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

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

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## ABSTRACT

Control of memory retention is important for proper responses to constantly changing environments, but the regulatory mechanisms underlying forgetting have not been fully elucidated. Since *C. elegans* possesses a simple nervous system and their memories usually do not persist more than hours, it is suitable to study the mechanisms of forgetting processes at the molecular level. Genetic screening and 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 these mutants, olfactory adaptation was extended from a few hours to a day, and the memory for salt chemotaxis learning was extended from 30 minutes to an hour. In olfactory adaptation to diacetyl, the prolonged retention phenotype of these mutants was rescued by expressing wild-type gene products in AWC sensory neurons. We also found that lack of or inactivation of AWC neurons caused prolonged retention of adaptation to diacetyl, although these neurons did not affect chemosensation or adaptation to diacetyl, suggesting that AWC sensory neurons only regulate forgetting of adaptation. In addition, inhibition of synaptic release from AWC sensory neurons prolonged retention of adaptation in wild type, although, hyperactivation of secretion in these neurons rescued the phenotype in the mutant animals. These results suggested that neurosecretion form these cells is important for the acceleration of forgetting. We, therefore, propose that these neurons accelerate forgetting through the TIR-1/JNK-1 pathway by sending signals that directly or indirectly accelerate 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 of behavioral responses to environmental stimuli. Most memories considered as short-term memories are vulnerable to disruption and are forgotten within hours if not consolidated into stable long-term memories (McGaugh, 2000).

Forgetting is important to eliminate superfluous memories and to prevent 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 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 the proteins 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). Since 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.

*Caenorhabditis elegans* has a simple neuronal network, and its connections have been well described (White et al., 1986). Despite neuronal simplicity, they show 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 hours (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, 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 in *C. elegans* are suitable models to study the forgetting of short-term memories at the molecular and neuronal levels.

Here, I show that, in *C. elegans*, forgetting of olfactory adaptation and salt chemotaxis learning is accelerated by the TIR-1/JNK-1 pathway. In forgetting of olfactory adaptation, TIR-1/JNK-1 pathway acts in a pair of neurons to send signals that induce forgetting. My results suggest that forgetting is actively regulated in the neuronal circuit, and leads to new 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 some 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 cGMP dependent kinase, EGL-4, a cGMP-gated channel β-subunit, TAX-2 (L'Etoile et al., 2002; Lee et al., 2010), and a TRPV (Transient Receptor Potential Vanilloid) 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 AWA (Figure 1A; Sengupta et al., 1996). Animals pre-exposed to 1:5000 diacetyl for 1.5 hours 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 hours demonstrated chemoattraction similar to naive animals (Figure 1B).

To examine whether recovery from adaptation is affected by the environment, I 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. I 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 (Figure 1D, E). 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 involved in the regulation of forgetting, I 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, I isolated the *tir-1(qj56)* mutant (see below). In wild-type animals, adaptation to diacetyl continued for less than 4 hours. In *tir-1(qj56)*, after conditioning with diacetyl, adaptation persisted for more than a day (Figure 2A), although naive *tir-1* animals showed an attractive response to diacetyl, and animals conditioned with diacetyl demonstrated a weaker response to diacetyl, as seen in wild type (Figure 2A), suggesting that *tir-1* mutant animals do not exhibit defects in the sensory response or in adaptation to diacetyl. To examine the possibility that this prolonged retention is caused by stronger adaptation than in wild-type animals; I 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 2B), 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.

We mapped the *qj56* mutation using a single nucleotide polymorphism between N2 and CB4858 (Hillier et al., 2008) that shows more N2-like behavior than the Hawaiian strain, CB4856. We then identified *qj56* as a deletion mutation in the *tir-1* gene (Figure 2C). In addition, other *tir-1* loss-of-function alleles exhibited prolonged retention of olfactory adaptation, just like *qj56* (Figure 2D). On the other hand, a gain-of-function mutant *tir-1(ok1052gf)* (Chuang and Bargmann, 2005) revealed a weak adaptation phenotype (Figure 2D). This implies that excess activation of TIR-1 prevents the establishment of olfactory adaptation.

*tir-1* encodes a highly conserved Toll/IL-1 resistance domain protein that is homologous to the mammalian adapter 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 3A). 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, I analyzed retention of adaptation in *unc-43*, *nsy-1*, *sek-1*, and *pmk-1* mutants after 4-hour recovery. All

mutants exhibited normal chemotaxis and adaptation to diacetyl, whereas *unc-43*, *nsy-1*, *sek-1*, but not *pmk-1*, exhibited prolonged retention of the olfactory adaptation, as seen in *tir-1(lf)* (Figure 3B). To confirm that TIR-1 functions upstream of NSY-1, I analyzed the phenotype of the *nsy-1; tir-1(ok1052gf)* double mutant (Figure 3C). The weak adaptation phenotype of *tir-1(ok1052gf)* was suppressed in *nsy-1; tir-1(ok1052gf)*, which showed a normal attractive response and adaptation to diacetyl, suggesting that in *tir-1(ok1052gf)*, the downstream signaling pathway is hyperactivated and, therefore, the adaptation to diacetyl 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), I analyzed the *jnk-1* mutant and found that *jnk-1* displayed prolonged retention of adaptation (Figure 3B). 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 3A). Since most of the long-term memory which last for more than 24 hours are CREB dependent in *C. elegans* (Rankin et al., 1990; Kauffman et al., 2011), we examine the memory retention in *crh-1* and *jnk-1; crh-1* double mutant animals. Prolonged retention phenotype was not suppressed in the *jnk-1; crh-1* double mutant, although *crh-1* mutant showed weak adaptation phenotype (Figure 3D), suggesting that prolonged retention of the memory in the TIR-1/JNK-1 pathways are CREB independent.

