THE GENETIC AND BEHAVIORAL ANALYSIS OF INSULIN SIGNALING IN CAENORHABDITIS ELEGANS LEARNING AND MEMORY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Abstract

Insulin signaling plays a prominent role in regulation of dauer formation and longevity in *Caenorhabditis elegans*. Here, I show that insulin signaling also is required in benzaldehyde-starvation associative plasticity, where worms pre-exposed to the odor attractant benzaldehyde in the absence of food subsequently demonstrate a conditioned aversion response towards the odorant. Animals with mutations in *ins-1, daf-2*, and *age-1* which encode the homolog of human insulin, insulin/IGF-1 receptor, and PI-3 kinase, respectively, have significant deficits in benzaldehyde-starvation associative plasticity. Using a conditional allele I show that the behavioral roles of DAF-2 signaling in associative plasticity can be dissociated, with DAF-2 signaling playing a more significant role in the memory retrieval than in memory acquisition. I propose DAF-2 signaling acts as a learning specific starvation signal in the memory acquisition phase of benzaldehyde-starvation associative plasticity but functions to switch benzaldehyde-sensing AWC neurons into an avoidance signaling mode during memory retrieval.
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<td>µL</td>
<td>microliter</td>
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<td>cm</td>
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<td>CTX</td>
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<td>normal growth medium</td>
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<td>SEM</td>
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<td>phosphoinositide 3</td>
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<tr>
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<td>phosphatase and tensin homolog</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3hydroxy-5-methylisoxazole-4-pro-pionic acid</td>
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<tr>
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Chapter 1
General Introduction
1 GENERAL INTRODUCTION

Learning is defined by a change in behavior as a result of experience and is presumably advantageous for survival of all animals. The ability to associate different sensory stimuli with environmental cues is particularly useful, as it provides an organism with information to avoid unfavorable environment encountered in the past, and helps the animal to search for environment previously associated to be favorable for survival. Memory refers to an organism’s mental ability to store, retain, and recall past experiences and presumably determines one’s behavior. Given that an individual’s behavior is essentially guided by his or her past experiences, much effort has been devoted to elucidate the mechanism involved in learning and memory. Although studies using vertebrate model organisms have helped the behavioral classifications of learning and memory, the complexity of the nervous systems in these higher organisms has limited their effectiveness as models to decipher the cellular and molecular mechanisms involved in learning and memory.

The goal of this project presented herein is to elucidate the nature of learning and memory at a cellular and molecular level using Caenorhabditis elegans as a model system. My thesis work describes the role of insulin signaling in mediating molecular processes involved in C. elegans associative learning and memory, and defines its behavioral role in learning acquisition versus memory recall.

In this chapter, I will first describe different behavioral mechanisms of learning and memory. Furthermore, by reviewing the existing literature in C. elegans associative learning,
this General Introduction will demonstrate the usefulness of *C. elegans* as an elegant platform from which to approach the greater question of how neural plasticity leads to emergent changes in animal behavior. Despite its role in glucose homeostasis, insulin signaling has been implicated in vertebrate learning and memory. This General Introduction will review the role of insulin signaling in *C. elegans* and findings that suggest insulin signaling’s role in learning and memory is conserved from vertebrate organisms to *C. elegans*.

1.1 Behavioral Mechanisms of Learning and Memory

Experience dependent behavioral plasticity (i.e. learning) can be divided into nonassociative and associative learning (Schwartz, 1984). Nonassociative learning is traditionally considered to be the simplest form of learning as it involves only a single sensory stimulus rather than multiple distinct stimuli. Associative learning, on the other hand, is a form of learning that requires an association between two sensory stimuli.

1.1.1 Nonassociative Learning

Nonassociative learning occurs when a single sensory stimulus experience alters an organism’s behavior, and can be further categorized into sensitization and habituation (Schwartz, 1984). Sensitization is defined as an increase in response towards stimuli due to exposure of a stimulus that raises the level of arousal. In contrast, habituation occurs when an organism decreases it response towards a given stimulus after repeated stimulation, and is considered critical for animal survival as it allows an animal to ignore irrelevant stimuli and focus on environmental cues important to survival. Although a decrement in sensory responses
can also be observed as in the case of sensory fatigue or adaptation, habituation is different from sensory fatigue or adaptation, as the habituated response can be rapidly reversed with the presentation of a novel stimulus, which is a phenomenon known as dishabituation (Rankin et al., 2009).

1.1.2 Associative Learning

Classical conditioning is a form of associative learning where an organism forms a link between two stimuli, thereby providing it the knowledge to predict the presence or absence of the more significant stimulus using the previously neutral stimulus (Schwartz, 1984). An example of classical conditioning is Pavlov’s dog where Ivan Pavlov discovered that the ringing of a bell (i.e. a previously neutral stimulus), through experience, can elicit a salivation response (i.e. a reflex response) normally triggered by the taste of food powder (i.e. a reflex-producing stimulus) (Pavlov, 1927). In behavioral neurobiology, classical conditioning is believed to be a result of neuronal plasticity that causes the internal representation of the conditioned stimulus (i.e. an initially neutral stimulus) to excite the internal representations of a salient unconditioned stimulus (i.e. a reflex-producing stimulus), such the conditioned stimulus (CS) alone can elicit a conditioned response (CR) that is normally triggered by the unconditioned stimulus (US).

The basic behavioral features of associative learning has been shown to be conserved across a variety of species, including single celled paramecia (Armus et al., 2006), honey bees (Behrends and Scheiner, 2009), fruit flies (Duerr and Quinn, 1982), Aplysia (Hawkins et al.,
1998), and fish (Braubach et al., 2009). This evolutionary conservation in behavioral expression of associative learning supports the hypothesis that associative learning is an important skill that is advantageous for the survival of all animals, and also implies that the underlying cellular and molecular mechanisms involved in associative learning might be conserved across phylogeny as well. Studies using vertebrate model organisms have helped the behavioral classifications of associative learning. Unfortunately, the complexity of the nervous systems in these higher organisms has limited their effectiveness as models to decipher the cellular and molecular mechanisms involved in learning and memory. Therefore, model organisms with simpler nervous systems will presumably be more suitable for the study of associative learning at a cellular level.

1.2 C. elegans as a Model System for the Genetic and Molecular Analysis of Associative Learning and Memory

With a compact 302-neuron nervous system, in which the complete synaptic connections have been mapped down to electronic microscope level (White et al., 1986), C. elegans emerges as an excellent invertebrate model for the molecular study of associative learning and memory. Forward genetic and reverse genetic techniques can be applied in C. elegans, as its entire genome has been mapped and sequenced. Most importantly, the nematode, despite its simple neural wiring, demonstrates behavioral expression classified as associative learning in a variety of learning paradigms. For example, C. elegans is capable of associating temperature or NaCl with the absence of food as demonstrated in thermotaxis learning and salt chemotaxis learning paradigms (Wen et al., 1997; Kodama et al., 2006; Tomioka et al., 2006). C. elegans also has the ability to associate the odorant butanone or
salt taste with the presence of food (Wen et al., 1997; Torayama et al., 2007). Furthermore, the nematode can associate sickness caused by ingesting pathogenic bacteria with odorants that are specific to only the pathogenic strain (Zhang et al., 2005). It has also been shown that the nematode’s dramatic change in odorant preference after exposure to a high concentration of benzaldehyde in the absence of food is a result of associative learning where the nematode forms an association between benzaldehyde and starvation (Nuttley et al., 2002).

1.2.1 Thermotaxis Learning in C. elegans

Thermotaxis in C. elegans is a behavior observed when the nematode approaches or avoids a thermal stimulus within a thermal gradient (Mori et al., 2007). Interestingly, when subject to a temperature gradient, C. elegans cultivated at a constant temperature in the presence of abundant food source will track along a temperature to a 0.1°C precision that corresponds to its cultivation temperature known as isothermotracking (Hedgecock and Russell, 1975). On the other hand, if the nematode is subsequently exposed to its previous cultivation temperature in the absence of food, it will no longer track the temperature (Mori and Ohshima, 1995). The thermotaxis behavior is considered a good model for the study of associative learning and memory, as the nematode must associate the temperature with the feeding state (i.e. presence or absence of food), encode this information into memory, and later recall such memory when transferred to a thermal gradient to track or avoid a temperature properly. Therefore, thermotaxis learning has been extensively studied in hope of elucidating its underlying cellular and molecular mechanisms.
The neural circuitry involved in thermotaxis behavior has been well characterized. A pair of AFD sensory neurons have been identified to be important for the temperature sensation, and the interneurons AIY, on which AFD projects, are critical for driving thermophilic behavior, while interneurons AIZ, on which AIY projects, is necessary for normal cryophilic behavior (Mori et al., 1995). Simultaneous activation of AIZ and AIY is believed to drive counteractive behavioral out-put signals that are integrated and balanced within RIA interneurons for proper thermotaxis behavior (Mori et al., 1995). Interestingly, recent studies have implicated AWC, a sensory neuron for olfactory sensation, also senses thermal stimuli and affects thermophlic behavior through its connection with AIY (Kuhara et al., 2008).

Genetically, multiple genes have been implicated in thermotaxis learning. For example, a mutation in ncs-1, which encodes a mammalian neuronal calcium sensor homolog, disrupts the nematode’s ability to associate temperature with its feeding state, suggesting the importance of intracellular calcium in regulating thermotaxis learning (Gomez et al., 2001). A mutation in sel-12, which encodes a human presenilin gene homolog, perturbs AIY neuron morphology and disrupts thermotaxis learning (Wittenburg et al., 2000). Intriguingly, it has been shown that mammalian ncs-1 plays a role in mammalian neuronal plasticity (Genin et al., 2001), and that human presenlin gene plays a role in familial Alzheimer’s diseases (Sherrington et al., 1995). These examples further support the notion that the molecular components of learning and memory are well conserved from C. elegans to vertebrate organisms.
1.2.2 Salt Chemotaxis Learning in \textit{C. elegans}

\textit{C. elegans} chemotaxis refers to the behavior when the nematode migrates towards or away from a chemical substrate (Ward, 1973). \textit{C. elegans} has a strong preference for NaCl, as the nematode chemotaxis towards the salt when presented an environment that has a point source of NaCl (Saeki \textit{et al.}, 2001). Laser ablation studies have shown that ASE sensory neurons (i.e. ASER and ASEL) are the primary sensory neurons for sensing NaCl (Bargmann and Horvitz, 1991). Although ASER and ASEL are anatomically left-right homologs, the neurons possess asymmetric gene expression profiles and sense different water soluble ions (Pierce-Shimomura \textit{et al.}, 2001). Further demonstrating the asymmetry of these two neurons, it has been shown that ASEL is an ON-neuron that is stimulated by an increase in NaCl concentration while ASER is an OFF-neuron that is stimulated by a decrease in NaCl concentration (Suzuki \textit{et al.}, 2008).

Similar to theromtaxis behavior, salt chemotaxis behavior is subjected to plasticity, which depends on the nematode’s past experience with NaCl (Wen \textit{et al.}, 1997; Saeki \textit{et al.}, 2001). When cultured in a NaCl containing agar plate in the absence of food, the animal’s chemotaxis towards NaCl decreases significantly (Saeki \textit{et al.}, 2001). The absence of food source is a critical factor for this behavioral plasticity, as worms cultured in the presence of food in NaCl containing plates demonstrate a strong attraction towards NaCl (Saeki \textit{et al.}, 2001). Furthermore, application of serotonin, a neurotransmitter involved in food sensation behaviors in \textit{C. elegans}, during the animal’s exposure to NaCl in the absence of food partially suppresses the decrease in NaCl chemotaxis (Saeki \textit{et al.}, 2001). These observations have led to the conclusion that \textit{C. elegans} is capable of associating its feeding state (i.e. presence or
absence of food) with the taste cue NaCl, and can act according to this associative memory when later presented a point source of salt. This salt chemotaxis plasticity is referred as salt chemotaxis learning in the literature.

