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Forgetting in *C. elegans* Is Accelerated by Neuronal Communication via the TIR-1/JNK-1 Pathway

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SUMMARY

The control of memory retention is important for proper responses to constantly changing environments, but the regulatory mechanisms underlying forgetting have not been fully elucidated. Our genetic analyses in *C. elegans* revealed that mutants of the TIR-1/JNK-1 pathway exhibited prolonged retention of olfactory adaptation and salt chemotaxis learning. In olfactory adaptation, conditioning induces attenuation of odor-evoked Ca^{2+} responses in olfactory neurons, and this attenuation is prolonged in the TIR-1/JNK-1-pathway mutant animals. We also found that a pair of neurons in which the pathway functions is required for the acceleration of forgetting, but not for sensation or adaptation, in wild-type animals. In addition, the neurosecretion from these cells is important for the acceleration of forgetting. Therefore, we propose that these neurons accelerate forgetting through the TIR-1/JNK-1 pathway by sending signals that directly or indirectly stimulate forgetting.

INTRODUCTION

Animals acquire a tremendous quantity of information from their environment that is partially retained in their nervous systems. These memories lead to behavioral plasticity, in which experiences induce changes in behavioral responses to environmental stimuli. Most memories considered short-term memories are vulnerable to disruption and are forgotten within hours if they are not consolidated into stable long-term memories (McGaugh, 2000).

Forgetting is important for the elimination of superfluous memories and for the prevention of interference between old and new memories that might arise in a constantly changing environment (Kraemer and Golding, 1997). Despite the importance of forgetting, the regulatory mechanisms underlying this phenomenon are not fully understood. Several studies reported that the duration of memory retention can be altered by the hyperactivation or inactivation of kinases and phosphatases, suggesting that the balance between phosphorylation and dephosphorylation determines the retention or loss of memory. For example, expressing inhibitors of calcineurin and phosphatase 1A in mouse brain induces a prolonged retention of spatial memory (Malleret et al., 2001; Genoux et al., 2002), whereas the excess activity of calmodulin-dependent kinase II (CaMKII) causes the recall-induced loss of memories (Cao et al., 2008). Recently, the expression of an inhibitor or dominant-negative form of protein kinase M zeta, which is a constitutively active protein kinase C, was found to disrupt long-term memory through the regulation of postsynaptic receptor phosphorylation (Shema et al., 2007). Given that these experiments perturb or enhance specific activities of kinases or phosphatases, upstream pathways that lead to the regulation of memory retention and forgetting remain unclear. In addition, active regulation of early-memory forgetting by the small GTP-binding protein Rac has recently been observed in *Drosophila* (Shuai et al., 2010), suggesting that molecular machinery other than protein phosphorylation may participate in the regulation of memory retention. Therefore, unbiased forward genetic screens may lead to the identification of the molecular machinery involved in memory retention and forgetting.

C. elegans has a simple neuronal network, and its connections have been well described (White et al., 1986). Despite neuronal simplicity, it shows behavioral plasticities to various stimuli, including volatile (Colbert and Bargmann, 1995) or water-soluble chemicals (Saeki et al., 2001), temperature (Mori, 1999), and tapping (Rankin et al., 1990). Although a few types of behavioral plasticity in *C. elegans* persist for more than 24 hr (Rankin et al., 1990; Kauffman et al., 2011), most types of plasticity are sustained for less than a few hours and are considered short-term memories. For example, olfactory adaptation, in which animals pre-exposed to an attractive odor show weaker chemoattraction compared with those that have not been exposed (Colbert and Bargmann, 1995), lasts for a few hours. By contrast, salt chemotaxis learning, a type of associative learning in which animals conditioned with NaCl and starvation show avoidance to NaCl,

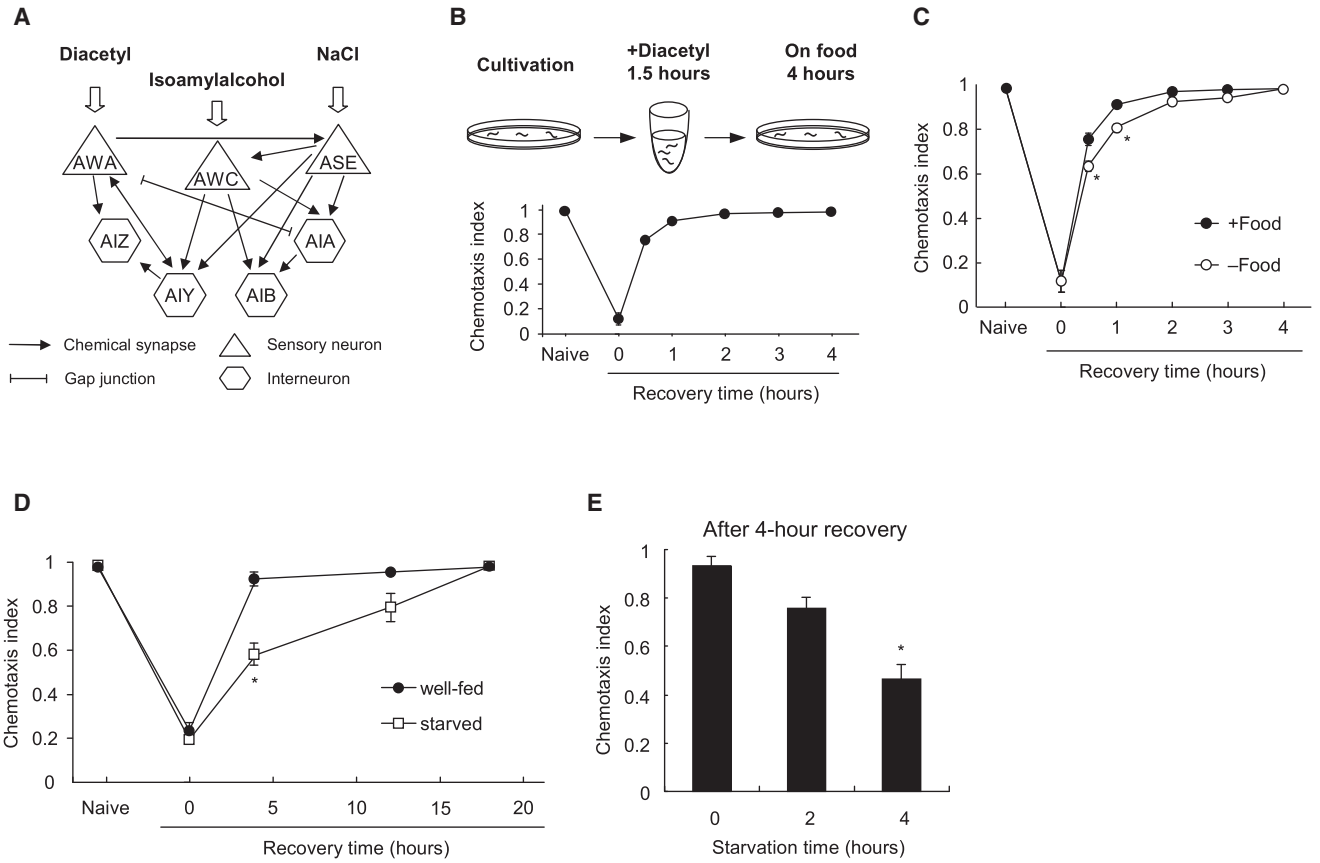

Figure 1. Forgetting of Olfactory Adaptation

(A) A neural network model for olfactory adaptation and salt chemotaxis learning (White et al., 1986). Odorant diacetyl and isoamylalcohol are sensed by AWA and AWC sensory neurons, respectively. NaCl is sensed by ASE sensory neurons.

(B) The time course of recovery from adaptation to diacetyl ($n \geq 6$). Animals conditioned with diacetyl for 1.5 hr showed adaptation. Chemotaxis to diacetyl gradually recovered on food without diacetyl.

