THE TRPV CHANNEL OSM-9 MEDIATES OLFACTORY LONG- TERM MEMORY CONSOLIDATION INDEPENDENT OF SLEEP

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SUMMARY

Memory is one of the most important abilities of the brain. It is defined as an alteration in behavior from an experience. For example, the *C. elegans* nematode will downregulate its chemotactic response to an innately attractive odor if it is not paired with food. This process is an example of olfactory classical conditioning. Through spaced training with this odor in the absence of food, *C. elegans* will maintain this memory of the odor for a prolonged period of time, akin to long-term memory formation. Although TRP channels are classically thought of as primary sensory receptors, intriguingly, it has been reported that the OSM-9/TRPV5/TRPV6 (TRP vanilloid) channel is required for odor short-term memory. We have discovered a new role for *osm-9/TRPV* in long-term olfactory memory formation (LTM). The *osm-9* mutant animals can acquire memory of the conditioned stimulus (odor) just like wild-type, but are unable to consolidate the memory for long-term maintenance. Interestingly, this TRPV-mediated long-term memory does not require sleep to form the long-lasting memory. Additionally, we found that genomic DNA and not cDNA is able to rescue the short-term odor memory defects seen in the null mutants. TRP channels are well studied in their ability to sense noxious stimuli, yet they are also implicated in memory and many diseases and disorders that limit neural plasticity, including Parkinson's disease, Alzheimer's disease, stroke, and sense disorders including anosmia. Our studies uncovered a role of TRP channels in neural plasticity, specifically, in olfactory long-term memory formation. These findings will broaden our knowledge of TRP channel function as a mediator of plasticity and memory, which could also potentially aid in understanding and treating the many diseases and disorders caused by TRP channel dysfunction.

INTRODUCTION

Transient receptor potential (TRP) ion channels were first discovered in the *Drosophila* eye photoreceptor cells (Cosens and Manning, 1969; Montel and Rubin, 1989; Minke, 2010), but have since been found in mammals and even in the single-celled yeast. Classically, TRP channels have been well established as magnesium-, sodium-, and calcium-permeable cation channels that can be activated by and simultaneously integrate multiple intracellular or extracellular stimuli to drive downstream signaling and membrane depolarization. These stimuli include, but are not limited to, pain, temperature, pH, injury, osmolarity, and cytokines (Yizheng Wang, 2017). There are seven TRP channel families, all sharing a similar topology, with six transmembrane helices, a short pore helix, and a cation-permeable pore loop region. The variability between the TRP families depends on the number of N-terminal ankyrin repeat domains and the presence of a C-terminal TRP box helix, which is a long helix parallel to the membrane (Muller et al., 2019). One TRP protein family is the TRP vanilloid-type (TRPV). TRPV proteins are generally considered as first-line nociceptive sensory receptors and include TRPV1-6, where TRPV1 is the canonical protein. TRPV1 is stimulated by vanillin, vanillic, endogenous lipids like the *N*-acylamides (e.g. endocannabinoids such as anandamide), high temperature (≥43°C), pain, ethanol, low pH, black pepper, garlic (allicin), cannabidiol (CBD), arachidonic acid, resiniferatoxin, and spicy foods, driven by the agonist capsaicin (De Petrocellis et al., 2011; Wang, 2017; De Petrocellis et al., 2017; Muller et al., 2019). The six ankyrin repeats in TRPV1 each contain two short anti-parallel helices and a finger loop and have been shown to interact with the proteins calmodulin and PI3K (Hellmich and Gaudet, 2014; Muller et

al., 2019). Upon activation, the TRPV1 channel allows calcium ions to enter the cell at the plasma membrane through the pore loop region, ultimately leading to desensitization of the channel and a subsequent refractory period (Muller et al., 2019). This desensitization is a large focus in the pain field with research focusing on alleviating acute and chronic pain. Although the sensory role of TRPV proteins has been well established, other roles of the TRPV channels, including the promotion of neural plasticity required for memory formation, are much less known.

Learning and memory are vital to survival. Organisms must update their knowledge of their surroundings based on environmental cues. One such way to promote learning and memory is through neural plasticity, which involves changes at the structural, molecular, and functional levels. Pioneering studies on the *Aplysia* gill-withdrawal reflex demonstrated the importance of synaptic plasticity on learning and memory through *in vitro* pharmacology and physiology experiments (Lent, 1976). Learning and memory are thought to be driven by two types of synaptic plasticity: the first is Hebbian, which includes synaptic long-term potentiation (LTP) and long-term depression (LTD; Fox and Stryker, 2017), and the second is non-Hebbian homeostatic compensation, which includes synaptic scaling (Turrigiano et al., 1998; Fox and Stryker, 2017). A large number of molecules implicated in these processes include factors that reorganize the actin cytoskeleton, affect trafficking, insertion and localization of receptors and cell adhesion molecules, localization of newly translated products, kinase function, and transcription of new genes (Bailey et al., 2015). As deep as, our understanding of these synaptic processes may be, we are still missing the roles of

some molecular players for memory in any intact organism. One protein type that is gaining new evidence for its role in memory is the TRP channel.

Although TRP channels have been studied thoroughly for their roles in sensation in the peripheral nervous system (Julius, 2013), they have more recently been studied for roles in the central nervous system, including neural plasticity (Sawamura et al., 2017). For example, TRPV channels have hippocampal expression and have been implicated in slow synaptic transmission (Eguchi et al., 2016), LTP (Marsch et al., 2007), and LTD of excitatory synapses on the interneurons (Gibson et al., 2008). Neural plasticity underlies learning and memory formation, e.g. through long-term potentiation and depression (LTP and LTD, respectively) of synapses in the mammalian hippocampus. Synaptic plasticity has been shown to underlie learning and memory, and TRP channels have recently been studied for a role in these processes. A TRPA channel (painless) has been observed to play a role in fly courtship associative long-term memory (Sakai et al., 2013). In the mammalian brain, the TRPCs function in the hippocampus to drive synaptic transmission and plasticity, and subsequent spatial working memory (Tai et al., 2008; Zhou et al, 2008). In addition to the hippocampus, expression of TRPCs has been uncovered in the temporal lobe, suggesting a role in neural plasticity, learning, and memory. TRPM4 has also been found to be expressed in the mammalian hippocampus and is important for plasticity and spatial working and reference memory (Bovet-Carmona et al., 2018). Several recent studies have implicated mammalian TRPV1 in memory formation, including fear memory consolidation behavior in mice (Genro et al., 2012; Marsch et al., 2007; Li et al., 2008). Acute stress was found to block spatial memory retrieval in young rats and also blocked LTP and drove LTD in

CA1 slices of the hippocampus, whereas TRPV1 agonists allowed spatial memory and LTP while blocking LTD, even in the presence of stress (Li et al., 2008). These TRPV1 memory studies were done with either pharmacological agent infusion to activate TRPV1 or by studying the behavior of TRPV1 knockout mice. Further dissection of the exact cellular or molecular interactions occurring in the behaviorally-defective mice would help gain a mechanistic understanding of how learning and memory are driven. An outstanding question to also answer is which stage of memory formation is mediated by TRP channels: acquisition, consolidation, or recall? In order to study the required genes in a live animal model as it is behaving, unbiased screens for genes required for learning and memory would need to be performed.

Using *C. elegans*, several unexpected genes have been identified more than 25 years ago in screens for short-term circuit plasticity, one being the transient receptor potential vanilloid 5 and 6 (TRPV5 and TRPV6) channel, OSM-9 (Colbert and Bargmann, 1995). The *C. elegans* nematode exhibits olfactory classical conditioning: prolonged exposure to innately attractive food-related odors in the absence of food elicits a downregulated response to the odor (Colbert and Bargmann, 1995). The *osm-9* gene is required for short-term olfactory memory in this paradigm. However, the mechanisms and dynamics of TRPV function in memory formation remain unknown. In addition, a role for *osm-9* in long-term memory has not been reported. Examination of the mechanisms and dynamics underlying TRPV-mediated memory in live *C. elegans* would help us understand how these channels act in an endogenous setting to integrate signaling over time to promote memory formation as the worms behave.

C. elegans provides a good model system to approach this problem. It is genetically-tractable and its nervous system is comprised of 302 neurons connected via 7,000 synapses. Also, it is the only organism where the entire connectome has been mapped (White et al., 1986; Cook et al., 2019, whereas the mammalian nervous system has 100 billion neurons connected by trillions of synapses and the entire neural connectivity map remains unknown (Kaiser, 2015). Additionally, it can form long-term associative memories of olfactory stimuli (Kaufmann et al., 2011). Of great help is that the neural circuit underlying olfaction is well established (White et al., 1986; Cho et al., 2016; Gordus et al., 2015). Importantly, it is transparent, making live cell imaging in the live animal a possibility. Thus, studying plasticity in the olfactory circuit in *C. elegans* has the potential to give us insight into the mechanisms by which experience changes complex neural circuits to promote memory formation.