Several mutant strains that are defective in salt chemotaxis learning have been isolated through forward genetic screens. The mutant strains JN603 and JN683 are the two strains first isolated from such a screen (Saeki et al., 2001). Unfortunately, the genetic mutations underlying the learning deficit in these two strains remain elusive. A mutation in hen-1, which encodes a secretory protein with an LDL motif, is found to disrupt salt chemotaxis learning (Ishihara et al., 2002). Recently, CASY-1, an ortholog protein of mammalian calsyntenins/alcadeins, has been identified to be critical for salt chemotaxis learning perhaps by acting as a learning-modulating neurohormone in C. elegans (Ikeda et al., 2008). Interestingly, a SNP at human calsyntenin-2 locus has been shown to have a strong genetic linkage with memory performance (Jacobsen et al., 2009), suggesting CASY-1’s role in learning and memory may be conserved in mammals as well.

1.2.3 Pathogen Avoidance Learning in C. elegans

C. elegans feeds on bacteria and can distinguish among different strains of microbes, as the nematode displays distinct innate olfactory responses to various bacterial strains (Shtonda and Avery, 2006). For example, C. elegans is repelled by the odor of bacterial strain Bacillus megaterium and pathogenic strain Microbacterium nematophilum. Although one would expect the nematode to have evolved mechanisms for avoiding all pathogenic food, C. elegans is
attracted towards the odor of infectious bacteria *Serratia marcescens* and *Pseudomonas aeruginosa* (Zhang et al., 2005). This apparent paradox is explained by the recent finding that the nematode can avoid pathogenic bacteria through olfactory associative learning (Zhang et al., 2005). *C. elegans* cultured on the standard laboratory food *E. coli* has a preference for the odor of pathogenic *P. aeruginosa* or *S. marcescens*. However, when fed the pathogenic *P. aeruginosa* or *S. marcescens* for four hours, the nematode will reverse its preference for the smell of pathogenic bacteria and avoid the odor instead (Zhang et al., 2005). This form of behavioral plasticity is considered associative learning, as the nematode learns to avoid the smell of pathogenic bacteria presumably through associating the sickness from ingesting the pathogenic food with the odor specific to the pathogens (Zhang et al., 2005). The pathogenic avoidance learning behavior has been shown to be mediated by serotonergic neurons ADF (Zhang et al., 2005). Furthermore, it is hypothesized that ADF releases serotonin in response to the aversive experience from ingesting pathogenic bacteria as a negative reinforcer, thereby modifying the olfactory circuitry to cause an avoidance response towards the smell of pathogenic bacteria (Zhang et al., 2005). Interestingly, serotonin in the mammalian intestine functions in malaise sensation such as the nausea feeling associated with chemotherapy (Gershon, 2003).

1.2.4 Butanone Enhancement Learning in *C. elegans*

*C. elegans* has the capacity to sense and chemotaxis towards or away from a variety of volatile odorants which are detected by three pairs of sensory neurons AWA, AWB, and AWC (Bargmann et al., 1993). While AWA and AWC neurons function to sense attractive odorants, AWB detects aversive odorant octanol (Bargmann et al., 1993). Laser ablations studies have
shown that AWA neurons are necessary for the sensation of odorant diacetyl and pyrazine. Interestingly, mis-expression of ODR-10 (diacetyl receptor protein) in AWB in mutant background lacking diacetyl receptors results in avoidance behavior towards otherwise attractive diacetyl in these mutants, suggesting each sensory neuron may be hardwired with signaling molecules either mediating attraction or avoidance responses (Troemel et al., 1997).

The two AWC neurons sense at least five odorants, including butanone, benzaldehyde, 2,3-pentanedione, isoamyl alcohol, and 2,4,5-trimethylthiazole (Bargmann et al., 1993). Similar to the ASE neurons, the two AWC neurons are functionally asymmetrical despite their similarity in anatomical structures, and are differentiated by their expression of the G-protein coupled receptor STR-2, which is randomly expressed in either the left or the right AWC neuron (Troemel et al., 1999). While the STR-2 expressing (AWC\textsuperscript{ON}) neuron senses butanone, the non-STR-2 expressing (AWC\textsuperscript{OFF}) neuron senses 2,3-pentanedione; benzaldehyde is sensed by both AWC\textsuperscript{ON} and AWC\textsuperscript{OFF} neuron (Wes and Bargmann, 2001). Similar to salt chemotaxis and thermotaxis, chemotaxis towards odorants is also subjected to plasticity.

*\textit{C. elegans} is modestly attracted towards butanone when tested to a point source of butanone (Colbert and Bargmann, 1995). However, after exposure to butanone in the presence of food, the nematode will exhibit an enhanced chemotaxis response towards the odorant (Torayama et al., 2007). This behavioral plasticity is termed butanone enhancement and is suggested to have properties similar to associative learning, where two sensory signals (i.e. butanone and food) are associated to lead to a change in behavior (i.e. enhanced butanone attraction) (Torayama et al., 2007). Butanone enhancement has been shown to be mediated by \textit{bbs-8}, which is a homolog of mammalian Bardet-Biedl syndrome gene, in the AWC\textsuperscript{ON} neuron.
Interestingly, without physically ingesting the food, animals exposed to butanone and the odor of food can show a partial enhancement in their chemotaxis towards butanone, raising the interesting hypothesis that sensory integration of food and butanone may occur solely within AWC^{ON} (Torayama et al., 2007).

1.2.5 Benzaldehyde-starvation Associative Plasticity in *C. elegans*

*C. elegans* will lose its chemotaxis response to the attractive odor sensory cue benzaldehyde after continuous exposure to the odorant (Colbert et al., 1995). This change in behavior was initially characterized as adaptation rather than muscle fatigue as the decreased response towards benzaldehyde was not found to be generalized to all odorants (Colbert et al., 1995). The notion of benzaldehyde adaptation was later challenged by the finding that the decreased benzaldehyde response after prolonged odorant exposure could be rapidly reversed by subjecting animals to centrifugation (i.e. dishabituation stimuli), suggesting that the decreased benzaldehyde response is a result of nonassociative learning (i.e. habituation) rather than sensory fatigue (i.e. adaptation) (Nuttley and van der Kooy, 2000). Interestingly, it was later found that benzaldehyde habituation could be suppressed by the presence of food during benzaldehyde exposure. Furthermore, a high concentration of serotonin, which is a neurotransmitter implicated in mimicking food consumption, was also sufficient to block benzaldehyde habituation (Nuttley et al., 2001). These observations have led to the conclusion that the decreased benzaldehyde response after odorant exposure is not due habituation, as the response decrement is not a simple reflection of the amount of benzaldehyde exposure, but rather is a result of associative learning, whereby animals associate benzaldehyde with the absence of food (Nuttley et al., 2002). In further support of the notion that benzaldehyde habituation is a form associative learning, it was found that benzaldehyde habituation was not pronounced if animals were exposed to benzaldehyde in the presence of salt but tested in the
absence of salt, suggesting that the perception of the taste cues (i.e. context) is required for the recall of the benzaldehyde-starvation associative memory (Law et al., 2004). In my thesis, this decrement response towards benzaldehyde after odorant exposure will be referred as benzaldehyde-starvation associative plasticity. Although several proteins, including EGL-4, a cGMP-dependent protein kinase, OSM-9, a TRP-like ion channel, and GOA-1, a G\textsubscript{o}\alpha protein, have been implicated in benzaldehyde-starvation associative plasticity (Colbert et al., 1997; L'Etoile et al., 2002; Matsuki et al., 2006), the molecular mechanisms underlying this behavioral plasticity remain unclear.

With the combination of a compact nervous system, genetic accessibility, and expression of associative learning behavior, C.elegans serves as an excellent platform for the study of associative learning and memory. Of particular interest to this project, the nematode can be utilized for elucidating the neural mechanisms of signaling pathways that have been implicated in vertebrate learning and memory through reverse genetic techniques.

1.3 The Role of Insulin Signaling in Vertebrate Learning and Memory

Despite its well known role for peripheral glucose homeostasis, insulin signaling in the central nervous system (CNS) has been implicated in vertebrate learning and memory (van der Heide et al., 2006). Although insulin/insulin receptor signaling can be transmitted through different transduction pathways, it has been suggested that the effect of insulin in learning and memory is likely mediated through the phosphoinositide 3 (PI3)-kinase transduction pathway (van der Heide et al., 2006). Activation of PI3-kinase leads to the phosphorylation of the
phosphatidylinositol to generate phosphatidylinositol trisphosphate (PIP$_3$), which activate downstream protein kinase B (PKB) (Vanhaesebroeck and Waterfield, 1999). PI3-kinase signaling is balanced by phosphatase and tensin homolog (PTEN), which dephosphorylates PIP$_3$ (Vanhaesebroeck et al., 1999). Emerging evidence has suggested that deterioration of CNS insulin signaling is associated with cognitive impairments and that insulin signaling modulate synaptic transmission and plasticity in vertebrate organisms.

1.3.1 Cognitive impairments associated with deterioration of CNS insulin signaling

It has been hypothesized that inadequate CNS insulin signaling causes cognitive impairments. Studies have shown that while long term hyper insulinemia increases the risk of dementia (Lu et al., 2009), insulin administration to healthy individuals can improve cognitive functions when glucose homeostasis is maintained (Craft et al., 1996). Furthermore, the number of brain insulin receptors decrease in aged cognitive impaired rodents (Zaia and Piantanelli, 2000). Similarly, in the brains of sporadic Alzheimer’s disease patients, a significant decrease in insulin and insulin receptors has been observed (Frolich et al., 1998). In type two diabetic patients with increased insulin resistance, verbal learning and memory performance tend to be worse than healthy individuals (Ryan and Geckle, 2000). Consistent with these findings, infusion of PI3-kinase inhibitors into rodent hippocampus was found to impair learning acquisition and memory recall (Barros et al., 2001b), whereas an increase in insulin receptors mRNA in rat hippocampus, a brain region associated with learning and memory processing, was observed after a spatial memory learning task (Zhao et al., 1999).
1.3.2 Insulin Signaling Modulates Synaptic Plasticity

The molecular basis underlying the role of insulin signaling in learning and memory has been attributed to its ability to affect glutamatergic and GABAergic transmissions through receptor trafficking (van der Heide et al., 2006). It has been shown that insulin facilitates the internalization of alpha-amino-3-hydroxy-5-methylisoxazole-4-pro-pionic acid (AMPA) receptors, thereby reducing glutamatergic excitatory transmission in hippocampal neurons (Man et al., 2000). Interestingly, studies have also demonstrated that insulin can facilitate the insertion of glutamate receptor 2 (GluR2), a subtype of AMPA receptor, and N-methyl D-aspartate (NMDA) receptors into plasma membranes possibly through activation of PI3-kinase (Man et al., 2003; van der Heide et al., 2005). Furthermore, insulin is shown to cause rapid integration of gamma-aminobutyric acid (GABA) receptor into plasma membranes of cells transfected with GABA receptors, and can also recruit receptors to the postsynaptic and dendritic membranes to augment GABA inhibition (Wan et al., 1997). Although emerging evidence has suggested insulin signaling plays a role in regulating different neurotransmission systems through receptor trafficking, its molecular mechanisms in mammalian learning and memory are far from completely understood.