(C) Retention curves of adaptation in the presence or absence of food during recovery ($n \geq 6$).

(D) Retention curves of adaptation to diacetyl in well-fed or 4 hr starved animals ($n \geq 6$).

(E) Chemotaxis to diacetyl after 4 hr of recovery in well-fed, 2 hr starved, and 4 hr starved animals ($n \geq 6$).

*$p < 0.01$; Student's t test (C and D) or Dunnett's test (E). Error bars represent SEM.

lasts for less than an hour (Saeki et al., 2001). Because of the simple nervous system and the availability of genetic approaches, these kinds of behavioral plasticity are suitable models to study the forgetting of short-term memories at the molecular and neuronal levels.

Here, we show that in *C. elegans* forgetting of olfactory adaptation and salt chemotaxis learning is accelerated by the TIR-1/JNK-1 pathway. In olfactory adaptation, odor-evoked Ca^{2+} responses in sensory neurons are reduced after conditioning, and the reduction is prolonged in the signaling pathway mutants, suggesting that the retention of olfactory adaptation is regulated at the level of sensory neurons. Furthermore, genetic analyses reveal that other neurons are required for the acceleration of forgetting, suggesting that forgetting processes are regulated in a cell-nonautonomous manner. Our results show that forgetting is actively regulated in the neuronal circuit and lead to insights into the study of learning and memory.

RESULTS

Retention of Olfactory Adaptation

Olfactory adaptation is a simple behavioral plasticity in which animals pre-exposed to an odorant show weaker chemoattraction to the odorant compared with naive animals. *C. elegans* shows attractive responses and adaptation to odorants sensed by two pairs of sensory neurons, AWA and AWC (Bargmann et al., 1993; Colbert and Bargmann, 1995). Olfactory adaptation in AWC is regulated by a cyclic guanosine monophosphate (cGMP)-dependent kinase, EGL-4, a cGMP-gated channel β subunit, TAX-2 (L'Etoile et al., 2002; Lee et al., 2010), and a transient receptor potential vanilloid (TRPV) channel, OSM-9 (Colbert et al., 1997). These proteins are not required for adaptation in AWA, indicating that the mechanisms of adaptation are distinct between these neurons. One of the attractive odorants, diacetyl, is mainly sensed by AWAs (Figure 1A; Sengupta et al., 1996). Animals pre-exposed to 1:5,000 diacetyl for 1.5 hr

showed much weaker responses to diacetyl than naive animals. This olfactory adaptation was fully reversible. Animals cultivated on food without diacetyl for an additional 4 hr demonstrated chemoattraction similar to naive animals (Figure 1B).

To examine whether recovery from adaptation is affected by the environment, we analyzed the retention of adaptation in the presence or absence of food. When worms were recovered in the absence of food, their chemotaxis indices were decayed more slowly than those of animals that were in the presence of food (Figure 1C). Therefore, retention of olfactory adaptation may be regulated by food signals. We also examined whether diet before conditioning affects the retention of olfactory adaptation and found that the starved animals showed prolonged retention compared with the well-fed animals (Figures 1D and 1E). These results suggested that the retention of olfactory adaptation was actively regulated by environmental and internal conditions. Therefore, retention of olfactory adaptation to diacetyl can be considered as a model to study the regulation of forgetting.

The TIR-1/JNK-1 Pathway Regulates Memory Retention for Olfactory Adaptation

To elucidate the molecular pathway for the regulation of forgetting, we devised a genetic screen for mutants that show longer retention of olfactory adaptation to diacetyl than wild-type animals by using the Mos1 transposon as a mutagen (Boulin and Bessereau, 2007). With this screen, we isolated the *tir-1(qj56)* mutant (see below). In wild-type animals, adaptation to diacetyl continued for less than 4 hr. In *tir-1(qj56)*, after conditioning with diacetyl, adaptation persisted for more than a day (Figure 2A), although *tir-1* mutant animals do not exhibit defects in the sensory response or in adaptation to diacetyl (Figure 2A). To examine the possibility that this prolonged retention is caused by stronger adaptation than in wild-type animals, we analyzed the retention curve after weaker conditioning. *tir-1* mutant animals conditioned with a lower concentration of diacetyl showed weaker adaptation compared to the wild-type animals conditioned with a higher concentration of diacetyl. Nonetheless, the mutants still exhibited prolonged retention (Figure S1A), suggesting that the longer retention in *tir-1* animals was not due to stronger adaptation. Taken together, *tir-1* mutant animals are not defective in chemosensation or adaptation to diacetyl but are defective in forgetting the adaptation.

Positional cloning by SNP mapping revealed that *qj56* is a deletion mutation in the *tir-1* gene (Figure 2B). In addition, other *tir-1* loss-of-function alleles exhibited prolonged retention of olfactory adaptation, just like *qj56* (Figure 2C). On the other hand, a gain-of-function mutant *tir-1(ok1052 gf)* (Chuang and Bargmann, 2005) revealed a weak adaptation phenotype (Figure 2C). This implies that excess activation of TIR-1 prevents the establishment of olfactory adaptation.

tir-1 encodes a highly conserved Toll/interleukin-1 resistance domain protein that is homologous to the mammalian adaptor protein SARM (sterile alpha- and Armadillo-motif-containing protein; Couillault et al., 2004). SARM is expressed mainly in the brain, although its function has not been fully determined (Kenny and O'Neill, 2008). In *C. elegans*, TIR-1 is known to regulate neuronal differentiation (Chuang and Bargmann, 2005) and the innate immune response (Shivers et al., 2009) via the p38 MAPK pathway (Figure S1B). In late embryogenesis, TIR-1 regulates asymmetric differentiation of a pair of AWC sensory neurons (AWC^{on} and AWC^{off}), in conjunction with UNC-43 (CaMKII), NSY-1 (MAPKKK), SEK-1 (MAPKK), and an unidentified MAPK (Chuang and Bargmann, 2005), whereas, in the innate immune response, TIR-1 functions in conjunction with NSY-1, SEK-1, and PMK-1 (MAPK; Shivers et al., 2009).

To determine whether the forgetting process is regulated by this pathway, we analyzed retention of adaptation in *unc-43*, *nsy-1*, *sek-1*, and *pmk-1* mutants after 4 hr recovery. All mutants exhibited normal chemotaxis and adaptation to diacetyl, whereas *unc-43*, *nsy-1*, and *sek-1*, but not *pmk-1*, exhibited prolonged retention of the olfactory adaptation, as seen in *tir-1(lf)* (Figure 2D). To confirm that TIR-1 functions upstream of NSY-1, we analyzed the phenotype of the *nsy-1; tir-1(ok1052 gf)* double mutant (Figure 2E). The weak adaptation phenotype of *tir-1(ok1052 gf)* was suppressed in *nsy-1; tir-1(ok1052 gf)*, which showed a normal attractive response and adaptation to diacetyl, suggesting that, in *tir-1(ok1052 gf)*, the downstream pathway is hyperactivated and, therefore, the adaptation is not as highly induced as in the wild-type. In addition, after conditioning, the double mutant showed the prolonged retention of olfactory adaptation, much like the *nsy-1* mutant. Therefore, NSY-1 may function downstream of TIR-1 in forgetting. Since SEK-1 can phosphorylate and activate JNK-1, as well as PMK-1 (Tanaka-Hino et al., 2002), we analyzed the *jnk-1* mutant and found that *jnk-1* displayed prolonged retention of adaptation (Figure 2D). Therefore, the forgetting process may be regulated by the TIR-1/JNK-1 pathway, which is distinct from the pathways that determine neuronal cell fate or those that regulate the innate immune response (Figure S1B).