To determine when the signaling pathway regulates the retention of olfactory adaptation, I 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 3E), suggesting that the signaling pathway regulates the retention of the olfactory adaptation in the mature nervous system.

**The TIR-1/JNK-1 pathway regulates forgetting in sensory neurons not involved in chemosensation**

Next, I analyzed neuronal circuit that TIR-1/JNK-1 pathway regulates forgetting of adaptation. We performed cell-specific rescue experiment by expressing wild type gene constructs under several promoters. First, I confirmed that expression of wild-type TIR-1 by a pan-neuronal promoter (Shioi et al., 2001), can rescue the prolonged retention phenotype (Figure 4A). Next, I expressed wild type TIR-1 in AWA sensory neurons by the *odr-10* promoter (Sengupta et al., 1996), which are the primary neurons of diacetyl perception. Although adaptation to diacetyl is due to change in the sensory response in AWA sensory neurons (Inoue et al., 2013), the expression of wild-type TIR-1 in AWA could not rescue the phenotype of *tir-1(lf)* (Figure 4A). To identify the neurons that regulate the retention, I analyzed *tir-1(lf)* expressing wild-type gene products by various promoters. I 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 (Figure 4A and 4B). In addition, the expression of SEK-1 in AWC can rescue the phenotype of the *sek-1* mutant (Figure 4A). I also ascertained that the *jnk-1* promoter drives expression in AWC (Figure 4C), to be consisted with my model, whereas other genes in this pathway were reported to be expressed in AWC at the adult stage (Chuang and Bargmann,

2005).

To confirm that the TIR-1/JNK-1 pathway regulates the retention of olfactory adaptation in AWC, I examined whether the AWC specific expression of the dominant negative form of SEK-1 or JNK-1 caused the prolonged retention. Expression in AWC 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 4D). To examine whether the JNK-1 activity is important for the forgetting of adaptation to diacetyl, I 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 AWC sensory neurons caused the prolonged retention (Figure 4D), suggesting that JNK activity in AWC 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). This result raised the possibility that $AWC^{off}$ neurons are important for the proper retention of olfactory adaptation to diacetyl. To examine this, I analyzed the retention of olfactory adaptation in animals with $2AWC^{on}$ or $2AWC^{off}$ (Bauer et al., 2007), and found that either $AWC^{on}$ or $AWC^{off}$ were sufficient for the acceleration of forgetting (Figure 4E). I 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 4F). Therefore, the abnormal asymmetric differentiation of AWC may not cause the acceleration of forgetting.

**Sensory neurons regulate the forgetting by neuronal secretion**

To confirm the role of AWC sensory neurons in forgetting, I analyzed the phenotype of the *ceh-36* mutants, which lack functional AWC 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 5A). Therefore, AWC are not involved in chemosensation or adaptation in these conditions, but are involved in accelerated forgetting. To examine how AWC regulate forgetting, I analyzed animals with hyperpolarized AWC sensory neurons by expressing constitutively active UNC-103 $K^+$ channels [UNC-103(gf); Gruninger et al., 2008; Shinkai et al., 2011] (Figure 5B). These animals exhibited normal chemosensation and adaptation to diacetyl, but the prolonged retention of adaptation, suggesting that inhibition of AWC neuronal activities causes longer retention. Taken together, I propose that AWC sensory neurons are not required for the acquisition and retention of the olfactory adaptation to diacetyl, but only regulate the forgetting process.

I then examined whether synaptic transmission and/or neurosecretion by AWC 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* mutant animals expressing PKC-1(gf) in AWC showed chemotaxis and adaptation to diacetyl as in wild-type, *tir-1* mutant animals. However, they did not show the prolonged retention of olfactory adaptation (Figure 5C), raising the possibility that this pathway regulates the neurosecretion. To test this, I 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 non synaptic release of diffusible neurotransmitters by cleaving the synaptobrevin (Whim et al., 1997). Wild-type animals expressing TeTx in AWC showed prolonged retention of olfactory adaptation (Figure 5D). Taken together, I proposed that AWC sensory neurons send signals for forgetting through neurosecretion and/or synaptic transmission.

To examine whether environmental signals sensed in AWC affects the retention of olfactory adaptation, I analyzed the phenotype of *tax-4*. TAX-4 is a cyclic nucleotide-gated cation channel, which is essential for chemosensation in AWC (Coburn and Bargmann, 1996). Retention in *tax-4* is not distinguishable from that in wild-type animals (Figure 5E), suggesting that AWC regulate forgetting independent on their sensory response. I 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 5F). 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 5F). 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, I 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 6A). This phenotype was rescued by the expression of wild-type cDNA in AWC (Figure 6B), suggesting that forgetting of olfactory adaptation to isoamylalcohol is also mediated through the TIR-1/JNK-1 pathway in AWC.