1.4 Insulin Signaling in C. elegans

Insulin signaling is well conserved from vertebrate organisms to C. elegans, and its role in daur formation, life span extension, and stress resistance has been extensively studied. Recently, insulin signaling has also been implicated in associative learning in C. elegans. In C. elegans genome, 38 genes have been predicted to be similar to mammalian insulin/insulin-like growth factors (IGF) with ins-1 and ins-18 being the closest homolog to human insulin (Pierce
et al., 2001). These insulin-like peptides are believed to bind onto the sole insulin/IGF-like receptor homolog DAF-2 to initiate insulin signaling (Kurz and Tan, 2004). Like in the mammalian system, AGE-1, which is the homolog of mammalian PI3-kinase, is activated by DAF-2 receptors to generate PIP$_3$ that activates the mammalian PKB homolog PDK-1. The activity of AGE-1 is counterbalanced by DAF-18, which is the mammalian PTEN homolog, via dephosphorylating PIP$_3$.

1.4.1 The Role of Insulin Signaling in Dauer Formation, Longevity, and Stress Resistance

The insulin signaling pathway was first implicated in *C. elegans* dauer formation (DAF). *C. elegans* goes through four larval stages before molting into an adult worm (Riddle et al., 1997). When an young larva is exposed to unfavorable environment such as scarcity of food, overcrowding, and high temperature, it will enter an alternative larva stage called dauer. The dauer larva shows a different morphology and becomes highly stress resistant with a slow metabolism and extended life span. The *daf* genes are categorized into dauer constitutive (i.e. promoting dauer arrest under a favorable environment) and dauer defective (preventing dauer arrest under an unfavorable condition). Although it is well known that downregulation of DAF-2 signaling promotes dauer formation, as animals with mutations in the DAF-2 pathway are dauer constitutive, it remains unclear which insulin-like peptide regulates dauer formation due to the redundancy in insulin-like genes (Pierce et al., 2001). Interestingly, it has been reported that human insulin and INS-1 atagonize DAF-2 signaling to promote dauer arrest, but whether the antagonism is a direct or indirect effect is unknown (Pierce et al., 2001). In addition to regulating dauer entry, DAF-2 signaling is also involved in regulating life span, as majority of genes that confer life span extension act in the DAF-2 pathway (Kurz et al., 2004).
For example, age-1 mutants have been shown to live 65% longer than wild type animals at 25°C (Friedman and Johnson, 1988). Surprisingly, rescue experiments have suggested that DAF-2 is required in neurons rather than other somatic tissues to mediate life span, raising the interesting hypothesis that the nematode life span is regulated by its nervous system (Apfeld and Kenyon, 1999). Mutants in the DAF-2 signaling pathway are also resistant to cell-damaging stress such hypoxia (Scott et al., 2002), ultra violet light (Murakami and Johnson, 1996), and pathogenic bacteria (Garsin et al., 2003), providing indirect evidence that aging may be a result of cell damage accumulation.

1.4.2 The Role of Insulin Signaling in C. elegans Associative Learning

Despite its prominent role in regulating dau er formation, longevity, and stress resistance, the insulin signaling pathway has recently been shown to be involved in salt chemotaxis learning and thermotaxis learning. Mutants of ins-1, daf-2, and age-1 were shown defective in pairing starvation with NaCl when tested to the salt chemotaxis learning paradigm (Tomioka et al., 2006). Using rescue experiments, it has been demonstrated that age-1 acts in salt sensing ASER neuron and INS-1 acts in AIA interneurons, suggesting that INS-1 might be released from AIA and activate DAF-2 signaling in ASER to mediate the behavioral plasticity in salt chemotaxis learning (Tomioka et al., 2006). ins-1 mutants were also found defective in associating starvation with temperature when subjected to the thermotaxis learning assay (Kodama et al., 2006). In contrast to what was observed in salt chemotaxis learning, age-1 mutants have a superior thermotaxis learning ability compared to the wild type, suggesting INS-1 may regulate thermotaxis learning through atagonizing DAF-2 signaling (Kodama et al., 2006). Expression of INS-1 from various neurons can rescue the thermotaxis learning deficit in
ins-1 mutants, while the enhanced learning ability observed in age-1 mutants can be reversed by expressing AGE-1 in either AIY, AIZ, or RIA interneurons, suggesting that INS-1 may function as a neurohormone to modulate interneuron activity in thermotaxis learning (Kodama et al., 2006).

Although previous studies have highlighted the importance of insulin signaling in C. elegans associative learning, its role in mediating the changes in neural plasticity that underlies learning and memory remains poorly defined. It also is unclear behaviorally whether insulin signaling functions in C. elegans memory acquisition or retrieval. In the following chapter, I will present data demonstrating that insulin signaling mediates C. elegans benzaldehyde-starvation associative plasticity, and that its behavioral role can be dissociated into memory acquisition and retrieval. First, I show that insulin signaling is required in benzaldehyde-starvation associative plasticity, where worms pre-exposed to the odor attractant benzaldehyde in the absence of food subsequently demonstrate a conditioned aversion response towards the odorant. Second, I show that animals with mutations in ins-1, daf-2, and age-1 which encode the homolog of human insulin, insulin/IGF-1 receptor, and PI-3 kinase, respectively, demonstrated significant deficits in benzaldehyde-starvation associative plasticity. Third, I demonstrate that INS-1 can act from multiple neurons and AGE-1 acts in benzaldehyde-sensing AWC sensory neurons to direct benzaldehyde-starvation associative plasticity. Finally, using a conditional allele, I show that the behavioral roles of DAF-2 signaling in associative plasticity can be dissociated, with DAF-2 signaling playing a more significant role in the memory retrieval than in memory acquisition. These findings represent significant advances in the conceptual and mechanistic role of insulin signaling in C. elegans learning and memory, and
will undoubtedly raise hopes that the complete biological mechanisms involved in learning and memory of vertebrate organisms are coming within reach.
Chapter 2
Insulin Signaling Plays a Dual Role in *C. elegans* Memory Acquisition and Retrieval

Data from this chapter will be submitted for publication as:

2 INSULIN SIGNALING PLAYS A DUAL ROLE IN C. ELEGANS MEMORY ACQUISITION AND RETRIEVAL

2.1 Introduction

Learning is defined by a change in behavior as a result of experience and is advantageous for the survival of all animals. Given that animal behavior is regulated by the nervous system, any changes in behavior must involve neuronal plasticity and the interplay of multiple neurons. Studies using vertebrate model organisms have defined the behavioral classifications of learning. Unfortunately, the complexity of the nervous systems in higher organisms has limited their effectiveness as models to decipher the cellular and molecular mechanisms involved in learning and memory.

With a 302-neuron nervous system, C. elegans emerges as an excellent invertebrate model for the molecular study of behavioral plasticity. The nematode, despite its simple neural wiring, exhibits behavioral plasticity in a variety of learning paradigms. For example, C. elegans is capable of associating temperature or NaCl with the absence of food as demonstrated in thermotaxis learning and salt chemotaxis learning paradigms (Wen et al., 1997; Kodama et al., 2006; Tomioka et al., 2006). C. elegans also has the ability to associate the odorant butanone or salt taste with the presence of food (Wen et al., 1997; Torayama et al., 2007). Furthermore, the nematode can associate sickness caused by ingesting pathogenic bacteria with odorants that are specific to only the pathogenic strain (Zhang et al., 2005). It has also been shown that the nematode’s dramatic change in odorant preference after exposure to a high concentration of benzaldehyde in the absence of food is a result of associative learning where the nematode forms an association between benzaldehyde and starvation (Nuttley et al., 2002).
I will refer to this behavioral plasticity as benzaldehyde-starvation associative plasticity. While the molecular and cellular bases of this type of plasticity remains unclear, it presents a simple yet elegant platform from which to approach the greater question of how neural plasticity leads to emergent changes in animal behavior.

Recent work has shown that insulin signaling, well known for its role in regulating aging and growth in *C. elegans*, also mediates thermotaxis learning and salt chemotaxis learning in worms (Kodama *et al.*, 2006; Tomioka *et al.*, 2006). The components of insulin signaling, including the insulin homolog INS-1, its receptor DAF-2, and the PI3-kinase homolog AGE-1, are highly conserved between *C. elegans* and mammals. In mammals, insulin signaling pathways also have been implicated in learning and memory. For example, in mice, administration of insulin into the brain improved memory retention, whereas blocking insulin’s downstream signaling pathway by inhibition of PI3-kinase activity disrupted learning (Park *et al.*, 2000; Barros *et al.*, 2001a). Two lines of indirect evidence suggest this might be true in humans as well. First, deterioration of insulin receptor function in the central nervous system is linked to sporadic Alzheimer’s disease (Watson and Craft, 2003). Second, type 2 diabetic patients are more likely to show deficits in learning and memory tasks than non-diabetic controls (Ryan *et al.*, 2000). Although previous studies have highlighted the importance of insulin signaling in *C. elegans* associative learning, its role in mediating the changes in neural plasticity that underlies learning and memory are not well understood. It also is unclear behaviorally whether insulin signaling functions in memory acquisition or retrieval.
In this chapter, I present data demonstrating that worms with mutations in components of the insulin signaling pathways are defective in benzaldehyde-starvation associative plasticity, and that insulin signaling plays a more significant role in the retrieval than the acquisition of memory. INS-1 can act from multiple neurons and AGE-1 acts in benzaldehyde-sensing AWC sensory neurons to direct benzaldehyde-starvation associative plasticity. My findings dissociate the behavioral roles of insulin signaling in the regulation of learning versus memory recall and better elucidate the molecular mechanism involved in this associative plasticity in *C.elegans*.

### 2.2 Methods

#### 2.2.1 Strains and General Methods

The mutant strains used in this study were as follows: *ins-1(nr2091), age-1(hx546), age-1(mg305), daf-18(e1375), age-1(m333); daf-18(e1375), and daf-2 (e1370)*. Wild-type Bristol N2, *age-1(hx546), daf-2 (e1370), and daf-18(e1375)* strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. *ins-1(nr2091) and age-1(mg305)* were a generous gift from Dr. Gary Ruvkun. All experiments used well-fed adult animals cultivated at 20ºC (except in experiments using *daf-2 and age-1(mg305*) mutants where animals including controls were cultivated at 15ºC ) on nematode growth medium (NGM: 50 mM NaCl, 15 g/L agar, 20 g/L peptone, 1 mM cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 1 mM KH₂PO₄ pH=6.0) seeded with *Escherichia coli* strain OP50 under standard conditions unless otherwise specified (Brenner, 1974).
2.2.2 Odorant chemotaxis assay

Chemotaxis assays were carried out as previously described (Nuttley et al., 2002). Approximately 100 animals were transferred onto a 10cm Petri dish containing 6 mL of NGM agar. A 1 µL drop of testing odorant diluted in ethanol was added to one end and 1 µL of control odorant ethanol was added on the opposite end of the agar. 10 min prior to transferring the animals, 1µL of 1M NaN₃ was applied to the ends of the agar plates where the odorants would later be added in order to immobilize the animals once they reached the odorant spot. The dilution (V/V) for testing odorants was 1:100 for benzaldehyde and 1:500 for butanone. A chemotaxis index (C.I.) was calculated one hour (unless otherwise specified) after animals’ introduction to assess their preference for the test odorant. C.I. is calculated by the number of animals within 2 cm of the test spot minus the number of animals within 2 cm of the control spot divided by the total number of animals on the plate.