To determine when the signaling pathway regulates the retention of olfactory adaptation, we analyzed the effect of stage-specific expression of wild-type TIR-1 in *tir-1(lf)* animals. The expression of wild-type TIR-1 in the adult stage restored the phenotype, although expression at the larval stage did not (Figure 2F), suggesting that the signaling pathway regulates the retention of the olfactory adaptation in the mature nervous system.

The TIR-1/JNK-1 Pathway Regulates the Sensory Ca^{2+} Response

To determine whether behavioral plasticity is caused by a change in the sensory response of AWA neurons, we analyzed the diacetyl-evoked Ca^{2+} response in AWAs of animals expressing a Ca^{2+} indicator, YC3.60 (Nagai et al., 2004). These animals showed normal chemotaxis, adaptation, and recovery to diacetyl (Figure S2A). In naive wild-type animals, diacetyl stimulus induced a Ca^{2+} increase in AWA. In contrast, after pre-exposure to diacetyl for 1.5 hr, these Ca^{2+} responses were diminished. In wild-type animals, after 4 hr cultivation on food, the odor-evoked increase in Ca^{2+} was recovered (50.0% ± 8.5% of naive animals; Figures 3A, 3B, and S2B). This change in sensory response is consistent with the change in behavior. Therefore, the change in behavioral response to diacetyl observed after conditioning and recovery may be due to an altered sensory response to diacetyl in AWA. We then examined the sensory response in

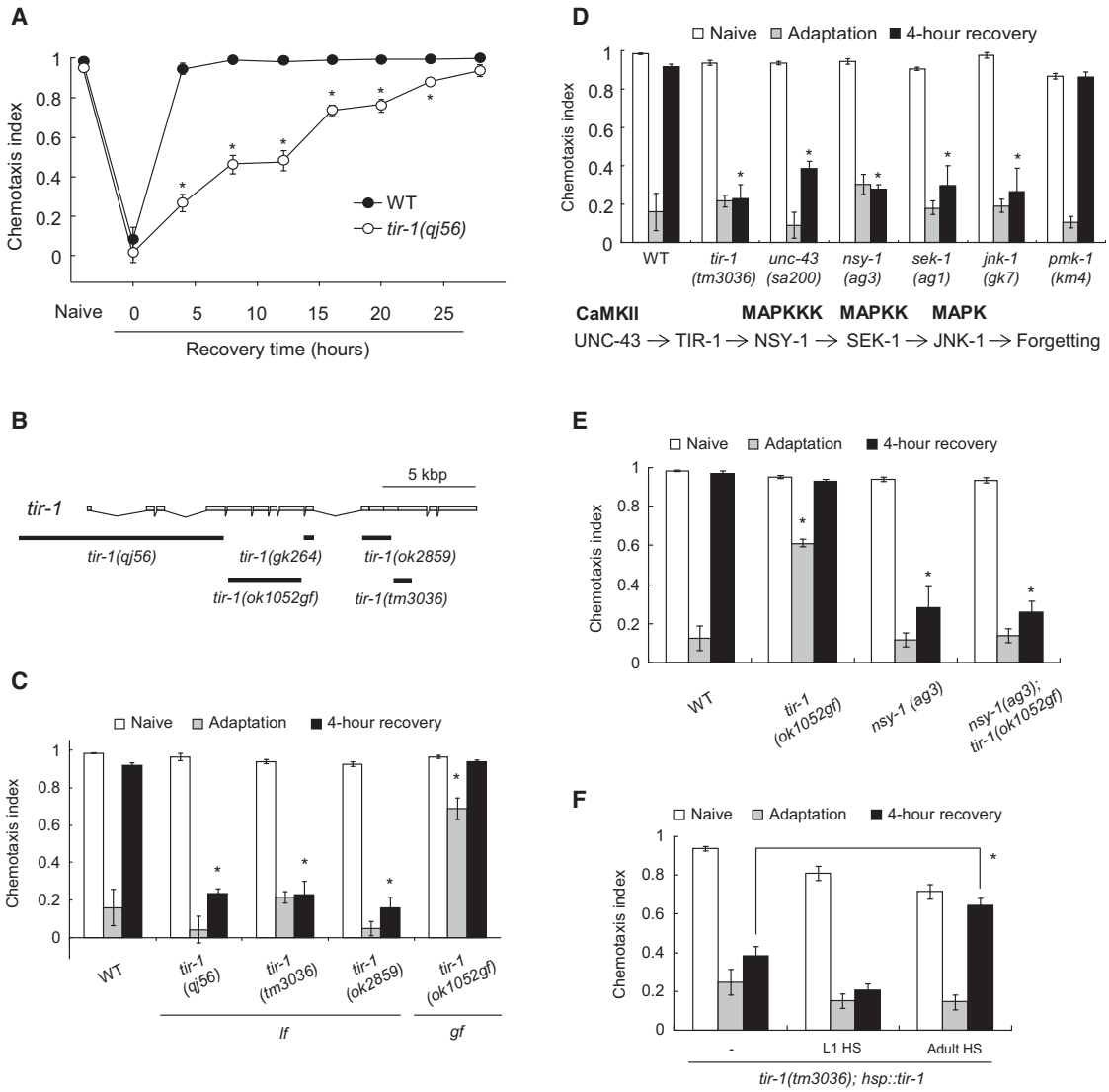

Figure 2. Forgetting of Adaptation to Diacetyl Is Regulated through a TIR-1/JNK-1 Pathway

(A) Retention curves of adaptation to diacetyl in wild-type and in *tir-1(qj56)*, which was isolated by our genetic screen ($n \geq 5$).

(B) Schematic depiction of the *tir-1* gene and of lesions in *tir-1* mutants. Deleted regions are indicated by solid bars. The *tir-1(qj56)* mutation was an approximately 13 kb deletion that included the start codon.

(C) Retention of the adaptation to diacetyl in *tir-1* mutant animals. Chemotaxis of naive, adapted, and 4 hr recovered animals was analyzed ($n \geq 4$).

(D) Retention of the adaptation to diacetyl of the CaMKII and TIR-1/JNK-1 pathway mutants ($n \geq 4$). A signaling pathway model for forgetting of adaptation is shown below.

(E) Genetic interaction between *tir-1(ok1052 gf)* and *nsy-1(ag3)* ($n \geq 6$).

(F) Heat-shock rescue experiment in *tir-1(tm3036)* carrying *hsp16.2::tir-1*. Animals were heat-shocked at larval (L1) or adult stages ($n \geq 6$).

*$p < 0.01$; Student's t test (A) or Dunnett's test (C–F). Error bars represent SEM. See also Figure S1.

tir-1 and *jnk-1* mutants. In naive mutant animals, the Ca^{2+} increase in response to diacetyl was weakened after exposure to diacetyl for 1.5 hr, as in wild-type animals. However, after 4 hr cultivation on food, Ca^{2+} responses to diacetyl in both mutant animals did not recover to the same extent as in wild-type animals (19.8% ± 4.7% of naive *tir-1* animals and 18.7% ± 7.0% of naive *jnk-1* animals, respectively; Figures 3A, 3B, and S2B). This phenotype can be rescued by the expression of the wild-type gene products (Figures 3C and S2A). These results suggest that prolonged retention of olfactory adaptation in the mutants is mainly due to decreased recovery of the sensory response to diacetyl following conditioning.