The *osm-9* gene is an ortholog of human TRPV5 and TRPV6. TRPV5 and TRPV6 not like the rest of the TRPV family, as they are not ligand-gated, nor are they thermosensitive (Vennekens et al., 2000; Nilius et al., 2000). TRPV5 and TRPV6 are localized to epithelial cells in the kidney and intestine (van Goor et al., 2017) and are calcium-selective channels constitutively open at physiological membrane potentials and are modulated by calmodulin in a calcium-dependent manner (Dang et al., 2019). Initially, *osm-9*/TRPV5/TRPV6 was first discovered in a screen for *C. elegans* mutants defective for sensing high osmolarity. It was subsequently studied for its role in short-term associative olfactory memory where the nematodes learn to ignore food-related odors when in the absence of food (Colbert and Bargmann, 1995; Colbert et al., 1997). Later studies revealed that *osm-9* also has a role in sensing noxious stimuli, causing

SDS avoidance behavior through expression in the ASH sensory neurons (Tobin et al., 2002). This avoidance behavior can be phenocopied through heterologous expression of mammalian TRPV1 in ASH and application of the capsaicin agonist (Tobin et al., 2002), where calcium is likely driven into the cell through the pore loop region to elicit depolarization (Lindy et al., 2014). Polyunsaturated fatty acids, including the TRPV1 endogenous activator arachidonic acid, function upstream of *osm-9* to mediate sensory transduction (Kahn-Kirby et al., 2004). The endogenous agonist nicotinamide was discovered to activate an OSM-9 and OCR-4 heteromer expressed heterologously in *Xenopus* oocytes, and nicotinamide was sufficient to phenocopy *osm-9* mutant nose-touch defect behavior (Upadhyay et al., 2016). The *osm-9* gene has also been shown to play a role in developmental programming and is downregulated transcriptionally post environmental stress exposure, causing avoidance of specific olfactory cues (Hall et al., 2010; Sims et al., 2016). These changes in *osm-9* expression were shown to be mediated by chromatin remodeling and endogenous siRNA pathways (Sims et al., 2016). Thus, *osm-9* has roles in sensory transduction in response to noxious stimuli, can be activated by polyunsaturated fatty acids, and plays a role in learning and memory, just like TRPV1. OSM-9 shares about ~25-26% protein sequence similarity to human TRPV1, TRPV5, and TRPV6, and also has intracellular N and C termini, six N-terminal ankyrin domains, and six transmembrane domains, with a calcium-permeable pore loop between the fifth and sixth. Thus, studies on *osm-9* may help to uncover conserved mechanisms of learning and memory.

In our classical conditioning paradigm, we focus on the *C. elegans* response to the food-related odorant butanone, which is specifically sensed by the AWC olfactory

sensory neurons (OSNs; Bargmann and Horvitz, 1991; Bargmann et al., 1993). The *C. elegans* nematode exhibits olfactory classical conditioning: one 80-minute exposure cycle to butanone in the absence of food causes the nematode to downregulate its chemotactic response to that odor. The second messenger for odor sensation within the AWCs is cGMP (L'Etoile and Bargmann, 2000; Birby et al., 2000). Prolonged odor stimulation leads to AIA interneuron circuit activity and PI3K signaling to the AWC neurons to drive accumulation of the cGMP-dependent protein kinase EGL-4 (L'Etoile et al., 2002) within the AWC nucleus. These events promote memory of the odor being profitless after 80 minutes of conditioning (O'Halloran et al., 2009; Lee et al., 2010; Cho et al., 2016). Loss-of-function mutations in *osm-9* cause defective learning that AWC-sensed odors can be profitless, and defective chemo-, osmo-, and thermosensation promoted by other sensory neurons (Colbert et al., 1997). Importantly, loss of *osm-9* restores function to mutants that ignore AWC-sensed odors as a consequence of expressing nuclear EGL-4. After EGL-4 nuclear translocation, nuclear endogenous RNAi promotes a dampened response to butanone by transcriptionally-downregulating the *odr-1* gene through heterochromatin remodeling via *hpl-2/HP1*, and the *osm-9* gene is epistatic to gain-of-function *hpl-2* or *egl-4* (O'Halloran et al., 2009; Juang et al., 2013). Thus, the TRPV channel *osm-9* is required downstream of both of these nuclear events and is the most downstream component of this paradigm currently known. However, the role of *osm-9* in this plasticity paradigm is still poorly understood. We were also curious if *osm-9* plays a role in long-term olfactory memory since its role in short-term olfactory memory is established. We wanted to understand the *osm-9* dynamics in plasticity

necessary for olfactory memory formation, and what the structural components of the gene are that allow it to drive this plasticity.

We previously found that the 80-minute butanone conditioned response is temporary and is lost after only 30 minutes recovery on food (Muñoz-Lobato et al., 2019). However, after odor spaced-training, *C. elegans* can exhibit long-term aversive memory phenotypes (Lakhina et al., 2015; Muñoz-Lobato et al., 2019). Worms subjected to three cycles of 80-minute exposure to the unprofitable odor in the absence of food with recovery periods on food in between each cycle will keep the memory and continue to ignore the odor for much longer periods of time (at least 16 hours after training; Muñoz-Lobato et al., 2019). Here, we find that the *osm-9* null mutants can actually be conditioned to ignore butanone after undergoing a spaced-training protocol, just like wild-type animals. Interestingly, we show that long-term memory consolidation, and not acquisition, requires *osm-9,* independent of sleep. Specifically, we find that *osm-9* mutants lose this odor memory after only 30 minutes, where it remains lost. The wild-type animals also lose the memory after 30 minutes, but regain it and keep it for at least 16 hours (Muñoz-Lobato et al., 2019). The wild-type trained animals undergo a period where the odor memory is fragile and is lost after only 30 minutes of recovery on food, only to be regained at 120 minutes of recovery and to last for at least 16 hours. The *osm-9* gene is not required for this 30-minute memory fragility period, but is required during the post 30-minute to 120-minute period where the memory is consolidated. Memory fragility in the period after conditioning is a hallmark of a consolidation (Dudai, 1996; Dudai, 2004). Thus, *osm-9* likely plays a role in olfactory long-term memory consolidation in addition to its previously defined role in short-term

memory. We previously found that wild-type animals, like mammals, require sleep during this fragile memory period to consolidate odor memory, but *osm-9* animals sleep just as much as wild-type, but are still unable to consolidate and mediate long-term memory. We also find that both the N- and C-terminus are required for long-term memory consolidation or maintenance. Finally, we show that the short-term memory defect is rescued only partially by *osm-9* cDNA expressed under its own promoters, but the defect may be more completely rescued when expressing its gDNA, suggesting a role for its introns in driving expression for memory.

Though TRP channels have been extensively studied as sensory receptors, they have not been well studied as integrators of prolonged stimulation. The goal of this work was to understand how the *osm-9* TRPV channel mediates long-term memory after prolonged exposure to odor, thereby elucidating TRP function in signal integration triggered by extended exposure to stimuli. We show that *osm-9* is important for the long-term memory consolidation step of memory formation. These data suggest the exciting possibility that a TRPV channel promotes long-term memory consolidation, and does this independent of sleep.

RESULTS

Although it has been previously shown that *osm-9* is required for olfactory short-term memory of food-related odorants (**Fig 3.1**, **Fig 3.S1**), its role in long-term memory formation hasn't been reported. We utilized a long-term memory spaced-training paradigm where we performed odor conditioning in three spaced intervals with recovery periods on food in between (**Fig 3.S1**). We tested *osm-9(ky10)* null mutants versus wild-type animals and found that although *osm-9(ky10) IV* animals are unable to form short-term odor memory after one cycle of conditioning (**Fig 3.1**), they can acquire memory immediately after spaced-training, like wild-type animals (**Fig 3.1**), shown by a chemotaxis index (**Fig 3.1A**) and learning index (**Fig 3.1B**). Wild-type animals lose the memory after 30 minutes of recovery on food, but regain it back after two hours of recovery (**Fig 3.2**, Muñoz-Lobato et al., 2019), and keep it for at least 16 hours post training (**Fig 3.3**). By contrast, *osm-9* animals lose the memory completely after only 30 minutes (**Fig 3.2**) of recovery on food and never gain it back, shown at two hours and 16 hours of recovery after training (**Figs 3.2** and **3.3**, respectively). This could indicate that *osm-9* is responsible for memory consolidation, but not acquisition, immediately post odor spaced-training. What is also interesting to note is that although *osm-9(ky10) IV* animals are able to acquire memory after three cycles of odor training, their chemotaxis behavior looks distinct from wild-type animals – *osm-9(ky10)* animals have an average chemotaxis index closer to zero (0.1, **Fig 3.1A**), which looks like wild-type animals after one-cycle of odor training (**Fig 3.1A**), whereas wild-type animals after three cycles of odor training look averse to butanone, with an average chemotaxis index of -0.3 (**Fig 3.1A**). Although odor spaced training can induce memory acquisition in

osm-9(ky10) animals as opposed to training them with one odor cycle, their behavioral state may be different from that observed in wild-type animals.