**The TIR-1/JNK-1 pathway regulates forgetting of associative learning**

To examine whether the signaling pathway also regulates forgetting in associative learning, I 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; Tomioka et al., 2006). In wild-type animals, the memory of salt chemotaxis learning was retained for less than 30 minutes, but in *tir-1(lf)*, the memory was prolonged to about an hour (Figure 7A). 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 7B). To examine where the signaling pathway regulates this forgetting, I analyzed mutant animals expressing wild-type *tir-1* cDNA using various promoters. The expression of TIR-1 in ASE, which are involved in the sensation of NaCl (Figure 1A; Bargmann and Mori, 1997), and in AWC could not rescue the phenotype (Figure 7C). The expression in a subset of neurons by the *zig-5* promoter (Yamada et al., 2010) is sufficient for

the rescue (Figure 7C), 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. I also noticed that these neurons are different 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*, I 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 studies revealed that forgetting of adaptation to diacetyl is induced by sensory neurons that are not involved in the retention of adaptation, indicating that forgetting is actively regulated in neuronal circuits.

### AWC sensory neurons secrete signals that accelerate forgetting in AWA sensory neurons

Olfactory adaptation is regulated at the early stages of chemosensation. Exposure to AWC-sensed isoamilalcohol neurons causes loss of $Ca^{2+}$ response to odor in AWC (Chalasani et al., 2010). Sawatari in my lab also revealed that, in wild-type animals, the $Ca^{2+}$ response to diacetyl was diminished after conditioning, and recovered after removal of diacetyl (Inoue et al., 2013), suggesting the change in behavioral response to diacetyl observed after conditioning and recovery may be due to an altered sensory response to diacetyl in AWA. Sawatari also revealed that in the *tir-1* and *jnk-1* mutants, the recovery of the $Ca^{2+}$ responses after conditioning were weaker than that in wild type (Inoue et al., 2013). These results, suggests that prolonged retention of olfactory adaptation in the mutants is mainly due to decreased recovery of the sensory response to diacetyl following conditioning.

I demonstrated that the TIR-1/JNK-1 pathway in AWC sensory neurons accelerates

forgetting of adaptation. I propose that AWC 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 AWC and was observed in wild-type animals expressing the dominant negative form of SEK-1 or JNK-1 in AWC. 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, the prolonged retention phenotype was also rescued by hyperactivated secretion from AWC by expressing PKC-1(gf), whereas impairment of the secretion in AWC caused the prolonged retention. Fourth, Sawatari revealed that the decreased recovery of the $Ca^{2+}$ response of AWA in the mutant animals was rescued by expressing the wild-type gene products in AWC (Inoue et al., 2013). I propose a model for the regulation of forgetting of olfactory adaptation to diacetyl (Figure 8).

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). I found that prolonged retention phenotype in *tir-1* was not observed when they were recovered without food, although *ceh-36* animals showed the phenotype even without food. Therefore, the forgetting signals from AWC may be inhibited by the food signals. My results suggest that sensory perception in AWC 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 like intestine to detect nutritional conditions. For animals, food signals are important information to survive, and hence those might accelerate to diminish behavioral change dependent on experience. Since my genetic studies suggested that TIR-1/JNK-1 pathway positively regulates the secretion of forgetting signal from AWC, one possibility is that the pathway negatively regulates the inhibition of forgetting signals by food signals. In addition, I predict that, in *tir-1(lf)*, core process for forgetting still works in AWA, because, even in the presence of food, *tir-1(lf)* eventually recovered from adaptation over an extended time (Figure 8).

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

Secreted factors are also important for the regulation of neuronal plasticity in mammals. BDNF is required not only for the induction of LTP (long-term potentiation) 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.

My 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 4C), although only *jnk-1* mutant animals are defective in forgetting. My 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 effecter or kinases. Since I did not succeed in direct detection of the activation and/or kinase activity of JNK-1 in AWC 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 AWC 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 as well as SARM, which is a homolog of TIR-1, have been reported to be expressed in the mammalian brain. JNK regulates various process 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 et al., 1995), and has been considered to be regulated at the level of olfactory receptors or at the downstream signaling level. I revealed that AWC sensory neurons send forgetting signals directly or indirectly to AWA sensory neurons. These signals seem to change sensory response 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). In *C. elegans*, 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, I found that in subsets of neurons, other than AWC, the signaling pathway also regulates salt chemotaxis learning, in which the memory is forgotten within an hour, 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; Cao et al. 2008). Therefore, forgetting signals might regulate the activity of protein kinases and phosphatases in AWA. I 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.

My 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 environments. Further studies of molecular mechanisms and neural networks will help further my understanding of forgetting in other organisms.

## Experimental Procedures

### Strains and culture

The strains used in this study were as follows: wild-type strain 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)*, *crh-1(tz2)*, *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 3 times with S-basal buffer [100 mM NaCl, 50 mM $K_2HPO_4$, (pH 6), 0.02% gelatin] and pre-exposed to 1:5000 diacetyl or 1:10000 isoamylalcohol in S-basal buffer with rotation for 90 minutes. 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 hours 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 hours at the L1 or adult stage, and allowed to recover at 20°C for 2 hours. 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 minutes. 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 minuets. I collected 1080 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 hours of recovery from adaptation to diacetyl. 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 for 4 times (F2-F5), worms that showed normal chemotaxis to diacetyl but were not attracted to diacetyl after 4 hours 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*,

3kb 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).