The TIR-1/JNK-1 Pathway Regulates Forgetting in Sensory Neurons Not Involved in Chemosensation

To identify the neuron(s) responsible for the regulation of forgetting, we performed neuron-specific rescue experiments. First, we confirmed that expression of wild-type TIR-1 by a

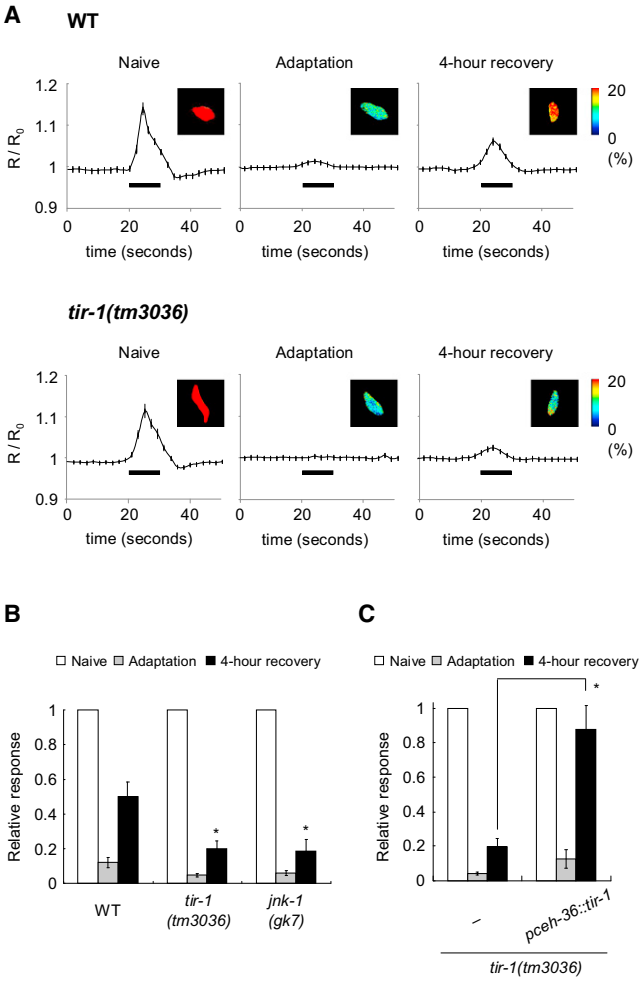

Figure 3. Ca^{2+} Responses of AWA Sensory Neurons after Adaptation and Recovery

(A) Averaged traces of Ca^{2+} response in AWAs in naive, diacetyl-adapted, and 4 hr recovered animals ($n \geq 20$). Timing of the diacetyl stimulation is shown as black bars. R represents the YFP/CFP ratio, and R_0 represents the averaged ratio of 16 s before diacetyl stimulation. Insets show pseudocolor images of AWAs after odor stimulation.

(B) Quantification of Ca^{2+} responses of wild-type, *tir-1(tm3036)*, and *jnk-1(gk7)* animals ($n \geq 8$). Data were normalized to Ca^{2+} responses in the naive animals (see also Figure S2B).

(C) Transgenic rescue of *tir-1(tm3036)* animals expressing wild-type gene products in AWCs ($n \geq 16$).

*$p < 0.01$; Dunnett's test (B) or Student's t test (C). Error bars represent SEM. See also Figure S2.

pan-neuronal promoter (Shinkai et al., 2011) can rescue the prolonged retention phenotype (Figure 4A). Although adaptation to diacetyl is due to change in the sensory response in AWA, the expression of wild-type TIR-1 in AWA by the *odr-10* promoter (Sengupta et al., 1996) could not rescue the phenotype of *tir-1(lf)* (Figure 4A). To identify the neurons that regulate the retention, we analyzed *tir-1(lf)* expressing wild-type TIR-1 by various promoters. We found that expression in AWC sensory neurons by the *odr-3* promoter (Roayaie et al., 1998) or the AWC-specific *ceh-36* promoter (Etchberger et al., 2007) can rescue the prolonged retention phenotype (Figures 4A and S3A). In addition, the expression of SEK-1 in AWC can rescue the phenotype of the *sek-1* mutant (Figure 4A). We also ascertained that the *jnk-1* promoter drives expression in AWC (Figure S3B), to be consistent with our model, whereas other genes in this pathway were reported to be expressed in AWC (Chuang and Bargmann, 2005).

To confirm that the TIR-1/JNK-1 pathway regulates the retention of olfactory adaptation in AWCs, we examined whether the AWC-specific expression of the dominant-negative form of SEK-1 or JNK-1 caused the prolonged retention. Expression in AWCs of SEK-1(DN), in which the two phosphorylation sites are substituted to Ala (SATA), caused the prolonged retention of olfactory adaptation to diacetyl (Figure S3C). To examine whether the JNK-1 activity is important for the forgetting of adaptation to diacetyl, we used a dominant-negative form of JNK-1(K148R), in which the Lys required for kinase activity is substituted (Li et al., 1996, Weber et al., 2000, Bae and Song, 2003). The expression of the JNK-1(DN) in AWCs caused the prolonged retention (Figure S3C), suggesting that JNK activity in AWCs is important for the acceleration of forgetting. Therefore, the TIR-1/JNK-1 pathway regulates forgetting of adaptation to diacetyl in AWC sensory neurons.

tir-1 and *sek-1* mutants exhibited a defect in the asymmetric differentiation of AWC sensory neurons ($2AWC^{on}$ phenotype). Our result raised the possibility that AWC^{off} neurons are important for the proper retention of olfactory adaptation to diacetyl. To examine this, we analyzed the retention of olfactory adaptation in animals with $2AWC^{on}$ or $2AWC^{off}$ (Bauer Huang et al., 2007) and found that either AWC^{on} or AWC^{off} was sufficient for the acceleration of forgetting (Figure S3D). We also analyzed *tir-1(gk264)* animals, because they show normal differentiation of AWCs ($1AWC^{on}$ $1AWC^{off}$; Chuang and Bargmann, 2005). *tir-1(gk264)* exhibited the same prolonged retention phenotype as *tir-1(tm3036)* (Figure 4B). Therefore, the abnormal asymmetric differentiation of AWCs may not cause the acceleration of forgetting.

Sensory Neurons Regulate Forgetting by Neuronal Secretion

To confirm the role of AWC sensory neurons in forgetting, we analyzed the phenotype of the *ceh-36* mutants, which lack functional AWCs owing to abnormal specification (Lanjuin et al., 2003). Although the *ceh-36* mutants showed normal chemotaxis and adaptation to diacetyl, after conditioning they exhibited the prolonged retention of adaptation to diacetyl, as seen in *tir-1(lf)* (Figure 4C). Therefore, AWCs are not involved in chemosensation or adaptation in these conditions but are involved in accelerated forgetting. To examine how AWCs regulate forgetting, we analyzed animals with hyperpolarized AWC neurons by expressing constitutively active UNC-103 K^+ channels (UNC-103[gf]; Gruninger et al., 2008; Shinkai et al., 2011) (Figure 4D). These animals exhibited normal chemosensation and adaptation to diacetyl but prolonged retention of adaptation, suggesting that inhibition of AWC neuronal activities causes longer retention. Taken together, we propose that AWC sensory neurons are not required for the acquisition and