Since sleep has been suggested to promote memory consolidation and is required for long-term memory in our paradigm (Muñoz-Lobato et al., 2019), we tested if the *osm-9(ky10)* mutants sleep after spaced training by quantifying their quiescence behavior in one hour post spaced training. To analyze movement, we utilized the WorMotel (Churgin et al., 2017), where we could take videos of individual worms as they recover for one-hour post training, and subsequently analyze their behavior with Matlab analysis. We measured sleep by testing if the worms exhibited any quiescence behavior for more than 30 seconds and generated raster maps from these data for visualization of the movement (**Fig 3.4A**). We then recorded the mean total quiescence (in minutes per hour) of each dataset. The *osm-9(ky10) IV* movements showed the same amount of quiescence as wild type, signifying that the *osm-9(ky10) IV* animals sleep as much as wild type animals, but are still unable to consolidate olfactory memory (**Fig 3.4B**).

We next tested extra *osm-9* alleles to uncover if this olfactory long-term memory consolidation defect is unique to the *osm-9(ky10)* mutants, and as a way to perform structure-function analysis. The *osm-9* gene has fourteen exons. The *ky10* allele is an early stop codon at the beginning of the fourth exon from the 5' end, occurring in the middle of the third ankyrin repeat (**Fig 3.5A**). We tested the *yz6* allele, also an early stop codon, at the end of the fifth exon, 3' of the six ankyrin repeats, thus leaving them intact, assuming the mRNA is translated (**Fig 3.5A**). We also examined the *ok1677* allele, a deletion of the twelfth, thirteenth, and part of the fourteenth exons, removing most of the

C-terminal intracellular tail, and the sequence that produces a non-coding RNA and tRNA (**Fig 3.5A**). The *ok1677* and *yz6* animals showed similar behavior to *ky10* since memory acquisition is still intact, but memory maintenance is defective, although the defect in these two alleles is not as severe as in *ky10* animals (**Figs 3.3** and **3.5B**). These data support that *osm-9* is not required for memory acquisition, but is important for memory consolidation or maintenance in long-term olfactory memory.

To study the expression requirements for *osm-9*/*TRPV5/TRPV6* in olfactory classical conditioning, we took a molecular genetic approach by performing rescue experiments with different *osm-9* transgenes under various promoters in the *osm-9(ky10)* mutant background versus their non-transgenic siblings and wild type as controls. We performed a short-term memory paradigm involving training with and without a food-related odorant for 80 minutes in the absence of food, as previously published (Colbert and Bargmann, 1995; Juang et al., 2013; **Fig 3.S1**). We were unable to rescue the *osm-9(ky10) IV* odor-learning defect (Colbert and Bargmann, 1995) when using a partial *osm-9* cDNA driven under its endogenous upstream and downstream promoters (**Fig 3.S4D**), which was previously shown to drive *osm-9* expression and localization (Colbert et al., 1997; Tobin et al., 2002). We also could not achieve rescue with the cDNA driven by an AWC promoter (*ceh-36*, **Fig 3.S4A**), the olfactory sensory neuron promoter (*odr-3*, **Fig 3.S4B** and **3.S4C**), under both of its own upstream and downstream promoters (**Fig 3.S4E**), or a ciliated neuron promoter (*nphp-4,* Jauregui and Barr, 2005; **Fig 3.S4G**). We also could not achieve rescue using a fosmid containing the whole *osm-9* gene (**Fig 3.S4F**). Partial rescue was achieved using the full-length *osm-9* cDNA under both the olfactory sensory neuron promoter *odr-3* and its

own downstream promoter (**Fig 3.6**). However, full rescue was obtained when expressing the *osm-9* genomic DNA under both its upstream and downstream promoters, although the extrachromosomal array silenced in all of the transgenic lines, precluding further rescue analysis (**Fig 3.S3**). The gDNA rescuing the short-term memory defects of *osm-9* mutants may signify that the introns contain something required for expression of the *osm-9* gene in order to mediate butanone short-term memory. The fosmid should have also rescued if the introns are indeed required, but it could have been an expression issue that prevented rescue with the fosmid.

In sum, we report here that the *osm-9/TRPV5/TRPV6* gene is required for long-term olfactory memory consolidation independent of sleep, and may require its introns for proper function in memory.

DISCUSSION

Transient receptor potential (TRP) channels are proteins best understood in their role as sensors of harmful environmental stimuli, yet they have a less understood role in regulating neural plasticity—a fundamental process in the brain that governs information storage and adaptation to environmental cues. We found that the *osm-9* TRPV channel, which has a known role in short-term olfactory learning (Colbert and Bargmann, 1995), requires its intronic regions for proper function in this paradigm. The requirement for genomic DNA to rescue the defective short-term memory in *osm-9(ky10) IV* mutants could signify a type of gene regulation required for proper odor learning. It has been reported that *osm-9* has endogenous small RNAs (endo-siRNAs) that are required for sensing aversive pheromones post-stress exposure (Sims et al., 2016). Endo-siRNAs or other RNAs produced from the *osm-9* locus could potentially also be required for this short-term associative odor memory. We were interested if *osm-9* also plays a role in long-term olfactory memory, and thus tested the mutants in a spaced-training paradigm that induces long-lasting memory in wild-type animals. The *osm-9(ky10)* mutants were able to acquire the odor memory after training, but lost it within 30 minutes of recovery on food, and it remained lost, whereas wild-type animals lose the memory at 30 minutes of recovery, regain it two hours later, and keep it at least 16 hours later. Thus, the *osm-9* TRPV channel is required for memory consolidation, a key component of long-term memory. Proper functioning TRPV channels may be necessary to drive the synaptic plasticity required for memory formation. Studies have shown that TRPV1 contributes to synaptic plasticity, specifically LTD induction across the hippocampus, mPFC, and NAc (Ruggiero et al. 2017; Gibson et al. 2008, Grueter et al. 2010, Lovelace et al. 2014).

Because these are regions required for long-term memory formation, it could be this alteration in synaptic plasticity that is required for proper memory consolidation. We tested different alleles of *osm-9* to perform structure function analysis for the long-term memory paradigm. The *ky10* allele is an early stop codon that removes the fourth to sixth and part of the third ankyrin repeats on the N-terminal tail, as well as the transmembrane domain, pore loop, and C-terminus. The *yz6* allele is also an early stop, but is directly after the ankyrin repeats, and removes the rest of the transcript like *ky10* does. To additionally test if the C-terminal tail is important for long-term memory, we tested the *ok1677* allele, which removes most of the C-terminus as well as a region that produces a non-coding RNA and tRNA. Both the *yz6* and *ok1677* animals behaved just like *ky10* mutants, which potentially shows a requirement for both the N- and C-terminus, including ankyrin repeats, the transmembrane domains, or the pore loop, in memory consolidation or maintenance.

Animals must respond and learn from their ever-changing surroundings to ensure survival. TRP channels emerged early in eukaryotic evolution to allow sensation of the environment. Over the course of evolution, they became coincidence detectors, sensing a range of mechanical to chemical cues, which are vital for learning and memory. Originally thought of as only having peripheral nerve expression, TRPV channel proteins are also found in the central nervous system throughout the brain. Could pleiotropy be occurring, where TRPV channels in the sensory neurons can respond to cues such as heat and protons, and then subsequently drive learning and long-term memory of those cues in the brain? The evolution of TRP channels could have

potentially driven survival through ambient sensation and simultaneous or subsequent triggering of memory formation.

TRP channels are found throughout the domain Eukaryota, in the animal, fungi, and protist kingdoms, meaning they evolved before multicellularity. TRPY1 is found in the single-celled fungus, *S. cerevisiae* and TRPA, C, M, L, and V channels, are found in the single-celled protist choanoflagellates (Peng et al., 2015), as well as in multicellular, bilaterally-symmetric animals, including protostome ecdysozoan invertebrates such as *Drosophila* and *C. elegans*, and deuterostome chordate vertebrates, including zebrafish, whale and Australian ghost sharks, rodents, and humans. TRPNs are found in *Hydra magnipapillata,* which is the last common ancestor of Cnidaria and Bilateria. Fourteen TRPA and TRPML channels were found in the sponge *A. queenslandica*, showing a diverse conservation of TRP channels since sponges are the oldest living metazoan phyletic lineage, diverging from other metazoans ~600 million years ago (Peng et al., 2015). TRPA1 was found to have evolved from a chemical sensor in a common bilaterian ancestor to invertebrates and vertebrates, conserved across ~500 million years of evolution (Kang et al., 2010). TRP channels themselves are likely even older than the nociceptive family of sensors, since fungi branched off from other life during the Proterozoic Era, about 1.5 billion years ago (Wang et al., 1999). These early TRP channels evolved the ability to sense multiple stimuli and drive distinct responses to ensure organismal survival. For example, the TRPA1 channel likely evolved through adaptive evolution via amino acid changes in response to the environment, explaining the temperature and compound sensitivities observed in different mammalian species (Chen et al., 2008; Xiao et al., 2008; Nagatomo et al., 2010; Peng et al., 2015). The

TRP channel predecessors were non-neuronally expressed, but as animals became more evolved, such as through development of a neural net or cephalization, TRP channels evolved neuronal roles such as sensory perception and polymodality. However, the distinct functions of TRP channels in higher level processes such as plasticity, learning, and memory are still being uncovered.