2.2.3 Odorant-starvation Plasticity Assay

The learning assays were carried out as previously described (Nuttley et al., 2002). In this assay, approximately 1000 worms are pre-exposed to the odorant of interest placed on the lid of the plate in the absence of food for 1 hour (unless specified otherwise) before being tested to the chemotaxis assay. Animals’ C.I. for the odorant after training is then measured using the chemotaxis assay as described above. In the benzaldehyde-starvation learning assay, animals were exposed to 2 µL of pure benzaldehyde placed on a piece of parafilm on the lid of the dish for 1 hour on a standard 10 cm Petri dish containing 6 mL of NGM agar and tested immediately to the chemotaxis assay after training. In the butanone-starvation learning assay,
animals were exposed to 2 μL pure butanone for 1 hour before being tested in the chemotaxis assay. Animals were trained and tested at 20°C unless otherwise specified.

2.2.4 Butanone enhancement assay

The butanone enhancement assay was performed as previously described (Torayama et al., 2007) with slight modifications. 1000 animals were placed on a NGM plate with a thick bacterial lawn and were exposed to 2 μL of butanone as described for the odorant-starvation assay. After 1 hour of exposure, animals were washed off the bacterial lawn and tested for chemotaxis to butanone using the chemotaxis assay.

2.2.5 Pathogen Avoidance Learning assay

The pathogen avoidance learning assay was used to measure the percentage of worms that were feeding on pathogenic bacteria at different time. To prepare the assay plates, 100 ul of saturated pathogenic strain Pseudomonas aeruginosa PA14 suspension and 100 ul of lab food E. coli OP suspension were spread on a NGM plate to make two small lawns that were approximately 40mm apart from each other and 10mm from the side of the plate. The plates were incubated at 20°C for 24 hours and 25°C for an additional 24 hours before use. For standard pathogen avoidance assay, approximately 250 animals were placed 5mm away from PA14 lawn and 5mm from the side of the assay plate. The plate was sealed with parafilm and the worms were allowed to move freely for four hours before being immobilized by placing the plate into -20°C for 10 minutes. The worms on and off the PA14 lawn were counted and the
percentage of worms on PA14 was calculated by the number of worms on PA14 lawn divided by the total number of worms on the plate.

2.2.6 PMA Treatment

PMA treatment was performed as described (Okochi et al., 2005). Briefly, adult animals were placed in standard culture plates containing 1 μg/ml PMA (PMA+) or DMSO solvent (PMA−) in the agar for 2 hr before being subject to chemotaxis assay to assess their chemotaxis to benzaldehyde.

2.2.7 Induction of Heat Shock

Heat-shock treatment was performed as previously described (Tomioka et al., 2006) with slight modifications. Animals were incubated at 33°C for 30 minutes in M9 buffer then transferred to a NGM plate seeded with OP50 E. coli at 20°C for 30 minutes before their examination in the benzaldehyde-starvation learning assay.

2.2.8 Generation of Rescue Strains

Germ-line transformation was performed by microinjection as described (Mello et al., 1991). pPD93.97(from A. Fire), which contained myo-3p::gfp or myo-3p::Venus were used as a co-injection marker for the rescue experiments. The DNA concentrations were 5 ng/μl for plasmid DNA carrying ins-1p::ins-1::Venus cDNA, along with 30 ng/μl myo-3p::GFP DNA and 65 ng/μl carrier DNA for ins-1 rescue experiments. The DNA concentrations were 30
ng/µl for plasmid DNA containing $daf-7p::ins-1::Venus$ cDNA, $glr-1p::ins-1::Venus$ cDNA, $ser-2p::ins-1::Venus$ cDNA, $odr-3p::ins-1::Venus$ cDNA, or $lin-11p::ins-1::Venus$ cDNA, along with 10ng/µl $myo-3p::Venus$ and 60ng/µl carrier DNA for $ins-1$ rescue experiments. The DNA concentrations were 70 ng/µl for plasmid DNA containing $ins-1(s)p::ins-1::Venus$ cDNA, along with 30ng/µl $myo-3p::GFP$ for $ins-1$ rescue experiments. The DNA concentrations were 70 ng/µl for plasmid DNA containing $ins-1(s)p::ins-1::Venus$ cDNA and 30 ng/µl for $daf-7p::ins-1::Venus$ cDNA, along with 30 ng/µl $myo-3p::GFP$ for rescue lines injected with both rescue constructs. The DNA concentrations were 50 ng/µl for plasmid DNA carrying $age-1$ cDNA under various promoters (except plasmid with $gcy-5p::age-1$ cDNA where 30 ng/µl were used), along with 50 ng/µl $myo-3p::GFP$ DNA and carrier DNA for $age-1$ rescue experiments. All the rescue constructs are generous gifts from Yuichi Iino lab.

### 2.2.9 Statistical Analysis

Two-way or three-way analyses of variance were used to examine group differences in all experiments. Bonferroni $t$ tests were used for post hoc analyses. T-tests were used for experiments with only two groups. The level of significance for all comparisons was $P < 0.05$. 
2.3 Results

2.3.1 Expression of *ins-1* is Required for Benzaldehyde-starvation Associative Plasticity

Wild type N2 naive (untrained) animals demonstrated a strong attraction response towards benzaldehyde that switched into an avoidance response after a one hour exposure to a high concentration of benzaldehyde in the absence of food (Fig. 1A). The avoidance response was more prominent when the odorant exposure time was extended to two hours (Fig. 1A). This switch in odorant preference results from associative learning where animals associate the conditioned stimulus (CS) benzaldehyde with the unconditioned stimulus (US) starvation, as the presence of food during benzaldehyde exposure suppresses this change in behavior (Nuttley *et al.*, 2002).

To better understand learning and memory at a cellular and molecular level, we searched for mutants that demonstrate deficits in benzaldehyde-starvation associative plasticity. Given the role of insulin signaling in learning and memory in both *C. elegans* and mammals, we examined insulin mutants and found that a mutation in the *ins-1* gene, a *C. elegans* homolog of mammalian insulin (Pierce *et al.*, 2001), completely suppressed the emergence of benzaldehyde avoidance after benzaldehyde training in the absence of food (Fig. 1A).

This lack of benzaldehyde-starvation learning in *ins-1(nr2091)* animals might be attributed to defects in sensing benzaldehyde (CS) or starvation (US), rather than in associative learning. To test the possibility that the *ins-1* animals’ plasticity deficit is a result of abnormal benzaldehyde sensation, we compared the naive chemotaxis of N2 and *ins-1* animals towards
1%, 0.1%, 0.05%, and 0.01% benzaldehyde and found no significant difference in naive benzaldehyde approach between the two strains (Fig. 1B). To determine if ins-1 animals can sense starvation, we examined the number of eggs laid on and off in ins-1 mutants. Consistent with the data reported previously that ins-1 mutants have normal starvation induced behavior (Kodama et al., 2006), we observed no difference in egg laying behaviors on or off food between ins-1 and N2 (Fig. 1C). The data suggest that mutations in ins-1 did not disrupt the animals’ ability to sense at least some aspects of starvation. To test the possibility that expression of INS-1 is required for the normal development of neurons involved in benzaldehyde-starvation learning, I performed a heat-shock rescue experiment. Rescue animals where ins-1 cDNA is under the control of a heat-shock promoter were generated, and the animals were heat-shocked at the adult stage. Expression of INS-1 during adult stage in ins-1(nr2091) animals rescued the defect in benzaldehyde-starvation learning (Fig. 1D), suggesting that the learning deficit observed in ins-1(2091) did not result from defects in neuronal development. Over-expressing INS-1 at the adult stage in wild type animals did not alter animals’ chemotaxis towards benzaldehyde (Fig. 1E).
Figure 1. *ins-1* mutants are defective in odorant-starvation associative plasticity

For all panels in the figure double asterisks represent significant differences from N2 within the same experiment (\(^{**}p < 0.05\) by Bonferroni \(t\)-test). Crosses represent significant differences.
between the indicated data points (+ p< 0.05 by Bonferroni t-test). Data represent means +/- s.e.m. (A) Benzaldehyde-starvation learning in wild type N2 and \textit{ins-1(nr2091)} worms. Animals were conditioned to 100% benzaldehyde in the absence of food for either 1 or 2 hours and immediately following conditioning, their chemotaxis to 1% benzaldehyde was examined. \textit{ins-1} mutants demonstrated significant deficits in benzaldehyde-starvation associative plasticity both after 1 hour and 2 hour of training. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(2, 35) = 147.12, P < 0.05$ (n = 6 plates for each data point). (B) Chemotaxis of N2 and \textit{ins-1(nr2091)} to various concentration of benzaldehyde. N2 and \textit{ins-1(nr2091)} were comparable in their chemotaxis to 1%, 0.1%, 0.05%, and 0.01% benzaldehyde, suggesting \textit{ins-1(nr2091)} animals sense benzaldehyde normally. A two-way analysis of variance revealed a main effect of benzaldehyde concentrations, $F(3,31) = 37.97, P < 0.05$, and no main effect of strain $F(1,31) = 0.078, P < 0.05$ (n = 4 plates for each data point). (C) Number of eggs of N2 and \textit{ins-1(nr2091)} laid in 1 hour on and off food. N2 or \textit{ins-1 (nr2091)} animal was placed on a standard OP50 food lawn or blank NGM agar and the number of eggs laid in 1 hour was scored. Starvation suppressed egg laying in both strains. A two-way analysis of variance revealed a main effect of food availability, $F(1, 35) = 155.71, P < 0.05$, and no main effect of strain $F(1,35) = 0.017, P < 0.05$ (n = 12 plates for each data point). (D) Rescue effect of \textit{ins-1} cDNA expression under a heat shock-inducible promoter in \textit{ins-1} animals. Animals were heat shocked at 33°C and allowed to recover before exposure to benzaldehyde in the absence of food. Expression of INS-1 under a heat shock promoter rescued the plasticity deficit in \textit{ins-1} mutants after heat shock. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(2, 35) = 32.06, P < 0.05$ (n = 6 plates for each data point). (E) The effect on chemotaxis by overexpressing INS-1 under a heat shock-inducible promoter in wild type animals. Expression of INS-1 under a heat shock promoter did not alter wild type animals’ chemotaxis towards benzaldehyde. A one-way analysis of variance revealed no significant difference between the strains, $F(2, 17) = 0.276, P = 0.763$ (n = 6 plates for each data point).
2.3.2 *ins-1* Mutants are Normal in Butanone-food Associative Plasticity and Pathogen Avoidance Learning

As with benzaldehyde, after exposure to butanone (an AWC-sensed odorant) in the absence of food, N2 wild type worms subsequently avoid butanone (Fig. 2A). However, after exposure to butanone in the presence of food, N2 wild type animals were more strongly attracted towards the odorant compared to naive (untrained) animals (Fig. 2A). This latter form of butanone-food associative plasticity has been termed “butanone enhancement” (Torayama *et al.*, 2007). To examine the extent of *ins-1(nr2091)*’s deficiency in odorant associative learning, I tested *ins-1* mutants in the butanone enhancement assay. *ins-1* animals demonstrated normal butanone-food associative plasticity when they were trained to pair the presence of food with butanone (Fig. 2A). Surprisingly, when *ins-1* animals were exposed to butanone in the absence of food, not only did *ins-1* mutants not demonstrate an avoidance behavior seen in the wild type, they exhibited an enhanced approach towards butanone similar to that seen in the butanone-food associative plasticity (Fig. 2A). The data imply that absence of INS-1 perhaps activates pathways that signal a “well-fed” state or the rewarding aspect of food. Therefore, even when exposed to butanone in the absence of food, *ins-1* mutants are still capable of associating the aberrant rewarding signal with butanone, thereby leading to an enhanced attraction towards the odorant. *ins-1* animals were also tested to pathogen avoidance learning assay to determine whether INS-1 mediates other behavioral plasticity. No significant difference was observed in the percentage of animals staying on pathogenic bacteria at four hours between *ins-1* and N2 animals (Fig. 2B), suggesting that INS-1 is not required for pathogen avoidance learning. Taken together, these data indicate that INS-1 functions specifically in associative plasticity where starvation is the US.
Figure 2. *ins-1* Mutants are normal in butanone-food associative plasticity and pathogen avoidance learning but defective in butanone-starvation associative plasticity