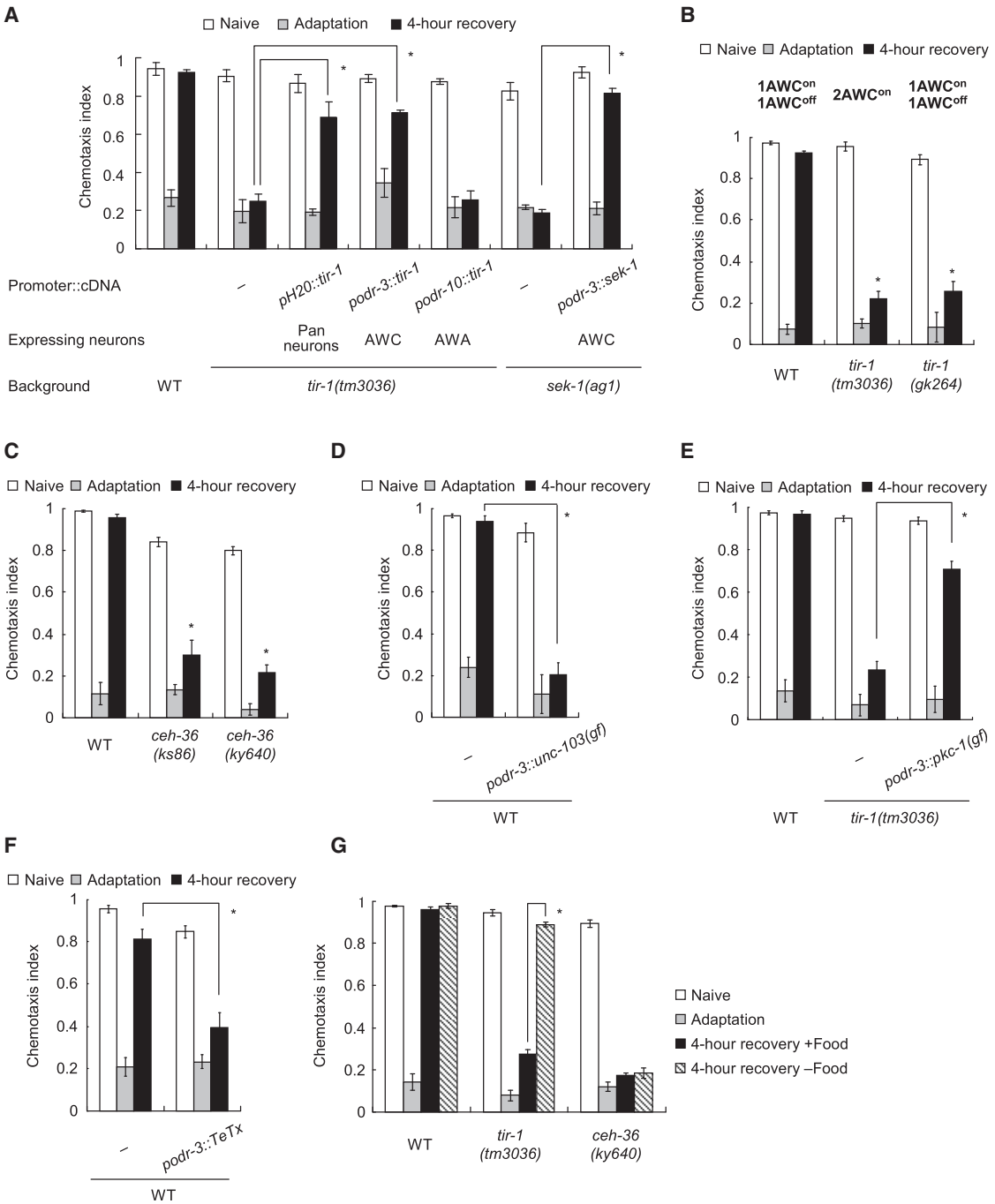

Figure 4. AWC Secretion Accelerates Forgetting through the TIR-1/JNK-1 Pathway

(A) Transgenic rescue of *tir-1(tm3036)* or *sek-1(ag1)* animals by expressing wild-type gene products under various promoters ($n \geq 4$).

(B) Wild-type, *tir-1(tm3036)*, and *tir-1(gk264)* animals were tested for the retention of adaptation to diacetyl ($n \geq 6$). *tir-1(gk264)* does not show a defect in AWC asymmetric differentiation.

(C) Retention of adaptation to diacetyl in *ceh-36* mutants defective in differentiation of AWCs ($n \geq 6$).

(D) Effect of the inhibition of AWC neuronal activity by expressing UNC-103(gf), which inhibit the neuronal activity ($n \geq 4$).

(E) Effect of PKC-1(gf) expression, which activates neural secretion. PKC-1(gf) was expressed in AWC sensory neurons of *tir-1(tm3036)* animals ($n \geq 5$).

(F) Effect of inhibiting secretion from AWC sensory neurons by expressing TeTx in wild-type animals ($n \geq 6$).

(G) Retention of adaptation to diacetyl in the presence or absence of food during recovery in wild-type, *tir-1(tm3036)*, and *ceh-36(ky640)* animals ($n \geq 6$).

*$p < 0.01$; Dunnett's test (A–C) or Student's t test (D–G). Error bars represent SEM. See also Figure S3.

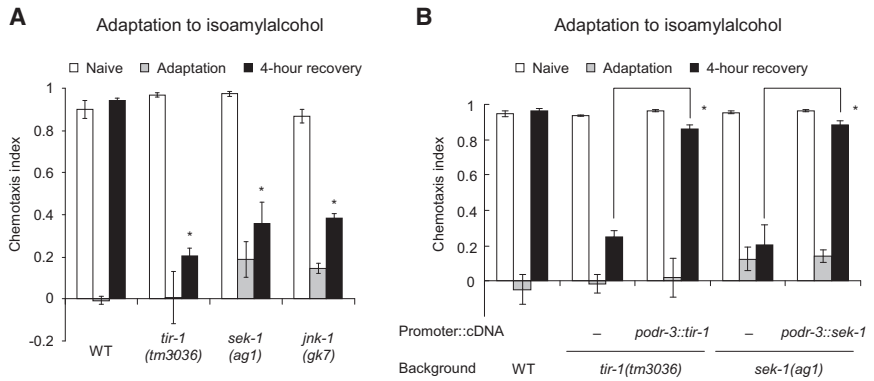

Figure 5. The TIR-1/JNK-1 Pathway in AWC Sensory Neurons Accelerates Forgetting of Adaptation to Isoamylalcohol

(A) Retention of olfactory adaptation to isoamylalcohol in wild-type, *tir-1(tm3036)*, *sek-1(ag1)*, and *jnk-1(gk7)* animals ($n \geq 4$).

(B) Transgenic rescue of *tir-1(tm3036)* and *sek-1(ag1)* mutants expressing wild-type gene products in AWCs ($n \geq 4$).

*$p < 0.01$; Dunnett's test (A) or Student's t test (B). Error bars represent SEM. See also Figure S4.

retention of the olfactory adaptation to diacetyl but only regulate the forgetting process.

We then examined whether synaptic transmission and/or neurosecretion by AWCs is involved in the acceleration of forgetting by using PKC-1(gf), which can be used as a tool for the enhancement of neuropeptide secretion (Sieburth et al., 2007). *tir-1* and *sek-1* mutant animals expressing PKC-1(gf) in AWCs showed chemotaxis and adaptation to diacetyl as in wild-type, *tir-1*, and *sek-1* mutant animals. However, they did not show the prolonged retention of olfactory adaptation (Figures 4E and S3E), raising the possibility that this pathway regulates the neurosecretion. To test this, we further examined whether the impairment of the neurosecretion affect the retention by using the expression of the Tetanus Toxin light chain (TeTx), which inhibits synaptic transmission (Schiavo et al., 1992) and possibly nonsynaptic release of diffusible neurotransmitters (Whim et al., 1997). Wild-type animals expressing TeTx in AWCs showed prolonged retention of olfactory adaptation (Figure 4F). Taken together, we proposed that AWC sensory neurons send signals for forgetting through neurosecretion and/or synaptic transmission.

To examine whether environmental signals sensed in AWCs affect the retention of olfactory adaptation, we analyzed the phenotype of *tax-4*. TAX-4 is a cyclic nucleotide-gated cation channel essential for chemosensation in AWCs (Coburn and Bargmann, 1996). Retention in *tax-4* is not distinguishable from that in wild-type animals (Figure S3F), suggesting that AWCs regulate forgetting independent of their sensory response. We also examined whether the food signals affect the prolonged retention in *tir-1(lf)*. When animals were recovered without food after adaptation, *tir-1* animals showed the shorter retention of olfactory adaptation, similar to wild-type animals (Figure 4G). In contrast, *ceh-36* mutant animals, in which the functional AWC neurons are not differentiated, show the prolonged retention even after the recovery without food (Figure 4G). Therefore, AWC neurons can accelerate forgetting of olfactory adaptation when animals are recovered on food.