Since TRPV channels are required for acute sensation and in plasticity, considered short and long-term processes, respectively, then how does its function and regulation differ between them? Distinct from the TRPV1-4 channels, TRPV5 and TRPV6 are highly-selective for calcium and mediate calcium signaling through their constitutive opening at physiologic membrane potentials, without the need for ligand activation. Potentially, the TRPV5 and 6 ortholog OSM-9 may also be constitutively open and mediating the calcium homeostasis necessary to drive long-term memory formation. Calcium is a known requirement for synaptic transmission. We previously showed that synaptic plasticity occurs in animals that maintain the memory and that slept post spaced-training (Muñoz-Lobato et al., 2019). Thus, OSM-9 may be involved in regulating the calcium levels required for this synaptic plasticity in spaced-trained animals. Another confound is understanding how TRPV1 mediates long-term memory. TRPV1-4 channel agonists cause a downstream signaling cascade to mediate sensation. Prolonged exposure causes desensitization of the channel. If the channel is similarly desensitized in plasticity, then how does its function continue to occur for long-term biological processes, including long-term memory? Understanding the agonists driving TRPV1-mediated plasticity that do not cause desensitization of the channel may help to answer this confounding question. The polymodality of TRP channels is alluring,

yet mystifying since it can be a number of individual or combinations of agonists or antagonists driving varied downstream responses. Yet, since Ca^{2+} signaling is important for synaptic plasticity, it could be that TRPV proteins are specifically required for regulating intracellular calcium to promote plasticity and thus learning and memory.

Besides being a current focus for chronic pain treatment (e.g. through topical capsaicin), the TRPVs are also emerging as a new drug target for diseases affecting neural plasticity. Indeed, a vast array of neurological diseases, including Alzheimer's disease (Di Marzo et al. 2008), Parkinson's disease, schizophrenia (Tzavara et al. 2006, Almeida et al. 2014, Madasu et al, 2015), epilepsy, and Charcot-Marie-Tooth disease (Morelli et al., 2013), as well as sense disorders (e.g. anosmia; Nilius, 2007) result from or are linked to defects in TRP function, with affected individuals exhibiting decreased neural plasticity. It has also been shown that TRPV1 could be a target for cognitive decline in Alzheimer's patients since applying its agonist capsaicin to neurons from AD rodents shows restoration of Aβ-induced degradation of hippocampal gamma oscillations (Balleza-Tapia et al., 2018). Rat models of biliary cirrhosis, which induces memory impairment, have decreased memory impairment when treated with capsaicin, which also increased TRPV1 and CREB mRNA in the CA1 area of the hippocampus (Bashiri et al., 2018). Recent research even suggests the use of TRPV1 agonists as therapeutics to aid in ameliorating the effects of schizophrenia since TRPV1 is linked to pain and cognitive defects in schizophrenic patients (Madasu et al. 2015). This could indicate that TRPV1-mediated plasticity defects may underlie some of these neurological disorders. Performing *in vivo* studies to understand how TRPV proteins regulate proper neurological function may uncover a role in learning and memory and

could potentially help to understand what happens in the TRPV1-mediated disease states. We aimed to elucidate how TRP protein functions at the molecular level to promote plasticity and memory formation. Understanding the molecular mechanisms and dynamics by which animals learn to ignore non-beneficial stimuli through TRP-mediated neural plasticity may be of importance to understanding not only how animals learn and form memories, but may also give insights into the etiology of TRP-mediated neurological diseases.

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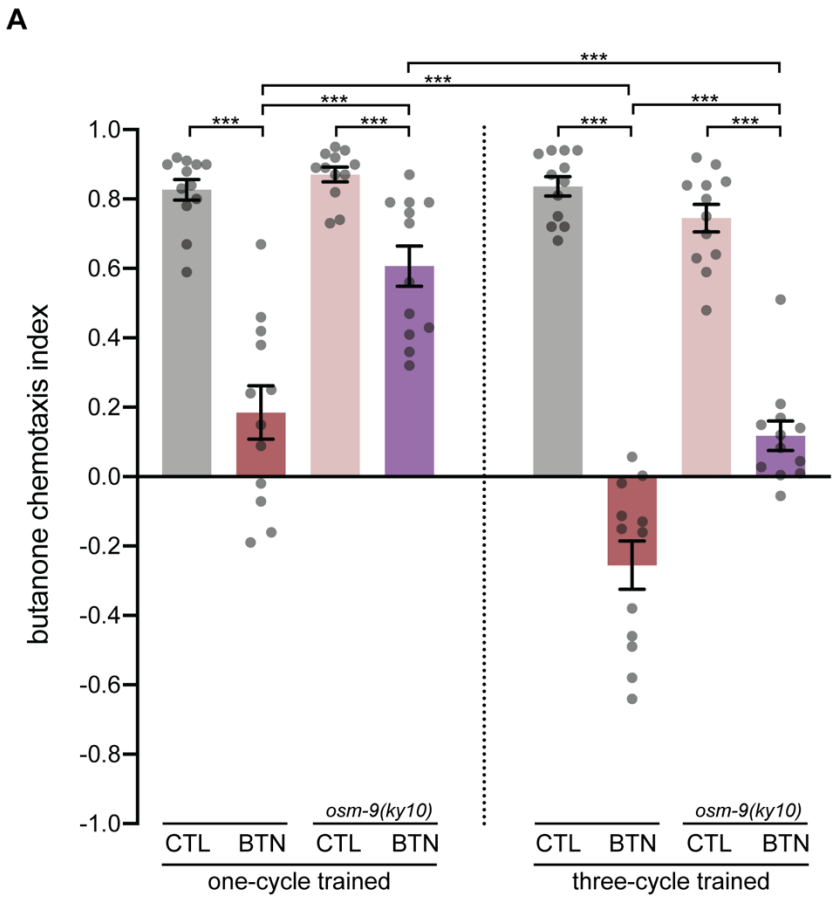
We would like to thank all past and present members of the L'Etoile lab. We thank Piali Sengupta for her gift of the *nphp-4* promoter and Cori Bargmann for her gift of the *osm-9::GFP5* construct. We would also like to thank Chris Fang-Yen and Matt Churgin for their help with the WorMotel and the Matlab analysis, and Joe Hill for the artwork used in Figs 3.S1 and 3.S2. We are grateful to the team behind wormbase.org (NIH grant #U24 HG002223), SMART (Schultz et al., 1998, EMBL), and Nikhil Bhatla for the wormweb.org exon-intron graphic maker used for Fig 3.7A. This work was funded by the NIH, specifically R01DC005991 (NDL), R01NS087544 (NDL), R25GM56847 (KB), R01 supplement NS87544 (KB), and F31DC014921 (KB).


A
butanone chemotaxis index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
***
CTL
BTN
osm-9(ky10)
one-cycle trained
three-cycle trained

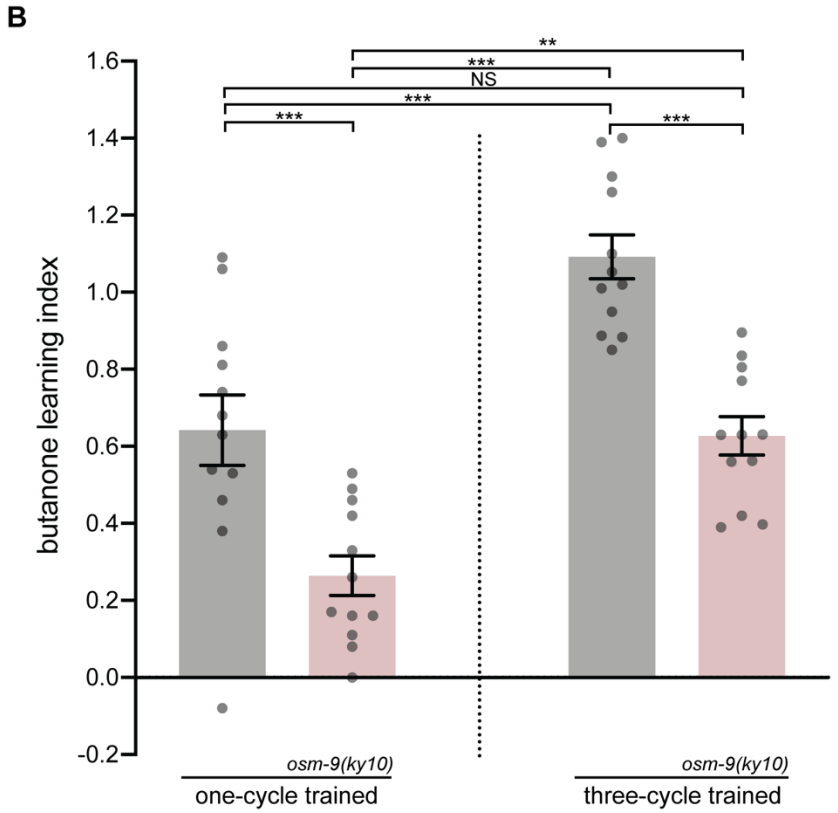

B
butanone learning index
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
**
***
NS
osm-9(ky10)
one-cycle trained
three-cycle trained