For all panels in the figure double asterisks represent significant differences from N2 within the same experiment (**p< 0.05 by Bonferroni t-test). Data represent means +/- s.e.m. (A) Butanone enhancement and butanone-starvation associative plasticity in N2 and *ins-1(nr2091)* mutants. Animals were conditioned to 100% butanone in the presence or absence of food for 1 hour and tested their chemotaxis to 0.5% butanone. *ins-1(nr2091)* mutants were normal in butanone enhancement but were defective in butanone-starvation associative plasticity. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(2, 35) =86.82, P < 0.05$ (n= 6 plates for each data point). (B) Pathogen avoidance learning in N2 and *ins-1* mutants. *ins-1* mutants avoided pathogenic bacteria as effectively as N2. A t-test revealed no significant difference between the two strains staying on pathogenic bacteria at the four hour time point, $t^{10} = 0.196, P= 0.848$ (n= 6 plates for each data point).
2.3.3 Animals with Mutations in the Components of DAF-2 Signaling are Defective in Benzaldehyde-starvation Associative Plasticity

Given that insulin can act as a neuropeptide to activate insulin/IGF-1 signaling in rodent neurons (Zhao et al., 2004), I examined mutants of daf-2, which encodes a homolog of the insulin/IGF-1 receptor, to determine whether a component of insulin signaling also regulates benzaldehyde-starvation associative plasticity. I found that daf-2(e1370) mutants demonstrated a strong defect in benzaldehyde-starvation associative plasticity (Fig. 3A). Consistent with the fact that daf-2(e1370) is a temperature sensitive allele, where the deficit in DAF-2 function is only apparent at temperatures above 20°C, I did not observe any plasticity defects when animals were trained and tested at 15°C (Fig. 3A). However, the plasticity defect in daf-2(e1370) became apparent when the animals were conditioned and tested at 20°C and the deficit was further exacerbated when the training and testing temperatures were raised to 23°C (Fig. 3A). The plasticity deficit observed in daf-2(e1370) likely is not due to sensory defects, as these mutants have normal benzaldehyde sensation and starvation induced behaviors (Murakami et al., 2005; Vellai et al., 2006).

Since AGE-1 (a homolog of PI3-kinase) is a known downstream target of DAF-2 for the regulation of daur formation and longevity in C. elegans (Baumeister et al., 2006), I sought to determine whether AGE-1 also plays a role in benzaldehyde-starvation associative plasticity. Two strains with hypomorphic alleles of age-1(hx546 and mg305) demonstrated a partial deficit in benzaldehyde-starvation associative plasticity (Fig. 3B). To examine whether the partial defects observed in age-1 mutants were due to the hypomorphic alleles, animals with a null age-1 mutation were investigated. Although null mutants of age-1 are lethal, the lethality can be suppressed by the absence of DAF-18 (a homolog of mammalian Phosphatase and
Tensin Homolog, PTEN). *age-1(m333null); daf-18 (e1375)* double mutants exhibited a similar partial deficit in benzaldehyde-starvation associative plasticity to those observed in *age-1(hx546)* and *age-1(mg305)* mutants (Fig. 3B). The partial learning deficit seen in *age-1* mutants cannot be attributed to sensory defects given these animals have normal benzaldehyde sensation and starvation behaviors (Murakami *et al.*, 2005; Vellai *et al.*, 2006). The data suggest that AGE-1 is likely a downstream target of DAF-2, but perhaps not the sole target activated by DAF-2 to mediate benzaldehyde-starvation associative plasticity.
A

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Chemotaxis Index

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Figure 3. Animals with mutations in the components of DAF-2 signaling are defective in benzaldehyde-starvation associative plasticity

For all panels in the figure double asterisks represent significant differences from N2 within the same group (**p< 0.05 by Bonferroni t-test). Crosses represent significant differences between the indicated data points (+ p< 0.05 by Bonferroni t-test). N.S. stands for not significant. Data represent means +/- s.e.m.  (A) Benzaldehyde-starvation associative plasticity in daf-2(e1370) mutants trained and tested at various temperatures. Wild type N2 and daf-2(e1370) mutants were conditioned and tested to benzaldehyde at 15°C, 20°C, and 23°C. daf-2 mutants exhibited defective benzaldehyde-starvation associative plasticity when trained and tested at 23°C (the temperature at which daf-2 is presumably defective in function) had normal benzaldehyde-starvation associative plasticity when trained at tested at 15°C (the permissive temperature). A three-way analysis of variance revealed a significant interaction between strain, conditioning, and temperature $F(2, 71) = 23.564, P < 0.05$ (n= 6 plates for each data point). (B) Benzaldehyde-starvation associative plasticity in mutants of the AGE-1 signaling pathway. age-1 mutants demonstrated partial plasticity defects, and daf-18 (e1375) animals exhibited significantly lower naive attraction towards benzaldehyde. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(4, 59) = 22.26, P < 0.05$ (n= 6 plates for each data point).
2.3.4 Animals with Hyperactive AGE-1 Signaling Exhibit Suppressed Attraction towards Benzaldehyde and Abnormal Butanone Olfactory Plasticity

To gain more insight into how AGE-1 signaling is required for benzaldehyde-starvation associative plasticity, mutants with hyperactive AGE-1 signaling were examined. Activation of AGE-1 leads to the generation of 3-phosphoinositide, whereas DAF-18 dephosphorylates 3-phosphoinositides (Ogg and Ruvkun, 1998). Animals with defective \textit{daf-18} therefore have an elevated level of 3-phosphoinositides and presumably hyperactive AGE-1 signaling. \textit{daf-18(e1375)} animals showed a significant decreased naive attraction towards benzaldehyde, but demonstrated a conditioned avoidance response to benzaldehyde similar to that of wild-type N2 after conditioning (Fig. 3B). The accumulation of 3-phosphoinositide with the \textit{age-1(m333null)} mutation fully reversed the decreased chemotaxis phenotype seen in \textit{daf-18} animals (Fig. 3B). When tested their approach to butanone, \textit{daf-18} animals showed a naive avoidance response towards butanone. Interestingly, \textit{daf-18} mutants exhibited enhanced butanone-starvation associative plasticity but impaired butanone-food associative plasticity (Fig. 4A). Treating \textit{daf-18} animals with β-phorbol ester phorbol 12-myristate 13-acetate (PMA), a pharmaceutical analog of diacylglycerol (DAG) known to increase synaptic transmission through activation of DAG signaling (Betz et al., 1998; Lackner et al., 1999), for two hours prior to testing significantly suppressed their attenuated naive attraction phenotype (Fig. 4B). Taken together, the data suggest that the level of AGE-1 signaling regulates the animal’s attraction to benzaldehyde, and perhaps imply that the level of insulin signaling establishes the extent and orientation of benzaldehyde chemotaxis through modulation of synaptic release.
Figure 4. daf-18 animals exhibit abnormal butanone chemotaxis and butanone olfactory plasticity.

For all panels in the figure double asterisks represent significant differences from N2 within the same group (**p< 0.05 by Bonferroni t-test). Crosses represent significant differences between the indicated data points (+ p< 0.05 by Bonferroni t-test). Data represent means +/- s.e.m. (A) Butanone olfactory plasticity in N2 and daf-18 (e1370) mutants. daf-18 mutants demonstrated an aversive naive response towards butanone and abnormal butanone-food associative plasticity and butanone-starvation associative plasticity. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(2, 35) =96.72, P < 0.05$ (n= 6 plates for each data point). (B) Naive benzaldehyde chemotaxis of N2 and daf-18 animals after 2 hr of PMA treatment. Treating daf-18 animals with PMA significantly reversed their decreased benzaldehyde chemotaxis. A two-way analysis of variance revealed a significant interaction between strain and PMA treatment, $F(1, 19) = 63.236, P < 0.05$ (n= 5 plates for each data point).
INS-1 Released from ASI and AIA Acts on Benzaldehyde-sensing AWC Sensory Neurons to Regulate Benzaldehyde-starvation Associative Plasticity

To better visualize and determine in which neurons INS-1 is required for benzaldehyde-starvation associative plasticity, I performed rescue experiments in ins-1(nr2091) by expressing INS-1 proteins fused with the reporter Venus. I found that the learning deficit was fully rescued when INS-1::VENUS was expressed from the ins-1 promoter (Fig. 5A), confirming that INS-1::VENUS was functional. Given that INS-1 has widespread expression in the nervous system but the expression is strongest and most consistent in AIA interneurons (Tomioka et al., 2006), it is hypothesized INS-1 is required in AIA interneurons for benzaldehyde-starvation associative plasticity. Surprisingly, expression of INS-1::VENUS in AIA only partially rescued the plasticity deficit observed in ins-1 mutants. Expression of INS-1::VENUS from different sets of neurons within the benzaldehyde-sensing AWC neural circuitry using glr-1, ser-2, daf-7, odr-3, lin-11 promoters also partially rescued the learning deficit of ins-1 mutants (Fig 5A-B). When ins-1 mutants were injected with both of the constructs that drive INS-1::VENUS under the ins-1(short) and daf-7 promoters, the expression of INS-1 in ASI and AIA was sufficient to fully rescue the learning deficit (Fig. 5A). Interestingly, close examination of ins-1p::ins-1::Venus expression and the rescue results revealed that expression of INS-1::VENUS from certain neurons that do not express INS-1 under ins-1 extrachromosomal array promoter could partially rescue the plasticity deficit in ins-1 mutants (Fig. 5A). Given that INS-1::VENUS is expressed in ASI and AIA under ins-1 promoter and that expression of INS-1 in ASI and AIA fully rescued the plasticity defect in ins-1 mutants (Fig 5A), INS-1 likely acts non cell autonomously from ASI and AIA either as a hormone or through synaptic delivery to mediate benzaldehyde-starvation associative plasticity under physiological conditions.
Since it is difficult to express DAF-2 from an extra-chromosomal array, I generated rescue lines of \textit{age-1}(hx546) injected with constructs driving \textit{age-1} cDNA in different subsets of neurons to identify the INS-1 targeting neurons in benzaldehyde-starvation associative plasticity. Expression of AGE-1 from neurons including interneurons AIY and AIZ using \textit{txx-3} and \textit{lin-11} promoters did not rescue the plasticity defect seen in \textit{age-1}(hx546) animals (Fig. 3D). However, when \textit{age-1} cDNA was expressed in AWC sensory neurons using \textit{odr-3} promoter, the plasticity deficit was rescued completely (Fig. 5C). In contrast, AGE-1 expression in other sensory neurons such as ASI, ASH, ADL, ASJ, AWB, and ASER using \textit{sra-6}, \textit{ser-1}, \textit{str-1}, and \textit{gcy-5} promoters did not rescue the learning deficit (Fig. 5C). These results indicate that AGE-1 functions in AWC sensory neurons, and INS-1 released from ASI and AIA might act on benzaldehyde-sensing AWC sensory neurons (perhaps through direct synaptic connections to AWC (White et al., 1986)) to regulate benzaldehyde-starvation associative plasticity.
**Chemotaxis Index**