The TIR-1/JNK-1 Pathway Regulates the Retention of Olfactory Adaptation to AWC-Sensed Odorant

To determine whether the TIR-1/JNK-1 pathway regulates forgetting of behavioral plasticities other than olfactory adaptation to diacetyl, we analyzed the retention of other olfactory adaptations. In *C. elegans*, isoamylalcohol is mainly sensed by AWC sensory neurons (Figure 1A; Bargmann et al., 1993), in which the regulatory mechanisms underlying the olfactory adaptation are distinct from those in AWA (L'Etoile et al., 2002; Palmitessa et al., 2005; Lee et al., 2010). Naive *tir-1*, *sek-1*, and *jnk-1* mutants exhibited a similar attractive response to isoamylalcohol to that in wild-type animals. After conditioning, however, they exhibited a prolonged retention of adaptation (Figure 5A). This phenotype was rescued by the expression of wild-type complementary DNA (cDNA) in AWCs (Figure 5B), suggesting that forgetting of olfactory adaptation to isoamylalcohol is also mediated through the TIR-1/JNK-1 pathway in AWCs. We also found that PKC-1(gf) in AWCs could rescue the phenotype (Figure S4). This result suggested that, similar to forgetting of adaptation to diacetyl, the neurosecretion and/or synaptic transmission is involved in the forgetting through autocrine or endocrine pathway, although the expression of PKC-1(gf) may cause other effects, because those animals showed a weak adaptation phenotype to isoamylalcohol.

The TIR-1/JNK-1 Pathway Regulates Forgetting of Associative Learning

To examine whether the signaling pathway also regulates forgetting in associative learning, we analyzed the retention curve of salt chemotaxis learning in *tir-1(lf)*. In this paradigm, by conditioning with NaCl and starvation, worms change their response to NaCl from attractive to repulsive (Saeki et al., 2001). In wild-type animals, the memory of salt chemotaxis learning was retained for less than 30 min, but, in *tir-1(lf)*, the memory was prolonged to about an hour (Figure 6A). Furthermore, *unc-43*, *nsy-1*, and *sek-1* mutants also exhibited prolonged retention of the memory, although *jnk-1* mutants had a weak phenotype (Figure 6B). To examine where the signaling pathway regulates this forgetting, we analyzed mutant animals expressing wild-type *tir-1* cDNA using various promoters. The expression of TIR-1 in ASE, sensory neurons for NaCl (Figure 1A; Bargmann and Mori, 1997), and in AWCs could not rescue the phenotype (Figure 6C). The expression in a subset of neurons by the *zig-5* promoter (Yamada et al., 2010) is sufficient for the rescue (Figure 6C), but expression in each of the neurons is not (data not shown). These results suggest that the TIR-1/JNK-1 pathway regulates different types of behavioral plasticity in different sets of neurons. We also noticed that these neurons are different

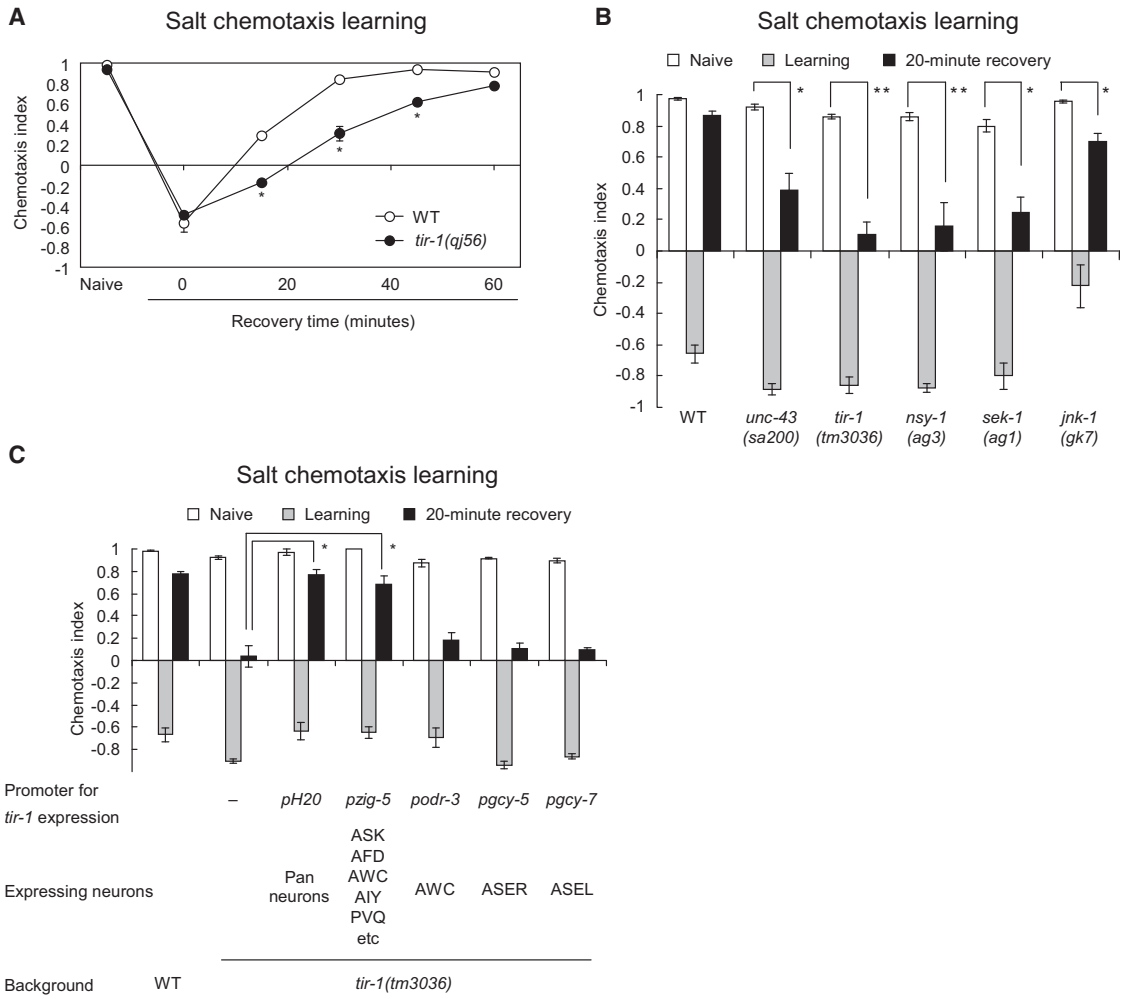

Figure 6. The TIR-1/JNK-1 Pathway Accelerates Forgetting of Associative Learning

(A) Retention curves of salt chemotaxis learning in wild-type and *tir-1(qj56)* animals ($n \geq 4$).

(B) Retention of salt chemotaxis learning in CaMKII and TIR-1/JNK-1 pathway mutants. Chemotaxis of naive, learned, and 20 min recovered animals were analyzed ($n \geq 4$).

(C) Transgenic rescue of *tir-1(tm3036)* animals by expressing wild-type gene products under various promoters ($n \geq 4$).

*$p < 0.01$, **$p < 0.001$; Student's t test (A and B) or Dunnett's test (C). Error bars represent SEM.

from neurons involved in memory acquisition and retention of salt chemotaxis learning (Tomioka et al., 2006), implying that forgetting of salt chemotaxis learning is also regulated by neuronal communication.

DISCUSSION

Forgetting is an important stage of behavioral plasticity, although there is little evidence that it is actively regulated at the molecular and neuronal levels (Shuai et al., 2010). By using an unbiased genetic screen, in *C. elegans*, we identified the TIR-1/JNK-1 pathway as the molecular machinery mediating the forgetting of a simple behavioral plasticity involved in olfactory adaptation, and in a type of associative learning, salt chemotaxis learning. Genetic and imaging studies revealed that forgetting of olfactory adaptation occurred in primary sensory neurons and retention is regulated by other neurons, indicating that forgetting is actively regulated in neuronal circuits.