Fig 3.1. The TRPV channel *osm-9* is required for short-term memory, but is not required for memory acquisition post spaced training. (A) Chemotaxis indices for one-cycle odor conditioned and three-cycle odor wild-type vs *osm-9(ky10)* spaced-trained animals. The chemotaxis index (CI) is calculated as the number of worms at the diluent (200-proof ethanol) subtracted from the number at the butanone point source, divided by the total number or worms (excluding any at the origin). Wild-type one- or three-cycle buffer trained (CTL, grey bar), wild-type one- or three-cycle butanone-trained (BTN, red bar), *osm-9(ky10)* one- or three-cycle buffer-trained (CTL, pink bar) and *osm-9(ky10)* one- or three-cycle butanone-trained (BTN, purple bar) are shown, and are denoted this way throughout the rest of the manuscript. "Three-cycle trained" label means 0' recovery (fifth to eighth bars) and will be annotated this way throughout the paper. N = number of trials where all trials are done on independent days and each grey dot represents an individual assay day, with 50-200 animals/day. The Shapiro-Wilk`` normality test was performed to determine data distribution, and if data were non-normally distributed, the analysis was done with the Kruskal-Wallis test, an analysis of variance of multiple comparisons for non-parametric data. If pairwise comparisons were normally distributed, then p-values were generated with a Student's unpaired t-test. If any of the datasets were non-parametric, then p-values were generated with the Mann-Whitney u-test. Hochberg adjustment for multiple comparisons was performed on all p-values generated from data included in the same graph to control Type I statistical error. If the data were normally distributed, then one-way ANOVA was performed, followed by Bonferroni correction of pairwise comparisons. Error bars represent S.E.M. Statistical significance was reported as ***$p<0.001$, ** $p< 0.01$, *$p<0.05$, and NS is $p>0.05$. Behavioral data throughout the paper are represented in this same way with the same numbers of animals on independent days. Additionally, throughout the paper, the same statistical analysis was performed on the data. Note that for every figure in this manuscript, that the Kruskal-Wallis or one-way ANOVA test was performed and yielded ***$p<0.001$, unless otherwise noted. The Kruskal-Wallis test was performed. The u-test was performed for one-cycle CTL vs BTN, one-cycle *osm-9(ky10)* CTL vs *osm-9(ky10)* BTN, three-cycle trained *osm-9(ky10)* CTL vs *osm-9(ky10)* BTN, one-cycle *osm-9(ky10)* BTN vs three-cycle *osm-9(ky10)* BTN, and three-cycle BTN vs *osm-9(ky10)* BTN. The t-test was performed for one-cycle BTN vs *osm-9(ky10)* BTN, three-cycle CTL vs BTN, and one-cycle BTN vs three-cycle BTN.
(B) Learning indices for one-cycle conditioned and three-cycle spaced-trained wild-type vs *osm-9(ky10)* animals. The learning index (LI) is calculated as the chemotaxis index of the BTN animals subtracted from the chemotaxis index of the CTL animals. The higher the learning index, the more the animals have learned and thus kept the BTN memory. Wild type is shown with grey bars and *osm-9(ky10)* with pink bars, and will be denoted this way throughout the manuscript. One-way ANOVA followed by Bonferroni correction was performed for one-cycle wild type vs *osm-9(ky10)*, one-cycle vs three-cycle wild type, one-cycle wild type vs three-cycle *osm-9(ky10),* one-cycle *osm-9(ky10)* vs three-cycle wild type, one-cycle vs three-cycle *osm-9(ky10)*, and three-cycle wild type vs *osm-9(ky10).*

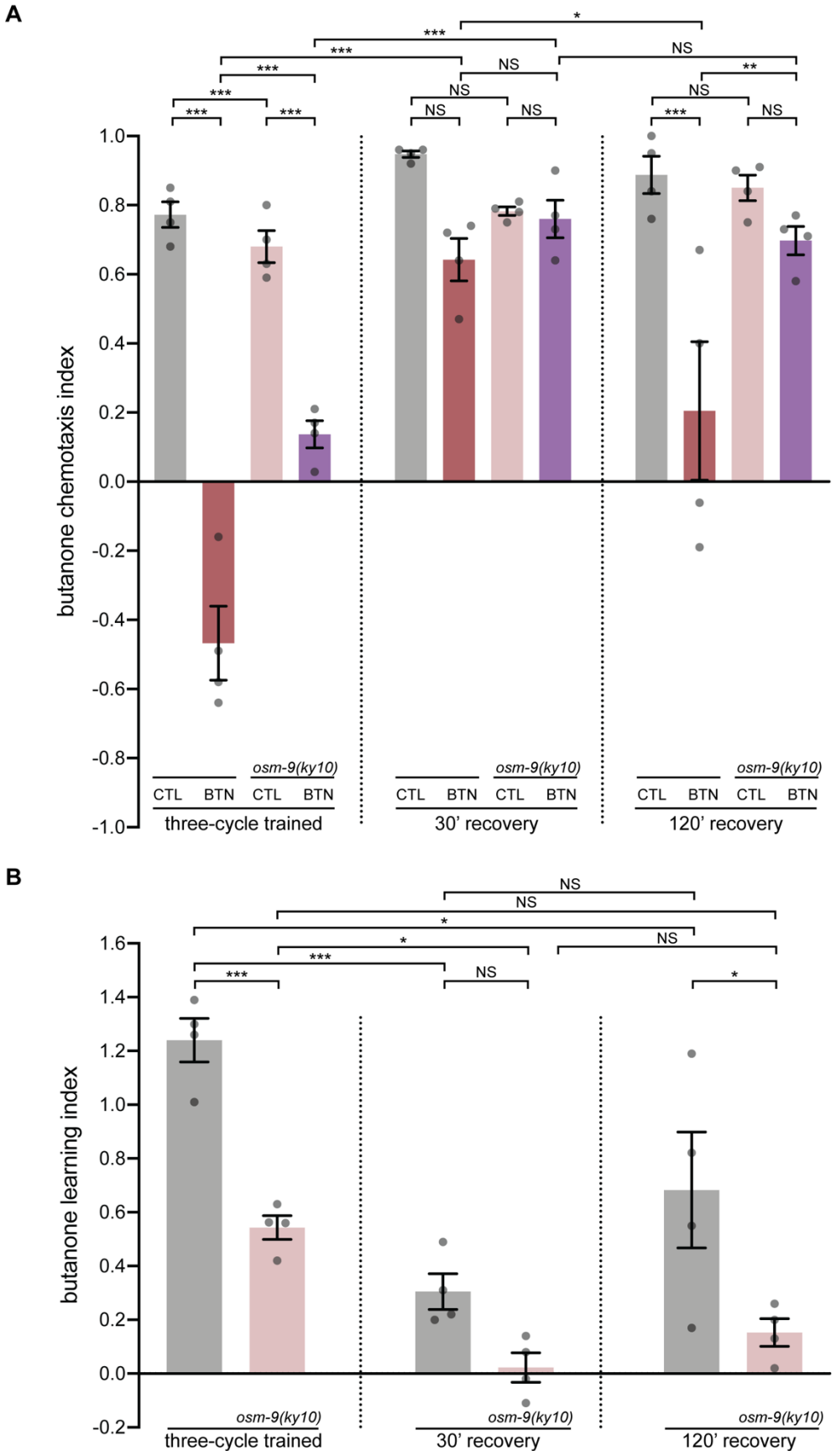
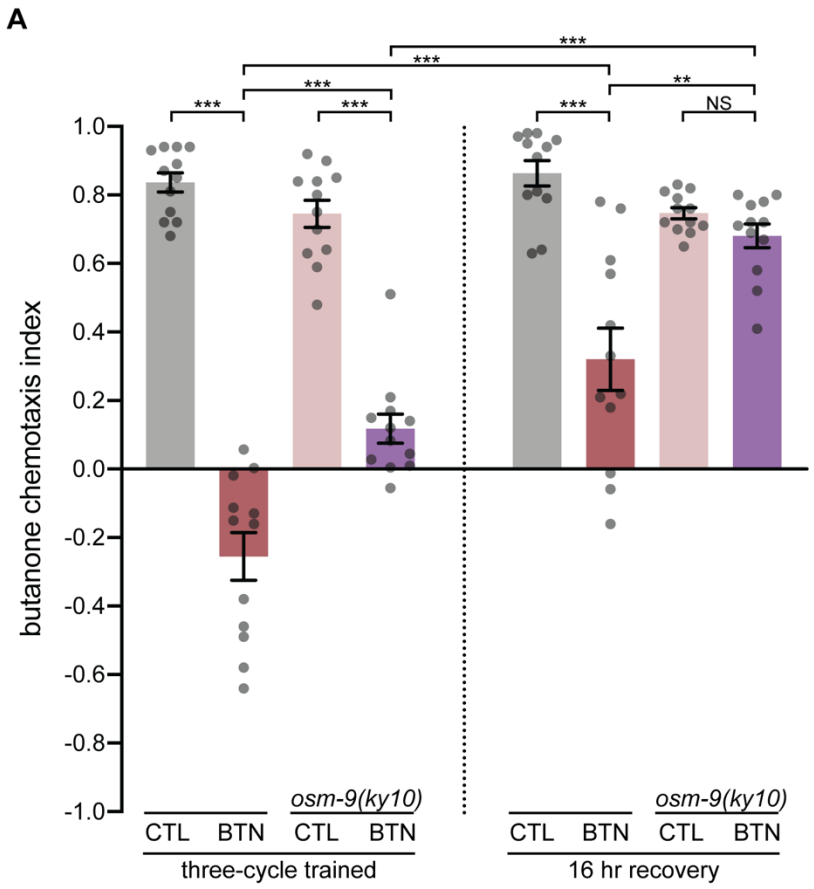