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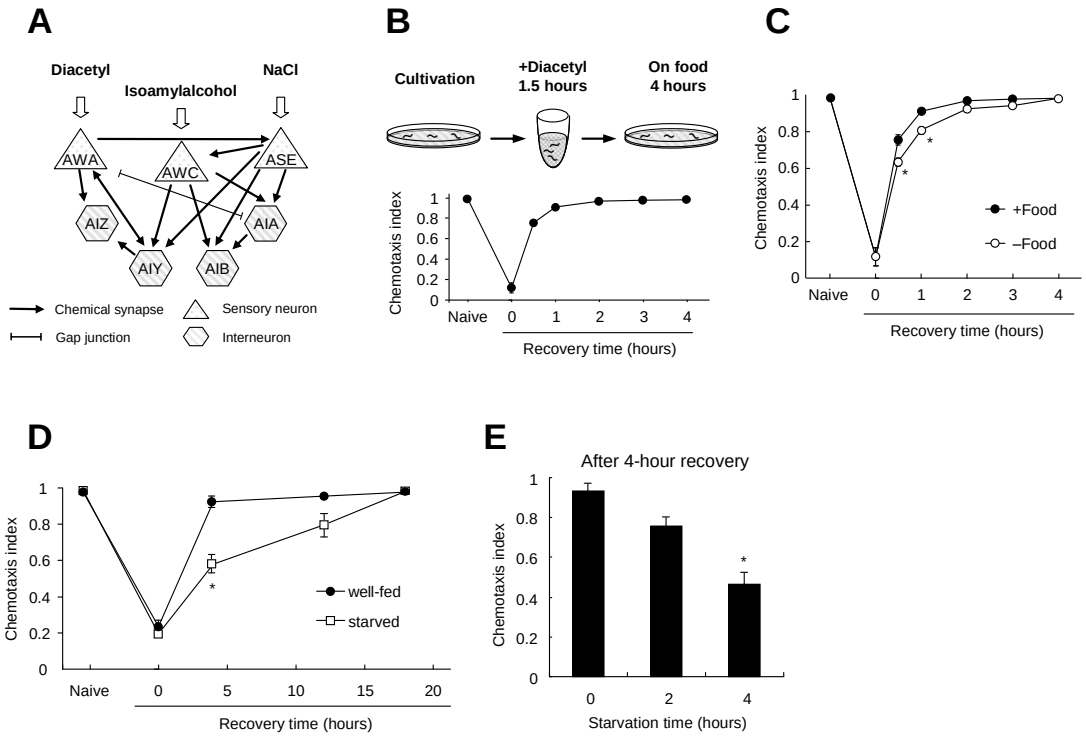


**Figure 1. Forgetting of olfactory adaptation**

(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 hours 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-hour starved animals ($n \geq 6$).

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

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

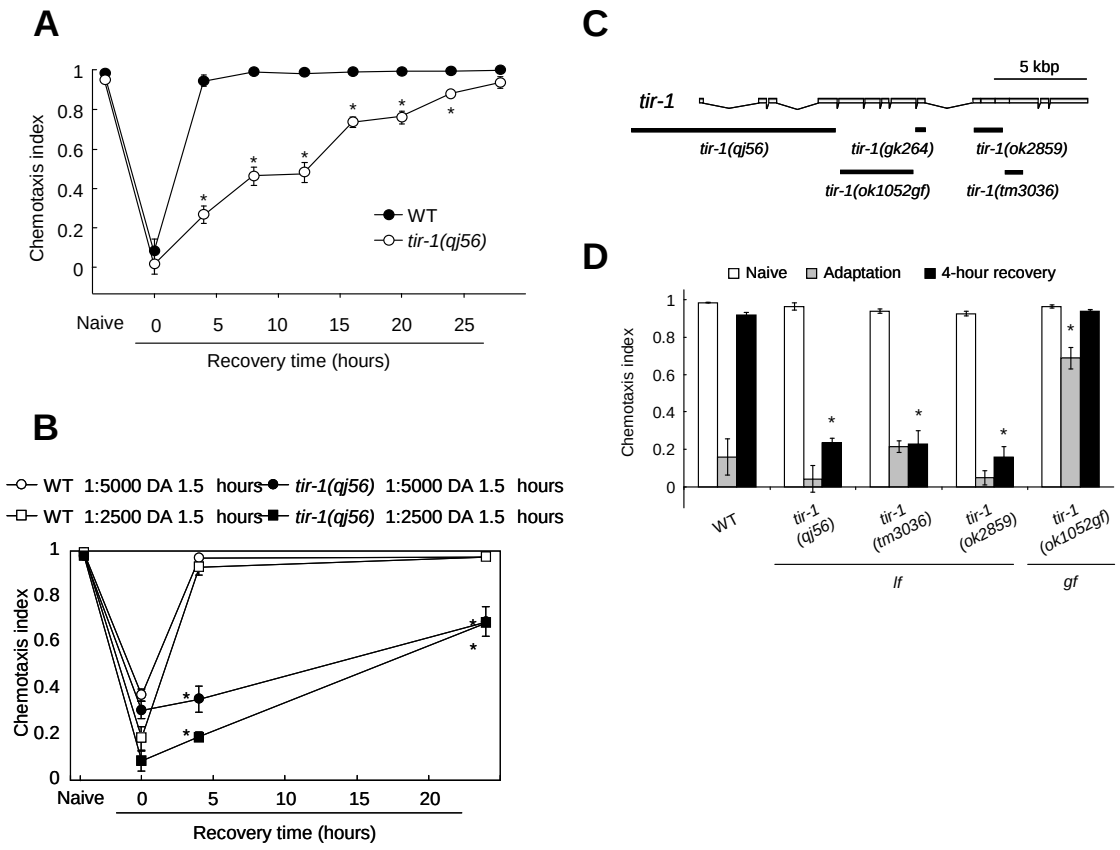


**Figure 2. TIR-1 regulate forgetting of adaptation to diacetyl**

(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) Retention curves of adaptation to diacetyl in wild-type and *tir-1(qj56)* animals conditioned with 1:5000 and 1:2500 diacetyl ($n \geq 4$). *tir-1(qj56)* animals conditioned with 1:5000 diacetyl, which exhibited weaker adaptation than wild-type animals conditioned with 1:2500 diacetyl, exhibited prolonged retention of adaptation. The recovery of *tir-1* animals was significantly different from that of the wild type (1:2500).