AWC Sensory Neurons Secrete Signals that Accelerate Forgetting in AWA Sensory Neurons

Olfactory adaptation in AWC sensory neurons is regulated at the early stages of chemosensation, because exposure to odor causes loss of Ca^{2+} response to odor removal in AWCs (Chalasani et al., 2010). In the present study, we revealed that olfactory adaptation and its retention in AWA sensory neurons are also regulated at the early stages of chemosensation. In wild-type animals, the Ca^{2+} response to diacetyl was diminished after conditioning and recovered after removal of diacetyl. By contrast, in *tir-1* and *jnk-1* mutants, the recovery of the Ca^{2+} response was weaker than that in wild-type. This result is consistent with their behavioral phenotype of prolonged adaptation.

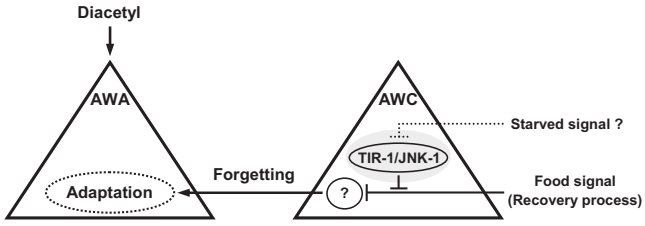

Figure 7. A Model for Regulation of Forgetting of Olfactory Adaptation to Diacetyl

AWA sensory neurons are important for perception of an attractive odorant such as diacetyl. Ca^{2+} response to diacetyl in AWAs is weakened after conditioning with diacetyl and is recovered after cultivation on food, suggesting that AWAs are also responsible for the adaptation. Since in *tir-1(lf)* and starved wild-type animals, the prolonged retention phenotype was only observed when they were recovered with food, we hypothesized that starved signals might inhibit the TIR-1/JNK-1 pathway, which accelerates forgetting. Although, *tir-1(lf)* animals do not show the prolonged retention phenotype when they were recovered without food, *ceh-36(lf)* animals, which do not have functional AWCs, showed the prolonged retention phenotype even without food. Thus, AWCs may regulate the acceleration of forgetting of olfactory adaptation by secreting signals to AWAs, which is inhibited by food signals during recovery. In addition, this inhibition may be also suppressed by TIR-1/JNK-1 signal. In this model, food signals may actively regulate the acceleration of forgetting.

We also noticed that in wild-type animals the Ca^{2+} response to diacetyl in AWA was not fully recovered to naive levels within 4 hr of cultivation on food, although the behavioral response to diacetyl was fully recovered. Therefore, other mechanisms may also participate in olfactory adaptation and in its retention.

Our results demonstrate that the TIR-1/JNK-1 pathway in AWC sensory neurons accelerates forgetting of adaptation in AWA sensory neurons. We propose that AWCs secrete signals for the acceleration of forgetting based on several reasons. First, the prolonged retention of olfactory adaptation in the pathway mutants was rescued by the expression of the wild-type gene products in AWCs and was observed in wild-type animals expressing the dominant-negative form of SEK-1 or JNK-1 in AWCs. Second, lack of or inactivation of AWC caused prolonged retention of olfactory adaptation to diacetyl, although these neurons did not affect chemosensation or adaptation to diacetyl. This finding also suggests that AWC neurons solely regulate forgetting. Third, imaging of the Ca^{2+} response of AWAs to diacetyl revealed that the decreased recovery of the sensory response in the mutant animals was rescued by expressing the wild-type gene products in AWCs. Fourth, the prolonged retention phenotype was also rescued by hyperactivated secretion from AWCs by expressing PKC-1(gf), whereas impairment of the secretion in AWCs caused the prolonged retention. We propose a model for the regulation of forgetting of olfactory adaptation to diacetyl (Figure 7).

AWC sensory neurons are key neurons for sensory perception in *C. elegans*. They sense attractive odorants as well as temperature (Bargmann et al., 1993; Kuhara et al., 2008). In addition, they are also important for adaptive responses, including food-induced physiological and behavioral changes. AWC neurons, in response to food deprivation, negatively regulate sex muscle excitability through insulin signaling (Gruninger et al., 2008) and also regulate local search behavior by secreting neuropeptide NLP-1 signals (Chalasani et al., 2010). We found that a prolonged retention phenotype in *tir-1* was not observed when the *tir-1* animals were recovered without food, although *ceh-36* animals showed the phenotype even without food. Therefore, the forgetting signals from AWCs may be inhibited by the food signals. Our results suggest that sensory perception in AWCs is not important for forgetting of olfactory adaptation, and, hence, AWC neurons are activated by other neurons such as aminergic neurons that are activated by food sensing or starvation (Chase and Koelle, 2007), or by other tissues such as intestine to detect nutritional conditions. For animals, food signals are important information to survive, and hence the food signals might accelerate to diminish behavioral change dependent on experience. Since our genetic studies suggested that the TIR-1/JNK-1 pathway positively regulates the secretion of forgetting signal from AWCs, one possibility is that the pathway negatively regulates the inhibition of forgetting signals by food signals. In addition, we predict that, in *tir-1(lf)*, the core process for forgetting still works in AWAs, because, even in the presence of food, *tir-1(lf)* eventually recovered from adaptation over an extended time (Figure 7).

Our genetic analyses suggested that the neurosecretion and/or synaptic transmission are important for the proper regulation of forgetting. Since AWCs do not make direct synapses to AWAs (White et al., 1986), neurosecretion may regulate the proper forgetting, although the synaptic transmission from AWCs can indirectly regulate AWAs. Identification of signals and their receptors will reveal the regulation of AWAs by AWCs.

Secreted factors are also important for the regulation of neuronal plasticity in mammals. Brain-derived neurotrophic factor is required not only for the induction of long-term potentiation (LTP) in the hippocampal CA1 region (Minichiello, 2009), but also for memory storage (Bekinschtein et al., 2007). In contrast, activin is important for the persistence of LTP (Ageta et al., 2010), suggesting that these signals enhance the maintenance of memories. Although absence of these signals after conditioning causes a deficit in the maintenance of memory storage without affecting memory formation, signals that weaken memories have not been identified (Minichiello, 2009). These kinds of signals as well as signals that induce retention of memories may be important to regulate the memory retention depending on environmental stimuli even in higher organisms.

Our results demonstrate that the TIR-1/JNK-1 pathway is involved in the secretion of signals for the acceleration of forgetting in *C. elegans*. The acceleration of forgetting is regulated by a partly different pathway from that for neural differentiation and the immune response. JNK-1 and PMK-1, both of which are the target of SEK-1, are expressed in AWC sensory neurons (Figure S3B), although only *jnk-1* mutant animals are defective in forgetting. Our genetic analyses revealed that TIR-1/JNK-1 signaling acts in the mature nervous system for the regulation of forgetting by activating the neuronal secretion. Thus, in the forgetting of adaptation, JNK-1 may selectively activate the neuronal secretion, which is distinct from PMK-1 signaling. This difference may be due to the distinct substrate-effector specificities of JNK-1 and PMK-1 and/or different subcellular localization of substrate effecters or kinases. Since we did not

succeed in direct detection of the activation and/or kinase activity of JNK-1 in AWCs during forgetting, further molecular analyses will be needed to clarify the regulatory mechanisms of JNK-1 activity and to identify the JNK-1 targets. Furthermore, TIR-1(gk264), which has a small deletion but has the TIR-1 domain, can regulate the differentiation of the AWCs but not the forgetting of olfactory adaptation, although TIR-1(tm3036), which lacks TIR-1 domain, cannot properly regulate both processes. These results raise the possibility that the deleted region of TIR-1(gk264) is important for selective activation of the downstream kinase JNK-1 and PMK-1. JNK and SARM, which is a homolog of TIR-1, have been reported to be expressed in the mammalian brain. JNK regulates various processes of nervous systems, such as development, repair, learning, and memory (Haeusgen et al., 2009), although the function of SARM has not been fully revealed (Thomas and Huganir, 2004). The TIR-1/JNK-1 pathway might also have similar functions to adapt to environmental changes in higher organisms.