A
butanone chemotaxis index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
***
***
***
***
***
*
NS
NS
NS
NS
NS
NS
**
***
NS
osm-9(ky10)
CTL
BTN
CTL
BTN
three-cycle trained
30' recovery
120' recovery
B
butanone learning index
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
***
***
*
NS
NS
NS
*
*
osm-9(ky10)
three-cycle trained
30' recovery
120' recovery

Fig 3.2. The TRPV channel *osm-9* is required for long-term olfactory memory consolidation post spaced training. (A) Chemotaxis indices for three-cycle spaced-trained animals after 0', 30' or 120' post recovery on plates seeded with OP50 as a food source. One-way ANOVA followed by Bonferroni correction was performed for CTL vs BTN, *osm-9(ky10)* CTL vs *osm-9(ky10)* BTN, CTL 30' vs BTN 30', *osm-9(ky10)* CTL 30' vs *osm-9(ky10)* BTN 30', CTL 120' vs BTN 120', *osm-9(ky10)* CTL 120' vs *osm-9(ky10)* BTN 120', CTL vs *osm-9(ky10)* CTL, BTN vs *osm-9(ky10)* BTN, CTL 30' vs *osm-9(ky10)* CTL 30', BTN 30' vs *osm-9(ky10)* BTN 30', CTL 120' vs *osm-9(ky10)* CTL 120', BTN 120' vs *osm-9(ky10)* BTN 120', BTN vs BTN 30', *osm-9(ky10)* BTN vs *osm-9(ky10)* BTN 30', BTN 30' vs BTN 120', and *osm-9(ky10)* BTN 30' vs *osm-9(ky10)* BTN 120'. (B) Learning indices for three-cycle spaced-trained animals after 0', 30', or 120' recovery on food. One-way ANOVA followed by Bonferroni correction was performed for wild-type vs *osm-9(ky10)* after 0' recovery (compare first two bars), 30' recovery (compare third and fourth bars), 120' recovery (compare fifth and sixth bars), wild-type 0' vs 30' *osm-9(ky10)* 0' vs 30', *osm-9(ky10)* 30' vs 120', wild-type 0' vs 120', *osm-9(ky10)* 0' vs 120', and wild-type 30' vs 120', all after three-cycle spaced-training.


A
butanone chemotaxis index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
***
***
***
***
***
**
NS
osm-9(ky10)
osm-9(ky10)
CTL
BTN
CTL
BTN
CTL
BTN
CTL
BTN
three-cycle trained
16 hr recovery

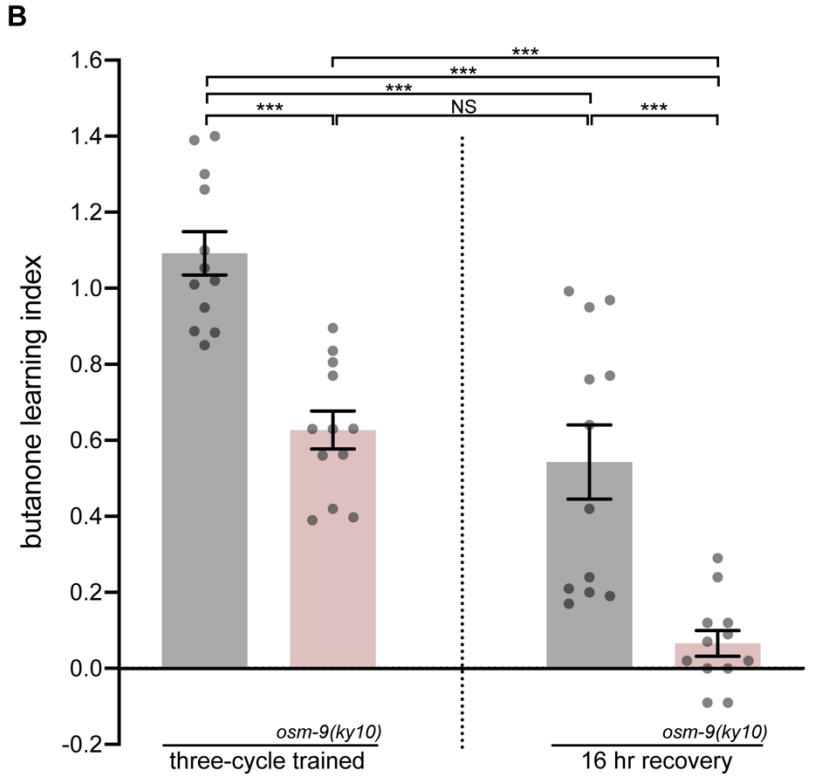

B
butanone learning index
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
***
***
***
***
NS
***
osm-9(ky10)
osm-9(ky10)
three-cycle trained
16 hr recovery

Fig 3.3. *osm-9* animals do not maintain the memory 16 hours after training. (A) Chemotaxis indices for three-cycle trained animals after 0' vs 16 hours of recovery on food. The Kruskal-Wallis test was performed. The u-test was performed for *osm-9(ky10)* CTL vs *osm-9(ky10)* BTN, CTL 16 hr vs BTN 16 hr, *osm-9(ky10)* BTN vs *osm-9(ky10)* BTN 16hr, and BTN vs *osm-9(ky10)* BTN. The t-test was performed for CTL vs BTN, BTN 16 hr vs *osm-9(ky10)* BTN 16 hr, *osm-9(ky10)* CTL 16 hr vs *osm-9(ky10)* BTN 16 hr, and BTN vs BTN 16 hr.
(B) Learning indices for three-cycle trained animals after 0' vs 16 hours of recovery on food. The Kruskal-Wallis test was performed. The u-test was performed for wild type 16 hr vs *osm-9(ky10)* 16 hr, wild type 0' vs wild type 16 hr, and *osm-9(ky10)* 0' vs wild type 16 hr. The t-test was performed for wild type 0' vs *osm-9(ky10)* 0', *osm-9(ky10)* 0' vs *osm-9(ky10)* 16 hr, and wild type 0' vs *osm-9(ky10)* 16 hr.