(C) 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.

(D) Retention of the adaptation to diacetyl in *tir-1* mutant animals. Chemotaxis of naive,

adapted, and 4-hour recovered animals was analyzed ($n \geq 4$).

*$P < 0.01$; Student's *t*-test (A) or Dunnett's test (B and D). Error bars represent SEM.

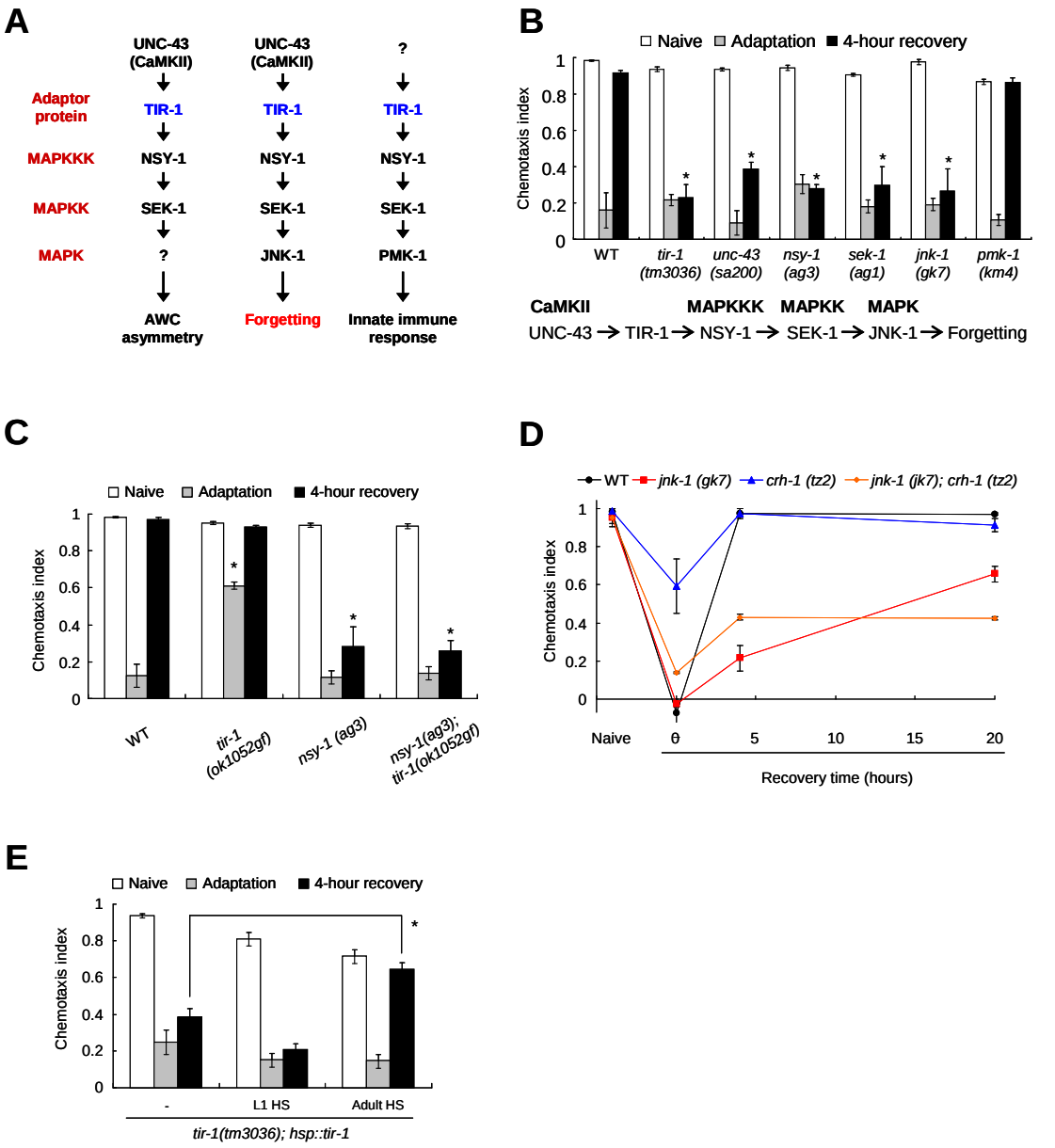


**Figure 3. Forgetting of adaptation to diacetyl is regulated through a TIR-1/JNK-1 pathway**

(A) Signaling pathways that act in conjunction with TIR-1 in *C. elegans*. In AWC sensory neurons, TIR-1 regulates asymmetric differentiation ($AWC^{on}$ and $AWC^{off}$) of a pair of AWC sensory neurons. In this cascade, TIR-1 acts in conjunction with UNC-43(CaMKII), NSY-1(MAPKKK), SEK-1(MAPKK), and an unidentified MAPK. In the intestine, TIR-

1 functions in conjunction with NSY-1, SEK-1, and PMK-1(MAPK) to protect from pathogens. In forgetting of olfactory adaptation, TIR-1 acts in conjunction with UNC-43, NSY-1, SEK-1, and JNK-1 in AWC sensory neurons.

(B) 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.

(C) Genetic interaction between *tir-1(ok1052gf)* and *nsy-1(ag3)* ($n \geq 6$).