**A**

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**B**

- **Ins-1(nr2091)**
- **Benzaldehyde**

**C**

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Figure 5. INS-1 can act from multiple neurons and AGE-1 acts in AWC to regulate benzaldehyde-starvation associative plasticity at the adult stage

For all panels in the figure double asterisks represent significant differences from N2 within the same group (**p< 0.05 by Bonferroni t-test). N.S. stands for not significant. Data represent means +/- s.e.m. (A) Rescue effect of INS-1::VENUS expression under different promoters in ins-1 animals. Expression of INS-1::VENUS from multiple sets of neurons partially rescued the plasticity deficit in ins-1 mutants, and expression of INS-1::VENUS in ASI and AIA was sufficient to fully rescue the deficit. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(9, 119) = 22.69, P < 0.05$ (n= 6 plates for each data point). (B) Part of the neural circuitry involved in benzaldehyde sensation (White et al., 1986). Arrows indicate synaptic connections; H shapes indicate gap junctions; triangles and hexagons represent sensory neurons and interneurons respectively. (C) Rescue effect of AGE-1 expression using various promoters in age-1(hx546) animals. The learning defect is rescued by the expression of AGE-1 in AWC neurons using the odr-3 promoter. Expression of AGE-1 in AIY interneurons using ttx-3, in subsets of neurons including AIZ using lin-11 promoter, in ADL and ASJ neurons using the sre-1 promoter, in ASER neurons using the gcy-5 promoter, in AWB neurons using str-1 promoter, or in ASH and ASI neurons using promoter sra-6 did not rescue the plasticity defect in age-1(hx546) animals. A two-way analysis of variance revealed a significant interaction between strain and conditioning, $F(8,107) = 52.39, P < 0.05$ (n= 6 plates for each data point).
2.3.6 DAF-2 Signaling Plays a More Significant Role in the Memory Retrieval than in the Memory Acquisition of Benzaldehyde-starvation Associative Plasticity

Given that a short-term learned event can be divided up into two components, memory acquisition and memory retrieval, we investigated whether DAF-2 signaling is required for the learned acquisition or the memory retrieval of benzaldehyde-starvation associative plasticity. Since DAF-2 is likely impaired in \textit{daf-2(e1370)} background, but normal at 15°C and also no plasticity deficit was observed when \textit{daf-2(e1370)} animals were trained and tested at 15°C (Fig. 6A), I examined \textit{daf-2(e1370)} animals’ learning ability when conditioned at 15°C but tested at 23°C and vice versa. This temperature shift assay permitted me to examine whether \textit{daf-2} animals would exhibit a deficit in behavioral plasticity by disrupting insulin signaling specifically during the acquisition or the recall phase of benzaldehyde-starvation associative plasticity. \textit{daf-2} animals trained at 15°C and tested at 23°C demonstrated a similar benzaldehyde attraction response compared to that of naive \textit{daf-2} animals (and thus no evidence of any memory performance), while \textit{daf-2} mutants conditioned at 23°C and tested at 15°C showed some degree of learning relative to that of the wild type (Fig. 6A). These data suggest that DAF-2 signaling is only partially involved in memory acquisition, but is essential in memory retrieval. Even when the conditioning time was extended to two hours to facilitate the acquisition of benzaldehyde-starvation associative memory, the same trend in memory performance by \textit{daf-2} mutants were observed where the plasticity deficit was more prominent when insulin signaling was disrupted during memory retrieval (Fig. 6B).

The memory retrieval deficit caused by a disruption of DAF-2 signaling can either reflect an erasure of benzaldehyde-starvation memory or a block of access to or output from the
otherwise intact memory. To distinguish between these two possibilities, I devised a conditioning protocol where *daf-2(e1370)* animals were trained at 15°C for 1 hour, washed and left in 23°C M9 buffer for 10 minutes to transiently disrupt DAF-2 signaling, and subsequently tested at 15°C to restore insulin signaling. If the disruption of DAF-2 signaling blocks the access to or output from the intact memory, then *daf-2* animals should show normal memory retrieval after insulin signaling is restored. On the other hand, if the retrieval deficit in *daf-2* mutants is caused by an erasure of memory, then the animals should not demonstrate any change in odorant preference (i.e. memory performance) after a temporary disruption of DAF-2 signaling. *daf-2* animals demonstrated comparable benzaldehyde-starvation associative plasticity to that of wild type animals when subjected to the temporary disruption of insulin signaling before testing (Fig. 6C), suggesting that the momentary disturbance in DAF-2 signaling did not impair memory performance of *daf-2* animals. To determine whether 10 minutes of 23°C up-shift is sufficient to disrupt insulin signaling, *daf-2* animals were trained at 15°C and tested to benzaldehyde at 23°C for 10 minutes rather than the standard 60 minutes testing period used in other experiments. *daf-2* mutants tested at 23°C for 10 minutes demonstrated a significant plasticity deficit compared to that of N2 worms, while the mutants tested at 15°C for 10 minutes did not exhibit any significant defect (Fig. 6D), suggesting that 10 minutes of temperature up-shift is sufficient to inactivate insulin signaling. Taken together, the data suggest the retrieval deficit observed in *daf-2* animals is not due memory erasure but rather due to a block in the access to or output from the memory.
Figure 6. DAF-2 signaling is partially involved in memory acquisition but essential in memory retrieval

For all panels in the figure double asterisks represent significant differences from N2 within the same group (**p< 0.05 by Bonferroni t-test). Crosses represent significant differences between the indicated data points (+ p< 0.05 by Bonferroni t-test). N.S. stands for not significant. Data represent means +/- s.e.m. (A) Benzaldehyde-starvation associative plasticity in daf-2(e1370) mutants when DAF-2 signaling is disrupted during memory acquisition or retrieval. Wild type N2 and daf-2(e1370) mutants were grown at 15°C, then conditioned at 15°C or 23°C and tested to benzaldehyde at 15°C or 23°C. Disruption of DAF-2 signaling during training partially blocked benzaldehyde-starvation associative plasticity while disruption of DAF-2 signaling during testing fully blocked this plasticity in daf-2(e1370) mutants. A three-way analysis of variance revealed a significant interaction between strain, conditioning, and temperature \( F(3,127) = 13.30, P < 0.05 \) (n= 8 plates for each data point). (B) Benzaldehyde-starvation associative plasticity in daf-2(e1370) mutants when the conditioning period is extended to 2 hours at two different temperatures. Disruption of DAF-2 signaling during training partially blocked benzaldehyde-starvation associative plasticity while disruption of DAF-2 signaling during testing fully blocked this plasticity in daf-2(e1370) mutants. A three-way analysis of variance revealed no significant interaction between strain, conditioning, and temperature \( F(1, 47) = 77.63, P < 0.05 \) (n= 6 plates for each data point). (C) Benzaldehyde-starvation associative plasticity in daf-2(e1370) mutants with transiently disruption of DAF-2 signaling after conditioning. Animals were conditioned at 15°C, washed and left in 23°C M9 for 10 minutes before testing at 15°C. Transient disruption of DAF-2 signaling did not block benzaldehyde-starvation associative plasticity in daf-2(e1370) mutants. A three-way analysis of variance revealed no significant interaction between strain, conditioning, and temperature \( F(1, 63) = \)
0.0019, \( P = 0.45 \) (n= 8 plates for each data point). (D) Benzaldehyde-starvation associative plasticity in \( daf-2(e1370) \) mutants when DAF-2 signaling is disrupted during memory retrieval. Animals were conditioned at 15°C and tested 15°C or 23°C for 10 minutes. \( daf-2 \) animals demonstrated a significant plasticity deficit when tested at 23°C for 10 minutes compared to that of wild type N2. A three-way analysis of variance revealed a significant interaction between strain, conditioning, and temperature \( F(1,47) = 7.08, P < 0.05 \) (n= 6 plates for each data point).

### 2.4 Discussion

In this study insulin signaling was shown to be crucial in regulating benzaldehyde-starvation associative plasticity in \( C. elegans \). Animals lacking functional INS-1, which is the closest human insulin homolog in \( C. elegans \) (Pierce et al., 2001), exhibited a severe deficit in benzaldehyde-starvation associative plasticity compared to wild type animals (Fig. 1A). Furthermore, animals with mutations in \( daf-2 \) and \( age-1 \) (which encode an insulin/IGF-1 receptor and a PI-3 kinase that are implicated in insulin signaling) are also defective in benzaldehyde-starvation associative plasticity (Fig. 3A-B). The rescue experiments suggest that INS-1 likely acts from ASI and AIA under physiological conditions and that DAF-2 signaling is required in the benzaldehyde-sensing AWC sensory neuron to mediate benzaldehyde-starvation associative plasticity (Fig. 5A-C). Given that ASI and AIA both synapse onto AWC (White et al., 1986), I speculate that INS-1 may act best through synaptic delivery to activate DAF-2 signaling in AWC for this associative plasticity.
2.4.1 The Attraction Signaling and Repulsion Signaling in AWC^{ON} may be Activated Simultaneously after Butanone Exposure

Since sensory preferences in *C. elegans* are modulated based on context and experience, it is intriguing that *ins-1* animals showed an enhanced attraction towards butanone after exposure to the odorant even in the absence of food (Fig. 2A). Butanone exposure in the presence of food enhances chemotaxis towards the odorant, while exposure to the same odorant in the absence of food results in an avoidance behavior (Torayama *et al.*, 2007). These behavioral changes have been suggested to be the result of switching between the competing attraction and repulsion activities in AWC^{ON} (Tsunozaki *et al.*, 2008). One possibility might be that when animals are exposed to a high concentration of butanone, pathways for both attraction and repulsion are activated in the AWC sensory neuron. In the absence of food, enhanced repulsion activities might antagonize the output of the attraction pathway, generating an avoidance response. On the other hand, the presence of food might inhibit repulsion pathways, thereby resulting in an enhanced attraction response. In such a scenario, INS-1 might function to induce the repulsion pathway in AWC. Therefore, in the absence of functional INS-1, the lack of a repulsion pathway enhances the attraction pathway in AWC by default, and leads to enhanced attraction towards butanone after butanone exposure in the absence of food. Alternatively, INS-1 perhaps suppresses pathways that signal a “well-fed” state or the rewarding aspect of food. Therefore, even when exposed to butanone in the absence of food, *ins-1* mutants are still capable of associating the aberrant rewarding signal with butanone, thereby leading to an enhanced attraction towards the odorant.
2.4.2 The Effect of AGE-1 Signaling Activation in Behavioral Plasticity may be Site Dependent

While *age-1* mutants are defective in salt chemotaxis learning and benzaldehyde-starvation associative plasticity (Tomioka *et al.*, 2006), these mutants have demonstrated enhanced learning ability when tested to thermotaxis learning (Kodama *et al.*, 2006), suggesting that AGE-1 can either promote or inhibit associative learning, depending on the paradigm. This paradox in AGE-1’s effect on learning perhaps can be explained by the neurons where AGE-1 signaling is activated in different learning paradigms. For instance, signaling through AGE-1 occurs in AIY, AIZ, or RIA interneurons to mediate thermotaxis learning (Kodama *et al.*, 2006), while AGE-1 signaling is required in ASER or AWC sensory neurons to regulate salt chemotaxis learning and benzaldehyde-starvation learning respectively (Tomioka *et al.*, 2006). It is possible that sensory neurons and interneurons possess dissimilar downstream effectors or different mediators that interact with AGE-1 signaling. Therefore, activation of AGE-1 singaling in different neurons could generate outputs that may either be inhibitory or stimulatory for behavioral plasticity. This hypothesis is similar to that attraction or repulsion induced by an odorant depends on the neuron in which the odorant receptor is expressed and activated. For example, activation of the ODR-10 receptor by diacetyl on AWC induced an attraction response, while stimulation with diacetyl of the same receptor on AWB generated an aversive response (Troemel *et al.*, 1997).