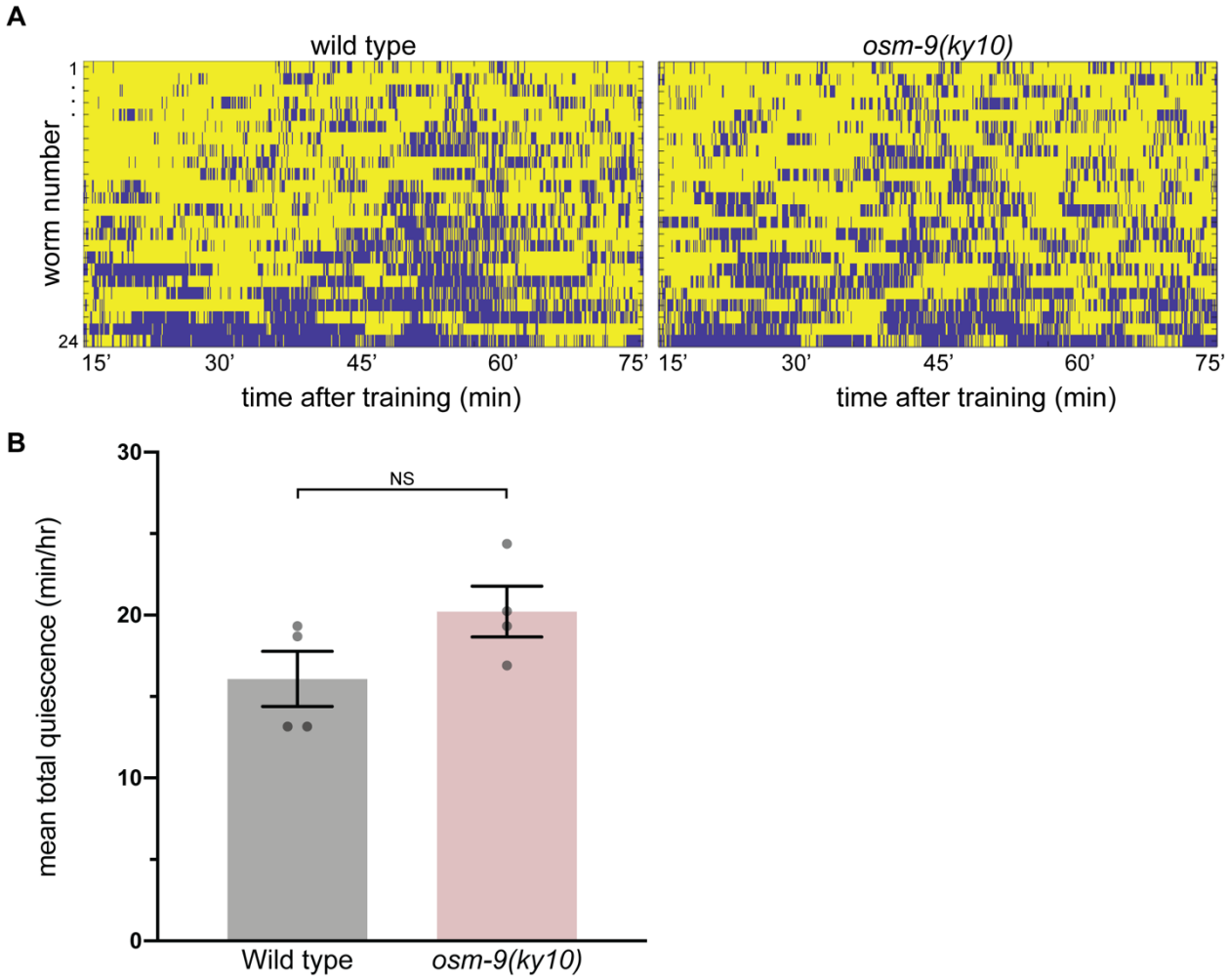


Fig 3.4. Spaced-training does not induce a difference in quiescence between wild type versus *osm-9(ky10)* in the hour post training. (A) Raster plot of activity of wild-type and *osm-9(ky10)* animals 15-75 minutes after three-cycle spaced training. Animals were placed in a WorMotel (Churgin et al., 2017) and videos were taken of their movement. Worm number is shown on the y-axis where each row represents one worm, with 24 worms total shown per plot. Yellow indicates activity and blue indicates quiescence, which was defined as no movement for more than 30 seconds.
(B) The mean total quiescence after spaced training is not significantly different between wild-type and *osm-9(ky10)* animals. Mean total quiescence in minutes per hour of animals in a WorMotel. Each grey dot represents 24 animals tested per condition on an independent day. The t-test was performed.

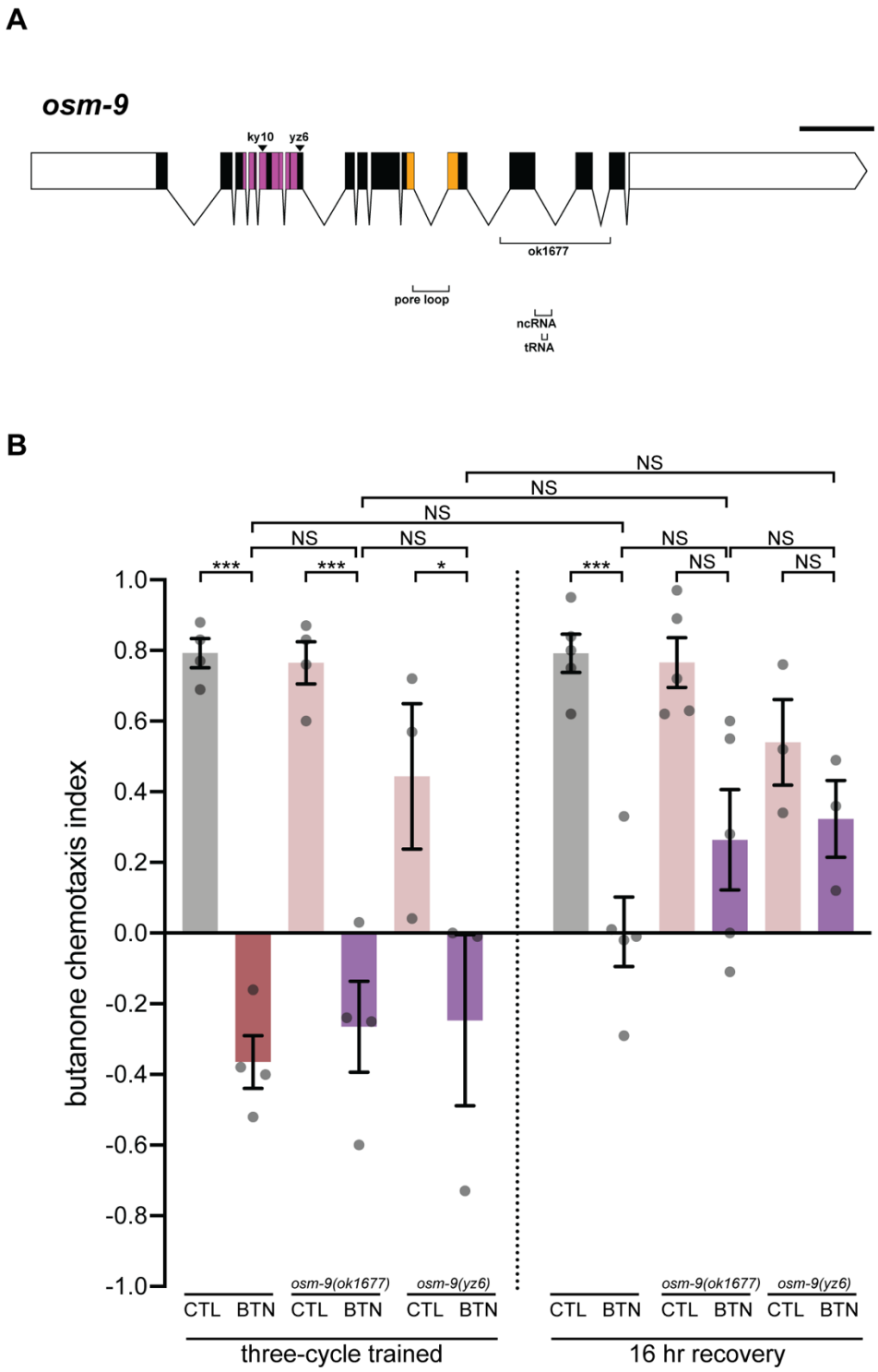
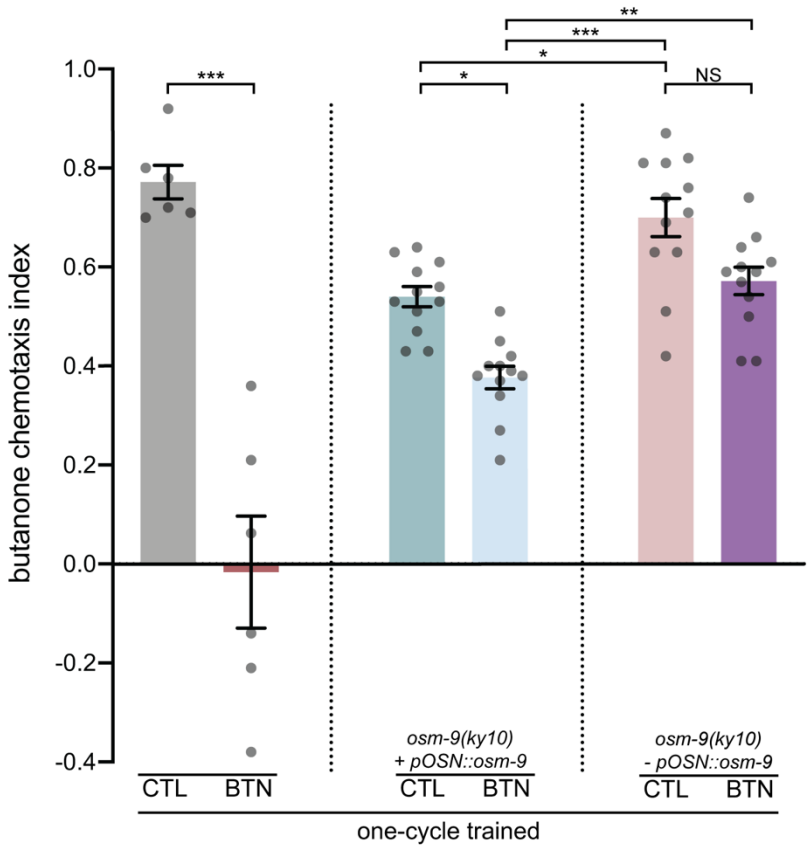