(D) Retention curves of adaptation to diacetyl of the wild type, *crh-1*, *jnk-1*, and *crh-1; jnk-1* ($n \geq 2$).

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

*$P < 0.01$; Dunnett's test (B-E). Error bars represent SEM.

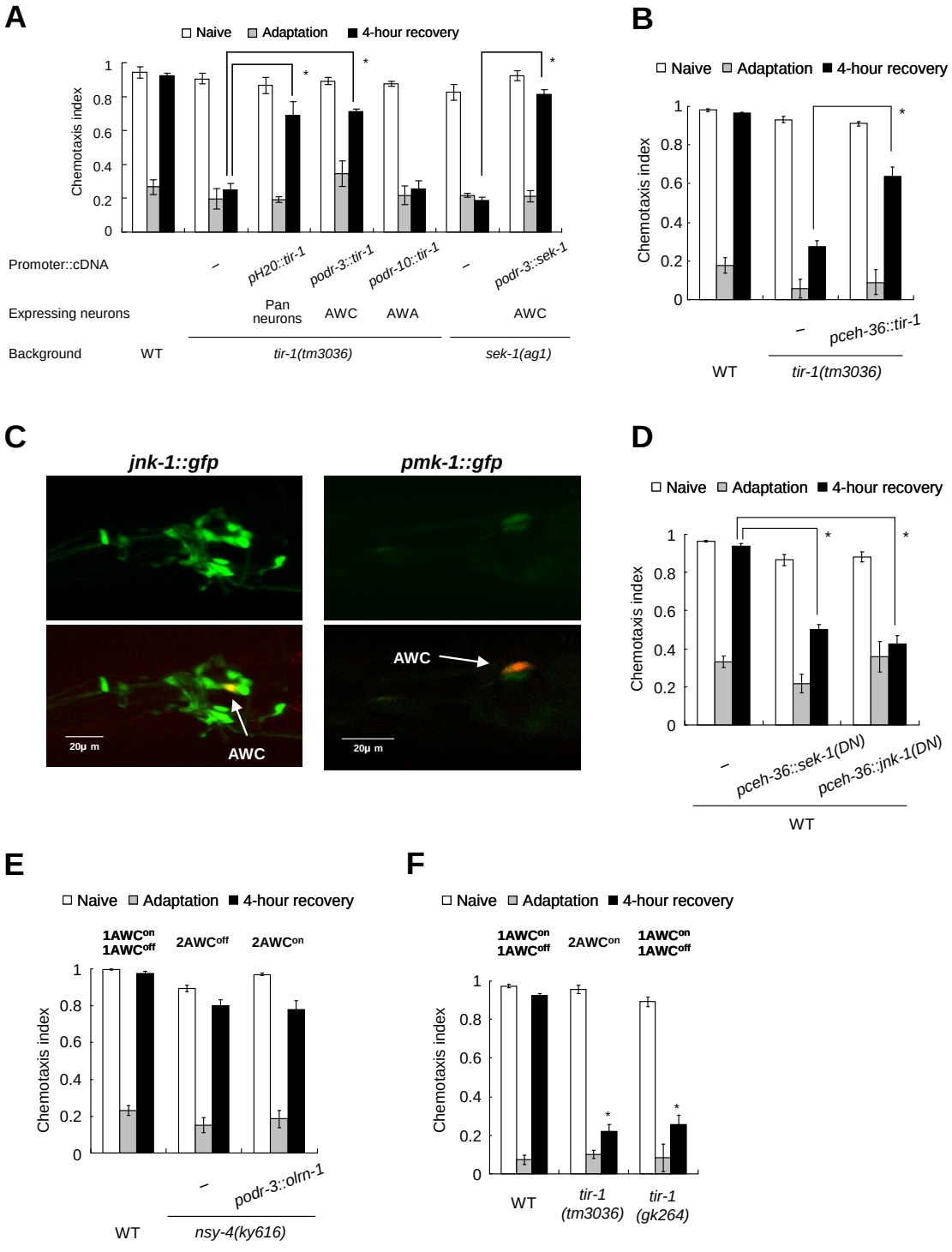


**Figure 4. TIR-1/JNK-1 pathway regulate forgetting of adaptation to diacetyl in AWC sensory neurons**

(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) Transgenic rescue of *tir-1(tm3036)* by the exclusive expression of wild-type gene products in AWC sensory neurons using *ceh-36* promoter (prom3; $n \geq 6$).

(C) Confocal view of wild-type animals expressing GFP driven by *jnk-1* (left) or *pmk-1* (right) promoters. The expression of GFP was colocalized with mCherry expressed in AWC by the *ceh-36* promoter (arrow) at the adult stage.

(D) Retention of the adaptation to diacetyl in wild-type animals expressing dominant negative form of *sek-1* or *jnk-1* in AWC ($n \geq 4$).

(E) Wild-type, *nsy-4(ky616)*, and *nsy-4(ky616);Ex[odr-3::olrn-1]* animals were tested for retention of adaptation to diacetyl ($n \geq 4$). *nsy-4(ky616)* mutant animals were used as animals with $2AWC^{off}$, and *nsy-4(ky616);Ex[odr-3::olrn-1]* were used as animals with $2AWC^{on}$ (Bauer et al., 2007).

(F) 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, and hence *tir-1(gk264)* animals show a $1AWC^{on}$ $1AWC^{off}$ phenotype as do wild-type animals.

*$P < 0.01$; Dunnett's test (A, D, and F) or Student's *t*-test (B). Error bars represent SEM.

