.00E+00	0.987	0.482	0.024	0.00E+00	AIA
17	*glc-3*	WBGene00001593	0.00E+00	0.696	0.367	0.015	0.00E+00	AIA
18	C46C2.3	WBGene00008111	0.00E+00	0.615	0.267	0.006	0.00E+00	AIA
19	*nlp-57*	WBGene00008589	6.30E-219	1.94	0.653	0.092	1.17E-214	AIA
20	*egl-3*	WBGene00001172	6.74E-17	0.564	0.773	0.515	1.25E-12	AIA
21	T22F7.4	WBGene00020703	6.98E-96	0.889	0.454	0.088	1.29E-91	AIA
22	R13H7.2	WBGene00020069	7.11E-234	0.964	0.438	0.039	1.31E-229	AIA
23	*pdi-6*	WBGene00015168	7.57E-06	0.4	0.426	0.309	1.40E-01	AIA
24	B0252.8	WBGene00044615	8.53E-74	0.67	0.51	0.122	1.58E-69	AIA
25	*cho-1*	WBGene00000501	8.86E-48	1.71	0.47	0.16	1.64E-43	AIA
26	*nlp-15*	WBGene00003753	9.33E-120	1.79	0.924	0.34	1.72E-115	AIA
27	*pgal-1*	WBGene00017671	9.43E-25	0.531	0.984	0.8	1.74E-20	AIA
28	*lmd-3*	WBGene00018700	9.75E-89	0.96	0.478	0.109	1.80E-84	AIA
29	*enpl-1*	WBGene00011480	1.02E-08	0.561	0.43	0.3	1.88E-04	AIA
30	*cdr-4*	WBGene00010470	1.08E-06	0.423	0.247	0.149	1.99E-02	AIA
31	*snt-4*	WBGene00004924	1.21E-10	0.39	0.853	0.629	2.24E-06	AIA
32	*nlp-1*	WBGene00003739	1.66E-74	0.903	0.466	0.114	3.06E-70	AIA
33	K02F2.5	WBGene00019324	1.74E-44	1.08	0.988	0.8	3.22E-40	AIA
34	F41E7.7	WBGene00009623	2.09E-122	0.624	0.382	0.051	3.85E-118	AIA
35	ZK380.6	WBGene00304812	2.13E-30	1.09	0.466	0.199	3.93E-26	AIA
36	*egl-21*	WBGene00001189	2.25E-16	0.554	0.964	0.829	4.15E-12	AIA

37	F22B5.4	WBGene00009042	2.35E-22	0.464	0.351	0.141	4.34E-18	AIA
38	*hsp-16.41*	WBGene00002018	2.64E-09	0.603	0.841	0.588	4.87E-05	AIA
39	*aip-1*	WBGene00000097	2.80E-05	0.603	0.386	0.287	5.18E-01	AIA
40	*tyra-3*	WBGene00006475	2.83E-48	0.397	0.231	0.044	5.22E-44	AIA
41	*glb-33*	WBGene00022284	3.46E-165	0.799	0.323	0.03	6.39E-161	AIA
42	*nlp-41*	WBGene00007316	3.92E-60	0.799	0.418	0.104	7.24E-56	AIA
43	*pdi-2*	WBGene00003963	4.26E-12	0.52	0.602	0.403	7.87E-08	AIA
44	*mab-21*	WBGene00003112	4.28E-38	0.385	0.211	0.044	7.92E-34	AIA
45	*casy-1*	WBGene00000403	4.39E-15	0.457	0.809	0.57	8.11E-11	AIA
46	F23A7.4	WBGene00009069	4.66E-08	0.475	0.155	0.069	8.62E-04	AIA
47	*dmsr-2*	WBGene00021275	4.77E-98	0.561	0.327	0.047	8.81E-94	AIA
48	*akap-1*	WBGene00016977	5.26E-16	0.499	0.311	0.143	9.72E-12	AIA
49	*hsp-16.2*	WBGene00002016	5.27E-13	0.467	0.809	0.497	9.73E-09	AIA

Table 3: Data generated by CeNGEN.

49 genes were found to be expressed in AIA interneurons. Part of listed genes were examined.