2.4.3 Insulin Signaling Plays a Partial Role in the Memory Acquisition of Benzaldehyde-starvation Associative Plasticity

It is of significant interest that the present data suggest that the behavioral roles of insulin signaling in benzaldehyde-starvation associative plasticity can be dissociated between
memory acquisition and memory retrieval, with insulin signaling playing a partial role in memory acquisition but an essential role in memory retrieval (Fig. 6A-C). INS-1 appears to mediate associative learning tasks that involve starvation as the US in *C. elegans*. Supporting this, *ins-1* mutants are defective in thermotaxis learning, salt chemotaxis learning, and benzaldehyde-starvation associative plasticity where starvation is the US (Kodama et al., 2006; Tomioka et al., 2006), but are normal in other associative learning tasks such as butanone-food associative plasticity and pathogen avoidance learning (Fig. 2A-B). Therefore, insulin signaling might function as one of the starvation signals necessary for the association process during the memory acquisition of benzaldehyde-starvation associative plasticity. This speculation is perhaps not surprising as insulin signaling is well known for its role in dauer formation, and food availability is one of the key cues to trigger dauer arrest (Baumeister et al., 2006). Even though we and others have observed that *ins-1* animals exhibit normal starvation behaviors such as the enhanced slowing response and starvation-suppressed egg laying (Kodama et al., 2006), it remains possible that multiple pathways might regulate different starvation responses, and that INS-1 transmits a learning specific starvation signal required for benzaldehyde-starvation associative plasticity. In this model, insulin signaling acts upstream of memory acquisition and perhaps functions as a part of the US pathways. However, it cannot be ruled out that INS-1 might be released from multiple neurons to maintain basal insulin signaling in neurons that are permissive for benzaldehyde-starvation associative plasticity, and that the disruption of this neuronal insulin signaling might have a detrimental effect that perturbs the machinery required for the memory acquisition.
2.4.4 Insulin Signaling is Essential in the Memory Retrieval of Benzaldehyde-starvation associative plasticity

Using the temperature shift assay, I found that functional DAF-2 signaling is critical during the retrieval phase of benzaldehyde-starvation associative plasticity (Fig. 6A). Furthermore, the benzaldehyde-starvation associative memory cannot be degraded by temporary disruption of DAF-2 signaling between training and testing (Fig. 6B), suggesting that continuous DAF-2 signaling is not required for the maintenance of memory. Therefore, I propose the associative plasticity deficit observed in DAF-2 animals is likely due to a block in the output of the memory.

My data suggest that the output of benzaldehyde-starvation associative memory may result from a switch in the signaling mode of AWC sensory neurons, depending on the level of insulin signaling in AWC. Animals with elevated AGE-1 signaling (i.e. daf-18 mutants) demonstrated an attenuated benzaldehyde naive attraction that could be reversed by increasing synaptic transmission through PMA treatment (Fig. 4B). Furthermore, AGE-1 is required in benzaldehyde-sensing AWC for benzaldehyde-starvation associative plasticity (Fig. 3B). Therefore, I propose that high levels of insulin signaling within AWC, a neuron associated with naive attractive responses towards benzaldehyde, perhaps switch the signaling mode of AWC from attraction to repulsion through decreasing synaptic release, and that the level of insulin signaling in AWC establishes the extent and orientation of benzaldehyde chemotaxis. Indeed, it has recently been proposed that AWCON neuron possesses the capacity to switch from an attractive to repulsive signaling mode by decreasing excitability or synaptic release through down regulation of gcy-28 activity and DAG/PKC signaling, thereby suppressing animals’ attraction towards benzaldehyde and generating an avoidance behavior towards the odorant
butanone (Tsunozaki et al., 2008). Further supporting the hypothesis that such a mechanism exists as an output of benzaldehyde-starvation associative memory, it has been suggested that elevation of insulin signaling might lead to suppression of neurotransmitter release from ASER sensory neurons (Tomioka et al., 2006). Additionally, the PI3-kinase pathway, which operates downstream of DAF-2 in the worm (Baumeister et al., 2006), has been shown to modulate the excitability of rodent olfactory receptor neurons (Spehr et al., 2002). The finding that $daf-18$ mutants avoided AWC sensed butanone under a naive condition (Fig 4A) is also consistent with the hypothesis that high levels of insulin signaling switches the signaling mode of AWC. It will be interesting to determine whether high levels of insulin signaling in AWC might lead to a decrease in excitability or synaptic release through lowering $gcy-28$ activity and DAG/PKC signaling, thereby switching the output signaling mode from attraction to repulsion. However, this hypothesis remains to be confirmed with further molecular and electrophysiological analyses in these pathways.
Chapter 3
General Discussion
3 GENERAL DISCUSSION

The goal of behavioral genetics is to identify and define the genetic mechanisms by which sensory information is encoded and processed to generate specific behaviors. To achieve such a goal, one needs a model system with sophisticated genetic tools and behavioral expression of learning to determine the genes important for experience dependent plasticity and the mechanism in which the genes act. The model organism *C. elegans* combines behavioral complexity, genetic accessibility, and a well characterized compact nervous system, and therefore serves as an excellent platform to understand the relationships between genes and neural plasticity that leads to emergent changes in animal behavior.

Even though insulin signaling has been implicated in learning and memory in mammals, fundamental questions remain on how it modulates neural plasticity, thereby leading to emergent changes in animal behavior. To solve such a question, others have studied the role of insulin signaling in learning and memory using *C. elegans* (Kodama et al., 2006). Although previous studies have highlighted the importance of insulin signaling in *C. elegans* associative learning, its role in mediating the changes in neural plasticity that underlies learning and memory remains poorly defined. It also is unclear whether insulin signaling functions in *C. elegans* memory acquisition or retrieval.

*C. elegans*’ dramatic change in odorant preference after exposure to a high concentration of benzaldehyde in the absence of food is a result of associative learning where the nematode forms an association between benzaldehyde (CS) and starvation (US). In the
previous chapter, I present data demonstrating insulin signaling is required in three neurons ASI, AIA, and AWC to regulate this associative learning task. Furthermore, the behavioral role of insulin signaling in benzaldehyde-starvation associative plasticity can be dissociated into memory acquisition and retrieval, where insulin signaling may act in the US pathway as a starvation signal during memory acquisition and in the memory output pathway during memory retrieval. In this chapter, based on my findings that memory acquisition and retrieval are mediated in three neurons, and with the understanding of insulin signaling’s behavioral role in benzaldehyde-starvation associative plasticity, I propose three plausible models that describe the role of insulin signaling in mediating the neural mechanisms underlying memory acquisition, storage, and retrieval in *C. elegans*.

### 3.1 Model 1: The Maintenance of High Levels of DAF-2 Signaling in AWC Functions as the Benzaldehyde-starvation Associative Memory

One possible model to account for my data is that the maintenance of high DAF-2 signaling in AWC functions as the benzaldehyde-starvation associative memory. In this scenario, INS-1 is released from AIA and ASI in the absence of food, and activates DAF-2 signaling in AWC to transmit the starvation signal, but the level of DAF-2 signaling in AWC is limited by the activity of DAF-2 or its downstream components, thereby maintaining the attraction signaling mode in benzaldehyde-sensing AWC under a naive condition. Sensory association of starvation signals and benzaldehyde sensed by AWC may result in a sustained increase in the synaptic integration of DAF-2 receptors or the activity of DAF-2 downstream components in AWC. Therefore, once the association processes have taken place, release of INS-1 can lead to high level of DAF-2 signaling in AWC and switch the neuron into a
repulsion signaling mode, thereby generating an avoidance behavior upon subsequent benzaldehyde exposure. Essentially, the model suggests the memory is stored as an activation of unknown machinery that maintains high level of synaptic integration of DAF-2 receptors or activity of DAF-2 downstream effectors in AWC. However, this model implies that AWC is in a predetermined repulsion mode after memory acquisition. It has been demonstrated that animals trained to associate benzaldehyde with starvation in the presence of salt lack benzaldehyde-starvation associative plasticity upon subsequent exposure to benzaldehyde in a salt free environment, suggesting that the memory retrieval of benzaldehyde-starvation associative plasticity follows an occasion-setting mechanism, in which context cues (i.e. salt) function in a hierarchical fashion to modulate memory recall by defining an appropriate setting (Law et al., 2004). Therefore, this current model cannot account for the context dependent aspect of memory retrieval, unless one argues that the retrieval of context memory may act downstream of the benzaldehyde-starvation associative memory output as a gatekeeper to allow the transmission of the repulsion signaling originated from AWC.
**A. Naive Attraction**

INS-1(US) → BZ (CS) → DAF-2

**AGE-1**

Low level of insulin signaling

AWC

Attraction  Repulsion

---

**B. Memory Acquisition**

BZ (CS) → DAF-2 → AGE-1

**Sense association/Memory encoding**

AWC

---

**C. Memory Retrieval/Conditioned Avoidance**

INS-1(US)

**AGE-1**

High level of insulin signaling

AWC

Absence of context memory retrieval  Repulsion
Figure 7. A model for the regulation of benzaldehyde-starvation associative plasticity by insulin signaling where memory is stored as a maintenance of high levels of insulin signaling.

(A) Benzaldehyde-sensing AWC neurons are in an attraction signaling mode under low levels of insulin signaling, thereby generating a naive attraction response towards benzaldehyde (BZ). INS-1 is released from AIA and ASI from the synapse in the absence of food to activate insulin signaling in AWC, but the level of insulin signaling in AWC is limited either through the number of DAF-2 receptors or activity of its downstream effectors under the naive condition.

(B) During the memory acquisition phase, benzaldehyde-sensing AWC receives US starvation signals (i.e. INS-1 and other unknown proteins) from ASI, AIA, and other unknown neurons. Under CS benzaldehyde exposure, the CS pathways and US pathways are associated along with the context, thereby inducing molecular changes in AWC to activate some unknown machinery that causes and maintains high levels of DAF-2 membrane integration or high activity of DAF-2’s downstream components. 