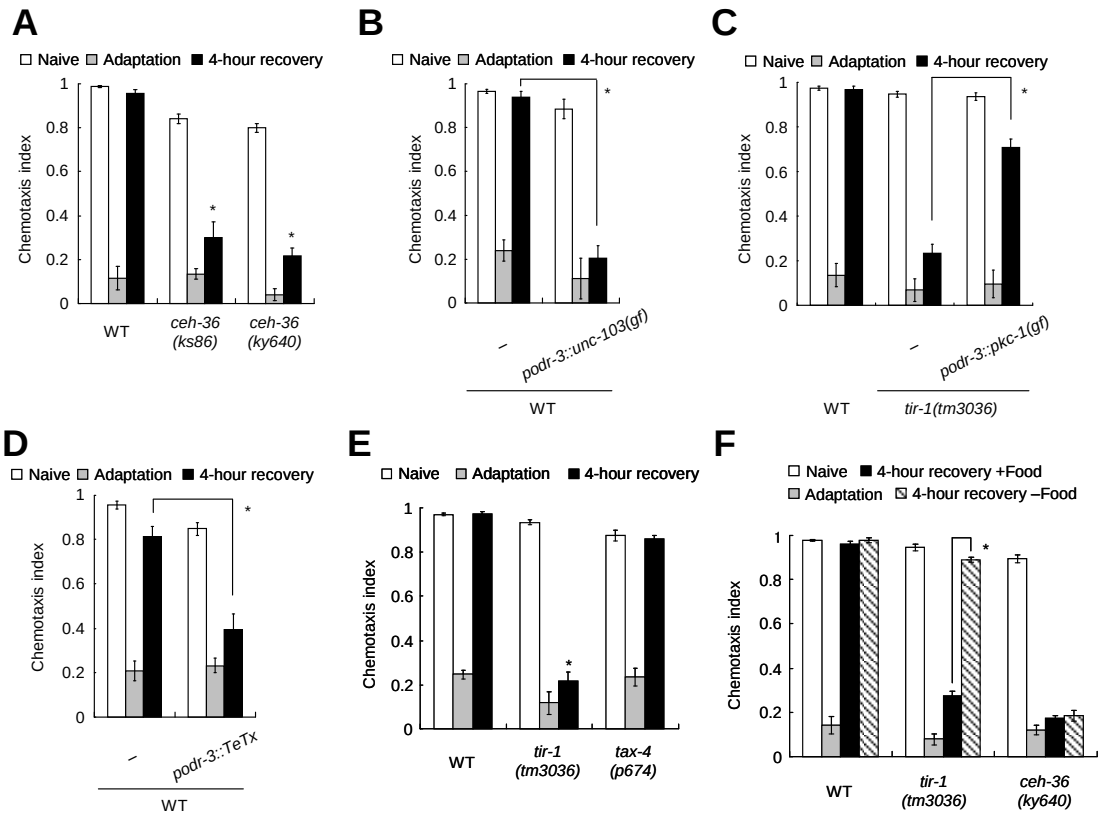


**Figure 5. AWC secretion accelerates forgetting through the TIR-1/JNK-1 pathway**

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

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

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

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

(E) Retention of the adaptation to diacetyl in wild-type, *tir-1(tm3036)*, and *tax-4(p674)* ($n \geq 6$).

(F) 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 and E) or Student's *t*-test (B, C, D, and F). Error bars represent SEM

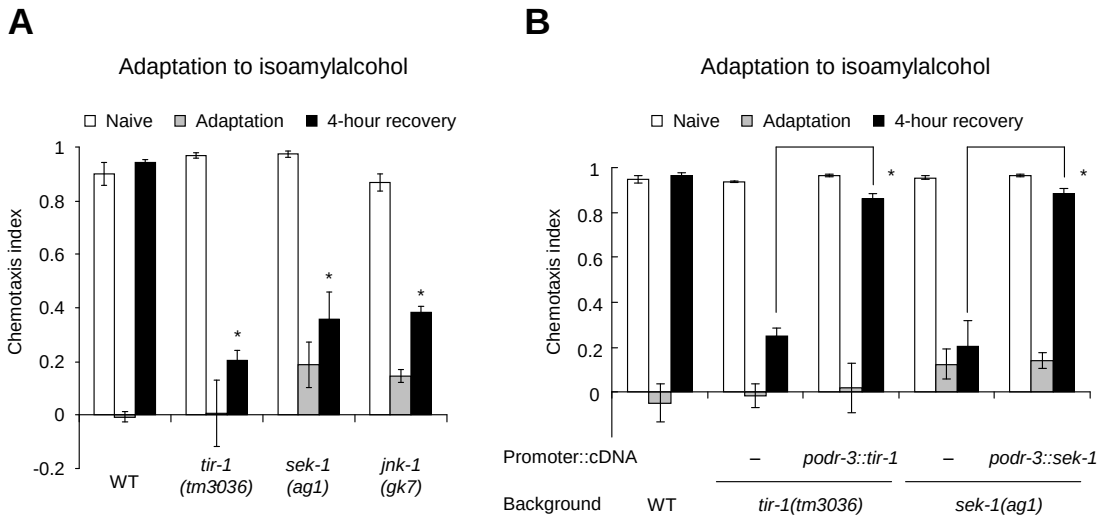


**Figure 6. The TIR-1/JNK-1 pathway in AWC sensory neurons accelerates forgetting of adaptation to isoamylalcohol**

(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 AWC sensory neurons ($n \geq 4$).