Regulatory Mechanisms of Forgetting

Olfactory adaptation is largely odor specific (Colbert and Bargmann, 1995) and has been considered to be regulated at the level of olfactory receptors or at the downstream signaling level. Our study suggests that the forgetting signal from AWCs directly or indirectly regulates the sensory response in AWAs. These signals seem to act at the receptor level, because *tir-1(lf)* did not show reduced response to pyrazine after conditioning with diacetyl and after recovery (date not shown). ODR-4 is required for proper localization of diacetyl receptor ODR-10 (Dwyer et al., 1998), and its function is considered as specific to the odorant receptor (Gimelbrant et al., 2001). Thus, the signals might control the functions of the olfactory receptors or signaling molecules, for example, by activating ODR-4 to change the membrane localization of receptors. In addition, we found that in subsets of neurons, other than AWCs, the signaling pathway also regulates salt chemotaxis learning, suggesting that downstream signaling is distinct between salt chemotaxis learning and olfactory adaptation. In mammals, the balance of activities of protein kinases and phosphatases is important for proper memory retention (Malleret et al., 2001; Genoux et al., 2002; Shema et al., 2007; Cao et al., 2008). Therefore, forgetting signals might regulate the activity of protein kinases and phosphatases in AWAs. We predict that suppressor screening of the pathway mutants may lead to the identification of the secretion and downstream factors.

In *Drosophila*, Rac regulates forgetting of the olfactory memory. Inhibition of Rac activity in the mushroom body causes prolonged retention of memory, whereas activation of Rac causes fast decay of memory probably through actin cytoskeleton remodeling (Shuai et al., 2010). These results indicate that Rac actively and cell-autonomously regulates memory retention in the mushroom body, a key region for olfactory learning. In *C. elegans*, Rac mutant (*rac-2*) animals did not show the prolonged retention of olfactory adaptation (data not shown), probably because olfactory adaptation in *C. elegans* does not require cytoskeletal remodeling.

Our study demonstrates that forgetting is actively regulated by neuronal communication, even in simple behavioral plasticity. This kind of active regulation of forgetting in neuronal circuits may be important for animals to properly change their behavior depending on their environments. Future studies of molecular mechanisms and neural networks will help further our understanding of forgetting in other organisms.

EXPERIMENTAL PROCEDURES

Strains and Culture

The strains used in this study were as follows: N2, CB4858, *tir-1(qj56)*, *tir-1(tm3036)*, *tir-1(ok2859)*, *tir-1(gk264)*, *unc-43(sa200)*, *nsy-1(ag3)*, *sek-1(ag1)*, *jnk-1(gk7)*, *pmk-1(km25)*, *ceh-36(ks86)*, *ceh-36(ky640)*, *tax-4(p674)*, CX8645(*kyIs140* I [*str-2::gfp*]; [*nsy-4(ky616*]; *kyEx1318* [*odr-3::olrn-1*]), EG1470(*oxEx229* [Mos1 Substrate, *pmyo-2::gfp*]), and EG2762 (*oxEx166* [HSP::MosTRANSPOSASE, *lin-15*{+}, *punc-122::gfp*]). All strains were cultured on NGM plates seeded with *E. coli* strain OP-50 (Brenner, 1974). EG1470 and EG2762 were grown at 25°C, although the other strains were grown at 20°C.

Behavioral Assay

Chemotaxis toward attractive odorants was performed as described previously with minor modifications (Bargmann et al., 1993). The changes included the use of the assay plate containing 50 mM NaCl and 1:100 dilutions of odorants. The chemotaxis index was calculated as (A – B)/N, where A was the number of animals within 1.5 cm of the odorant spot, B was the number of animals within 1.5 cm of the control spot, and N was the number of all animals. In the adaptation and recovery assays, adult worms were washed three times with S-basal buffer (100 mM NaCl, 50 mM K_2HPO_4 [pH 6], 0.02% gelatin) and pre-exposed to 1:5,000 diacetyl or 1:10,000 isoamylalcohol in S-basal buffer with rotation for 90 min. Next, the worms were washed once and allowed to recover on food. In the memory retention assay for the starved animals, washed animals were incubated in the S-basal buffer for 2–4 hr with rotation before pre-exposure. In the heat shock experiments, *tir-1* mutants carrying *hsp16.2::tir-1* were shifted to 32°C for 4 hr at the L1 or adult stage and allowed to recover at 20°C for 2 hr. The salt chemotaxis learning assay was performed as described previously (Saeki et al., 2001) with some modifications. To test chemotaxis toward NaCl, 6 cm assay plates were used. The chemotaxis index was calculated as (A – B)/N, where A was the number of animals within 1 cm of the peak of the salt gradient, B was the number of animals within 1 cm of the control spot, and N was the number of all animals. In the assay, worms were pre-exposed to conditioning buffer (20 mM NaCl, 5 mM K_2HPO_4 [pH 6], 1 mM $CaCl_2$, 1 mM $MgSO_4$), with rotation for 90 min. Next, the worms were washed once with wash buffer (5 mM K_2HPO_4 [pH 6], 1 mM $CaCl_2$, 1 mM $MgSO_4$) and allowed to recover from learning in the wash buffer. The results were statistically analyzed using Student's t test or Dunnett's test.

Screening for Mutants and Positional Cloning

Mos1-mediated insertional mutagenesis was performed as described previously (Boulin and Bessereau, 2007) with some modifications (Shinkai et al., 2011). Transgenic worms with both oxEx229 and oxEx166 were subjected to heat shock at 35°C for 75 minutes. We collected 1,080 F1 worms with oxEx229 that were allowed to lay F2 worms. In the screening, F2 worms that show normal chemotaxis to diacetyl were subjected to 4 hr of recovery from adaptation. After the recovery, worms that were not attracted to diacetyl were allowed to lay F3 worms, and F3 young adult worms were subjected to the same assays as F2 worms. After this protocol was repeated four times (F2–F5), worms that showed normal chemotaxis to diacetyl but were not attracted to diacetyl after 4 hr of recovery were chosen as candidates for mutants defective in forgetting. Since the Mos1 insertion site is not relevant to the phenotype, genetic mapping was performed using SNPs between N2 and CB4858 (Hillier et al., 2008), in which CB4858 shows normal adaptation and forgetting similar to N2.

DNA Constructs and Germline Transformation

odr-3::tir-1::DsRed and TeTx cDNA were provided by C. Bargmann and pLR73 (pDEST-*unc103[gf]*) were provided by L.R. Garcia. For the expression analyses of the *jnk-1* and *pmk-1*, 3 kb upstream promoter region of *jnk-1* and *pmk-1* were used. *jnk-1(DN)* was made by changing Lys in the ATP-binding domain to Arg.

Calcium Imaging

In an AWA imaging line, Cameleon YC 3.60 (Nagai et al., 2004) was expressed under the *odr-10* promoter (Sengupta et al., 1996). Calcium imaging was performed using "olfactory" chips (Chronis et al., 2007). Naive animals were picked from the NGM plates with food and animals pre-exposed to 1:1,000 diacetyl for 1.5 hr were used as adapted animals. Then, adapted animals were washed three times and then recovered by culture on food for 4 hr. Fluorescence images were obtained using an Olympus BX53-F microscope equipped with a 60× objective and an ORCA-D2 (Hamamatsu). Yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) intensity in AWA cell bodies was analyzed using AQUACOSMOS (version 2.6, Hamamatsu). $(R_{max} - R_0)/R_0$ 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 16 s interval preceding the first application of diacetyl.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.019.

LICENSING INFORMATION

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