(C) The increase in DAF-2 or the activity of its downstream effectors leads to high levels of insulin in AWC, which then suppresses the attraction signaling and promotes the repulsion signaling, thereby generating a conditioned avoidance response towards the odorant benzaldehyde upon subsequent exposure. In this model, the retrieval of the context memory presumably acts downstream of the retrieval processes of benzaldehyde-starvation associative memory by regulating the transmission of the repulsion signaling transmitting from AWC.
3.2 Model 2: The Level of Insulin Signaling in AWC is Regulated Presynaptically Where the Benzaldehyde-Starvation Associative Memory is Stored in the ASI and AIA Neurons

To account for the occasion-setting effect in benzaldehyde-starvation associative plasticity, a second alternative possibility is that the level of insulin signaling in AWC (i.e. the signaling mode of AWC) is regulated presynaptically by the increased release of INS-1, which acts as a memory output signal downstream of memory retrieval (Fig. 5). For example, AIA and ASI release low level of INS-1 that elicits low DAF-2 signaling in AWC to transmit the starvation signal under the naive condition. AWC senses benzaldehyde and receives the context signal (i.e. salt) from salt-sensing ASE, and these sensory signals are associated with starvation signals (INS-1 is one of the starvation signals) coming from ASI, AIA, and other unknown neurons. The association process could lead to changes in AWC that sends a memory encoding signal feeding back onto ASI and AIA to store the benzaldehyde-starvation associative memory. Upon subsequent reception of salt signals from salt-sensing ASE that define the appropriate context for memory recall and benzaldehyde signals from AWC, AIA and ASI release much greater INS-1 than when starvation is present alone. The increase in INS-1 in the synapses elevates DAF-2 signaling in AWC to switch the neuron into an avoidance signaling mode, thereby inhibiting benzaldehyde attraction and promoting benzaldehyde repulsion. In contrast to the previous model, this model suggests that sensory association occurs in AWC, and that memory storage and recall occur in AIA and ASI, while an increase in INS-1 release acts as a memory output signal to switch the signaling mode in AWC once the memory in AIA and ASI is recalled. In such a model, one must assume the increase in INS-1 release is memory retrieval dependent, as over expressing INS-1 under a heat shock promoter at the adult stage in wild type animals did not attenuate animals’ naive attraction towards benzaldehyde. However if such an assumption is true, then to account for
the partial rescue effect in ins-1 mutants when INS-1 is expressed from multiple sets of neurons other than AIA and ASI (Fig. 3A), one must assume memory storage and retrieval can also occur in these neurons that may or may not have direct synaptic connections with benzaldehyde-sensing AWC. Remarkably, such a model echoes the classical view of associative learning where the internal representation of the CS (i.e. benzaldehyde signaling) excites the internal representation of the US (i.e. INS-1 release) to elicit a conditioned response (i.e. avoidance) once the CS and US are associated.
**Naive Attraction**

- INS-1 (US)
- ASI
- AIA

- BZ (CS) → DAF-2 → AGE-1
- Low level of insulin signaling

- AWC
- Attraction → Repulsion

**Memory Acquisition**

- INS-1 (US)
- ASI Memory encoding
- AIA Memory encoding

- BZ (CS) → Sensory association
- Starvation signal (US)

- AWC
- Memory encoding signal

**Memory Retrieval**

- INS-1 (US)
- ASI Memory Retrieval
- AIA Memory Retrieval

- BZ (CS) → Benzaldehyde signaling
- Salt (Context)

- AWC

**Conditioned Avoidance**

- INS-1 (US)
- ASI
- AIA

- BZ (CS) → DAF-2
- AGE-1
- High level of insulin signaling

- AWC
- Attraction → Repulsion
Figure 8. A model for the regulation of benzaldehyde-starvation associative plasticity by insulin signaling where memory is stored in the ASI and AIA neurons

(A) Benzaldehyde-sensing AWC neurons are in an attraction signaling mode under low levels of insulin signaling, thereby generating a naive attraction response towards benzaldehyde (BZ). INS-1 is released from AIA and ASI from the synapse in the absence of food to activate insulin signaling in AWC, but the level of insulin signaling in AWC is low due to low release of INS-1 under the naive condition. (B) During the memory acquisition phase, benzaldehyde-sensing AWC receives context information (i.e. presence of salt) from salt-sensing ASE and US starvation signals (i.e. INS-1 and other unknown proteins) from ASI, AIA, and other unknown neurons. Under CS benzaldehyde exposure, the CS pathways and US pathways are associated along with the context, thereby inducing molecular changes in AWC and sending a memory encoding signal to AIA and ASI to encode the memory (AIA and ASI are connected through gap junctions). (C) During the memory retrieval phase, reception of benzaldehyde signals from AWC and appropriate context signals (i.e. presence of salt) from ASE causes active recall of the memory and leads to enhanced INS-1 release from AIA and ASI. (D) Once the memory is retrieved, the increase in INS-1 release leads to high levels of insulin in AWC which then suppresses the attraction signaling and promotes the repulsion signaling, thereby generating a conditioned avoidance response towards the odorant benzaldehyde. Essentially, the level of insulin signaling in AWC establishes the extent and orientation of benzaldehyde chemotaxis, but the change in the insulin signaling level is regulated by the molecular processes involved in memory acquisition and retrieval in AIA and ASI.
3.3 Model 3: The Level of Insulin Signaling in AWC is Regulated Postsynaptically Where the Benzaldehyde-Starvation Associative Memory is Stored in the AWC Neuron

Alternatively, a third model might be that INS-1 is released from AIA and ASI as a starvation signal, but the level of DAF-2 signaling in AWC (i.e. the signaling mode of AWC) after memory retrieval is regulated postsynaptically by the activity of DAF-2 or its downstream effectors rather than the amount of INS-1 at the synapse (Fig. 6). In this model, AWC senses benzaldehyde and receives context signals (i.e. salt) from ASE and starvation signals (INS-1 is one of the starvation signals) from ASI and AIA and other unknown neurons. The sensory signals are associated along with the context cues and leads to molecular changes that encode the associative memory within AWC rather than ASI or AIA. During the memory retrieval phase of benzaldehyde-starvation associative plasticity, the memory is recalled when AWC senses benzaldehyde under the appropriate context (i.e. salt). The retrieval of the associative memory subsequently leads to high levels of DAF-2 signaling in AWC either through an increase in synaptic integration of DAF-2 or activity of its downstream components, and switches AWC from an attractive to a repulsive signaling mode, thereby generating an avoidance behavioral output towards benzaldehyde. The proposed model places the increase in the number of DAF-2 receptors (or the activity of its post receptor signaling components) downstream of memory retrieval, and predicts that the secretion of INS-1 from any neurons should rescue the plasticity deficit in \textit{ins-1} animals as long as sufficient INS-1 is present at the synapse to augment DAF-2 signaling in AWC when the memory is retrieved. Indeed, the partial rescue effect observed in \textit{ins-1} animals when INS-1 is artificially expressed from multiple sets of neurons perhaps validates such a prediction. However, this model would predict that animals trained to associate benzaldehyde with starvation will not demonstrate an aversion response towards benzaldehyde when tested in the presence of abundant food, as a
lack of starvation should prevent the release of INS-1 into the synapses. This prediction will be
difficult to verify since *E. coli* releases a variety of odorants that may mask the smell of
benzaldehyde and therefore confound the results. Testing animals on a thin lawn of food may
resolve such a problem, but whether a thin lawn of bacteria is sufficient to suppress starvation
signals in the nematode will be difficult to address. Remarkably, such a model would suggest
that a single sensory neuron in *C. elegans* may possess the capacity of sensory association,
memory storage, and memory recall when such complex neural functions are believed to be
mediated by multiple neurons in specific brain regions in vertebrate organisms.
**A** Naive Attraction

- INS-1 (US)
- DAF-2
- AGE-1
- Salt

Low level of insulin signaling

Attraction

Repulsion

**B** Memory Acquisition

- INS-1 (US)
- DAF-2
- AGE-1

Salt

Memory encoding

**C** Memory Retrieval

- ASE
- BZ

Memory retrieval

**D** Conditioned Avoidance

- INS-1 (US)
- DAF-2

High level of insulin signaling

Attraction

Repulsion
Figure 9. A model for the regulation of benzaldehyde-starvation associative plasticity by insulin signaling where memory is stored in the AWC

(A) Benzaldehyde-sensing AWC neurons are in an attraction signaling mode under low levels of insulin signaling, thereby generating a naïve attraction response towards benzaldehyde (BZ). INS-1 is released from AIA and ASI from the synapse in the absence of food to activate insulin signaling in AWC, but the level of insulin signaling in AWC is limited either through the number of DAF-2 receptors or activity of its downstream effectors under the naive condition.

(B) During the memory acquisition phase, benzaldehyde-sensing AWC receives context information (i.e. presence of salt) from salt-sensing ASE and US starvation signals (i.e. INS-1 and other unknown proteins) from ASI, AIA, and other unknown neurons. Under CS benzaldehyde exposure, the CS pathways and US pathways are associated along with the context, thereby inducing molecular changes in AWC to store the associative memory. 

(C) During the memory retrieval phase, sensation of benzaldehyde and reception of the appropriate context signal (i.e. presence of salt) in AWC leads active recall of the associative memory that subsequently leads to either an increase in the membrane integration of DAF-2 or the activity of its downstream components in AWC. 

(D) Once the memory is retrieved, the increase in DAF-2 or the activity of its downstream effectors leads to high levels of insulin in AWC, which then suppresses the attraction signaling and promotes the repulsion signaling, thereby generating a conditioned avoidance response towards the odorant benzaldehyde.
3.4 Conclusion

In summary, the work presented in this thesis sheds light on the behavioral role of insulin signaling in *C. elegans* associative learning and memory, and pinpoints the location in which insulin signaling is functioning for the regulation of benzaldehyde-starvation associative plasticity. These data will help disclose how olfactory memories are integrated, encoded, and retrieved. Presumably, a single learned event can be divided into memory acquisition, storage, and retrieval. In all the models proposed here for benzaldehyde-starvation associative plasticity, benzaldehyde induced AWC activity activates the data storage location either for memory encoding or retrieval. The benzaldehyde-starvation associative memory is generated when the starvation data input, suggested here as the INS-1 from AIA and ASI, is released onto the AWC while AWC is activated by benzaldehyde, and the memory is subsequently encoded within AWC (model 1 and 3) or transferred to AIA and ASI for storage (model 2). During memory retrieval, benzaldehyde induced AWC activity activates these storage sites to generate repulsion signaling outputs from AWC. The neurons, on which AWC synapses, read these repulsion data outputs to cause an avoidance behavior towards benzaldehyde. Here, the repulsion signaling output from AWC is proposed to be the result of high levels of insulin signaling within AWC that can be achieved either through a presynaptic (model 2) or a postsynaptic mechanism (model 1 and 3). Whereas model 1 assumes that the maintenance of high levels of insulin signaling is the benzaldehyde-starvation associative memory, model 3 assumes that high levels of insulin signaling result from memory retrieval.

The three models proposed here provide preliminary working models that may facilitate the understanding of the neural mechanisms underlying benzaldehyde-starvation associative...
plasticity in *C. elegans*. Clearly, all three models require certain assumptions to fully account for the data. Behavioral plasticity mutants identified in future experiments may further elaborate on the three models by adding more genetic components into the acquisition, storage, and recall processes, and may help determine the correct model for benzaldehyde-starvation associative plasticity. For example, if the phenotype of a temperature sensitive behavioral plasticity mutant is found to be due to a deficiency in maintaining benzaldehyde-associative memory, performing rescue experiments in this strain might identify the memory storage site and therefore help differentiate model 1 and 3 from model 2 as the right model. Since model 1 and 3 differ on where the context memory is stored, a mutant deficient in occasion setting may help identifying the storage site of the context memory. *lrn-2* mutants have been demonstrated to be deficient in occasion setting, as these animals can always recall the benzaldehyde-starvation associative memory regardless of the context (Law *et al.*, 2004). Although the molecular identity of *lrn-2* remains unknown, cloning of *lrn-2* should allow rescue experiments in this mutant strain for the occasion setting deficit to locate the site of occasion setting processes. The findings from such an experiment should be useful in determining which model (i.e. model 1 or 3) is correct. If the correct model for benzaldehyde-starvation associative plasticity can be determined, it will certainly lead to better understanding of the biological mechanisms of learning and memory in more complex organisms such as vertebrate animals.
References