*$P < 0.01$; Dunnett's test (A) or Student's *t*-test (B). Error bars represent SEM.

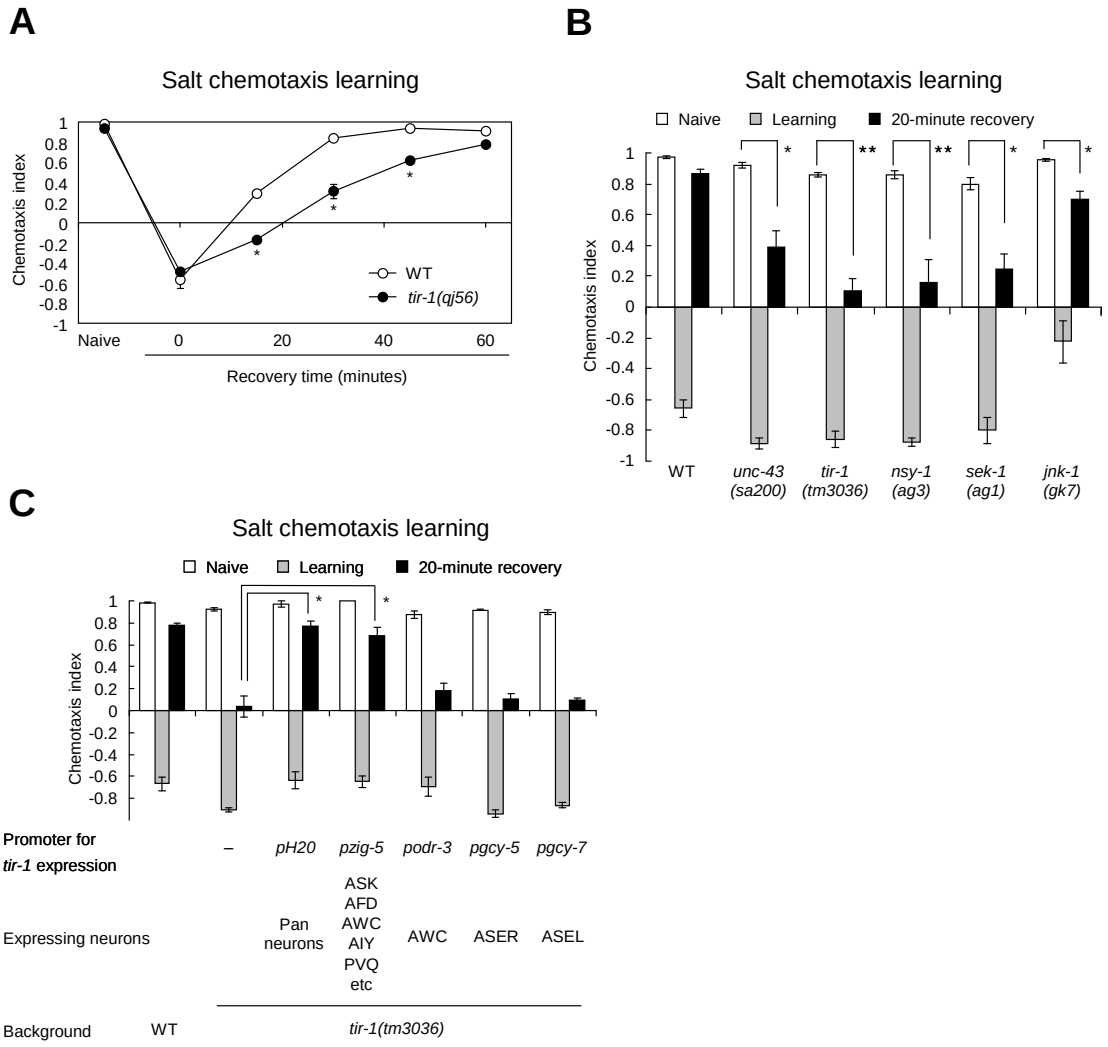


**Figure 7. The TIR-1/JNK-1 pathway accelerates forgetting of associative learning**

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

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

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

**P* < 0.01, ***P* < 0.001; Student's *t*-test (A and B) or Dunnett's test (C). Error bars

represent SEM.

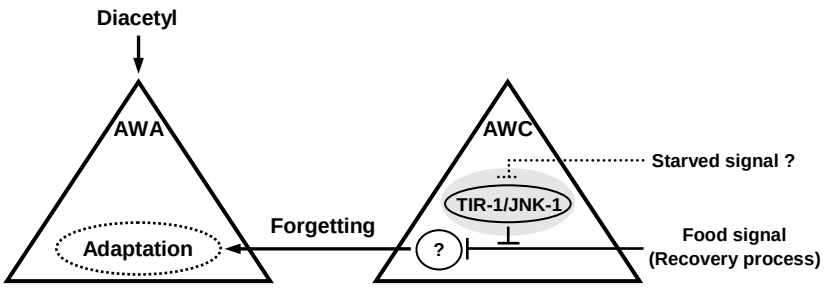


**Figure 8. A model for regulation of forgetting of olfactory adaptation to diacetyl**

AWA sensory neurons are important for perception of an attractive odorant such as diacetyl. $Ca^{2+}$ response to diacetyl in AWA is weakened after conditioning with diacetyl, and is recovered after cultivation on food, suggesting that AWA 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 AWC, showed the prolonged retention phenotype even without food. Thus, AWC may regulate the acceleration of forgetting of olfactory adaptation by secreting signals to AWA, 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.

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