Fig 3.5. The *osm-9(ok1677)* and *osm-9(yz6)* animals can acquire, but cannot maintain long-term memory. (A) Gene map of *osm-9.* Exons are denoted by black boxes and introns are black lines connecting the exons. Red-violet indicates ankyrin repeats (six total). Orange indicates a PKD channel domain. The 5' end white box represents an ~1.6 kb promoter region (Colbert et al., 1997). The 3' end white box

represents an ~3.2 kb promoter region (Colbert et al., 1997). Scale bar indicates 1000 bases. Black arrowheads indicate alleles with point mutations producing early stop codons (*ky10* and *yz6*). The bracket labeled *ok1677* denotes a deletion allele. The brackets labeled ncRNA and tRNA are predicted RNAs produced from these regions. The bracket labeled pore loop denotes the region permeable to calcium, located between the fifth and sixth transmembrane domains.
(B) Chemotaxis indices for three-cycle trained animals with no recovery or 16 hours recovery on food for wild-type, *osm-9(ok1677)* and *osm-9(yz6)* animals. Grey and red bars are wild-type buffer- and butanone-trained animals and pink and purple are *osm-9* buffer- and butanone-trained animals. One-way ANOVA, followed by Bonferroni correction was performed for three-cycle CTL vs BTN, three-cycle *osm-9(ok1677)* CTL vs *osm-9(ok1677)* BTN, three-cycle *osm-9(yz6)* CTL vs *osm-9(yz6)* BTN, 16 hr CTL vs BTN, 16 hr *osm-9(ok1677)* CTL vs *osm-9(ok1677)* BTN, 16 hr *osm-9(yz6)* CTL vs *osm-9(yz6)* BTN, three-cycle BTN vs three-cycle *osm-9(ok1677)* BTN, three-cycle *osm-9(ok1677)* BTN vs three-cycle *osm-9(yz6)* BTN, 16 hr BTN vs 16 hr *osm-9(ok1677)* BTN, 16 hr *osm-9(ok1677)* BTN vs 16 hr *osm-9(yz6)* BTN, three-cycle BTN vs 16 hr BTN, three-cycle *osm-9(ok1677)* BTN vs 16 hr *osm-9(ok1677)* BTN, and three-cycle *osm-9(yz6)* BTN vs 16 hr *osm-9(yz6)* BTN.

A


butanone chemotaxis index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
***
*
*
***
**
NS
CTL
BTN
osm-9(ky10)
+ pOSN::osm-9
CTL
BTN
osm-9(ky10)
- pOSN::osm-9
CTL
BTN
one-cycle trained

B

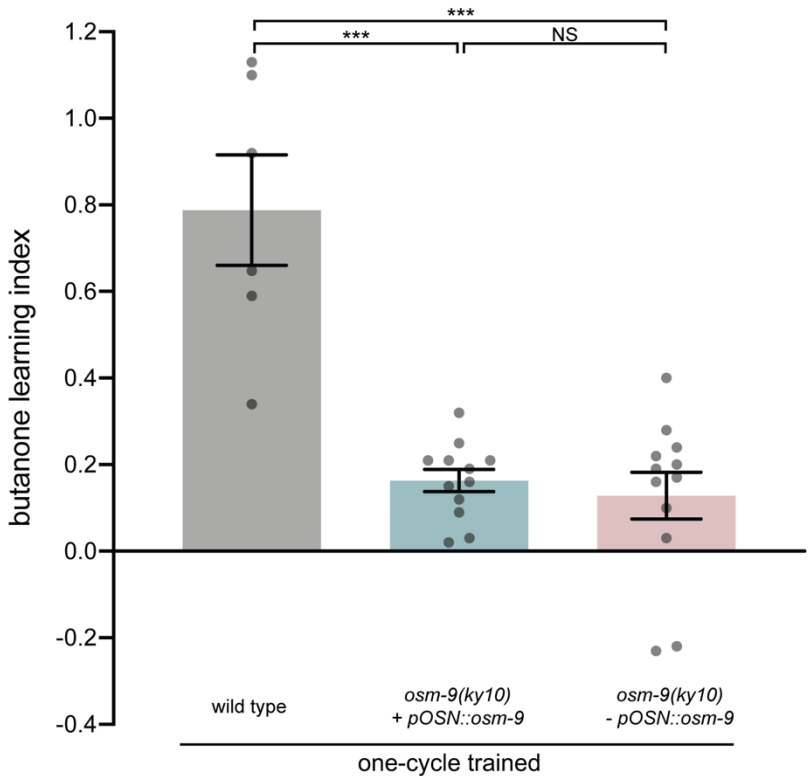

butanone learning index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
***
***
NS
wild type
osm-9(ky10)
+ pOSN::osm-9
osm-9(ky10)
- pOSN::osm-9
one-cycle trained

Fig 3.6. Expressing *osm-9* cDNA under the endogenous and OSN promoters in the *ky10* background only partially rescues short-term memory defects. (A) Chemotaxis indices of one-cycle trained animals. Chemotaxis indices of one 80-minute cycle buffer (CTL, first bar, grey) vs butanone-trained (BTN, second bar, red) wild-type animals, buffer-trained (CTL, third bar, teal) vs butanone-trained (BTN, fourth bar, light blue) *osm-9(ky10) IV; $_p$OSN::osm-9* animals, and buffer-trained (CTL, fifth bar, pink) vs butanone-trained (BTN, sixth bar, purple) non-transgenic siblings. The transgene is *KBC41/$_p$OSN::osm-9::UTR.* The *$_p$OSN* is the *$_p$odr-3* olfactory sensory neuron promoter, driving expression in the AWA, AWB, and AWC neurons, *osm-9* is the full length cDNA (~2.8 kb), and is also driven by the 3' UTR downstream endogenous promoter (~3.2 kb) shown to drive expression in various sensory neurons (Colbert et al., 1997). Transgenic data is from two combined lines. For Fig 1A, one-way ANOVA was performed, followed by the Bonferroni correction for multiple comparisons for CTL vs BTN, CTL vs BTN *osm-9(ky10); +$_p$OSN::osm-9*, CTL vs BTN *osm-9(ky10); - $_p$OSN::osm-9* (non-transgenic siblings), CTL *osm-9(ky10); +$_p$OSN::osm-9* vs CTL *osm-9(ky10); -$_p$OSN::osm-9*, BTN *osm-9(ky10); +$_p$OSN::osm-9* vs CTL *osm-9(ky10); -$_p$OSN::osm-9*, and BTN *osm-9(ky10); +$_p$OSN::osm-9* vs BTN *osm-9(ky10); -$_p$OSN::osm-9.*
(B) Learning indices for the odor-conditioned animals. One-way ANOVA followed by Bonferroni correction for wild type (first bar, grey) vs *osm-9(ky10); +$_p$OSN::osm-9* (second bar, teal), *osm-9(ky10); +$_p$OSN::osm-9* vs *osm-9(ky10); -$_p$OSN::osm-9* (third bar, pink), and wild type vs *osm-9(ky10); -$_p$OSN::osm-9*.

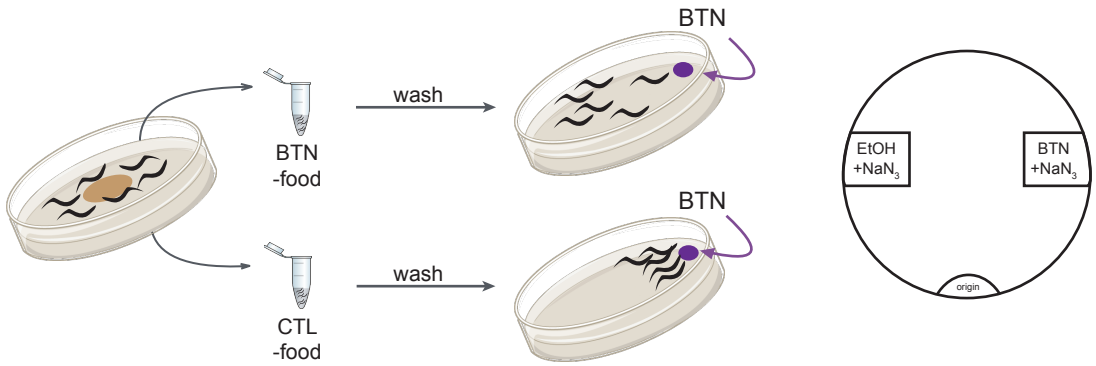


Fig 3.S1. Schematic of one-cycle odor-conditioning. Animals are conditioned to butanone as previously published (Juang et al., 2013). In brief, age-synchronized, one-day old adult animals are washed with buffer from NGM plates containing OP50 food to tubes with either buffer or butanone, both containing no food, for 80 minutes. After the 80-minute incubation, animals are washed and then plated onto chemotaxis assay plates. The chemotaxis index (See Fig 1 legend) is then calculated after at least two hours of the animals roaming.

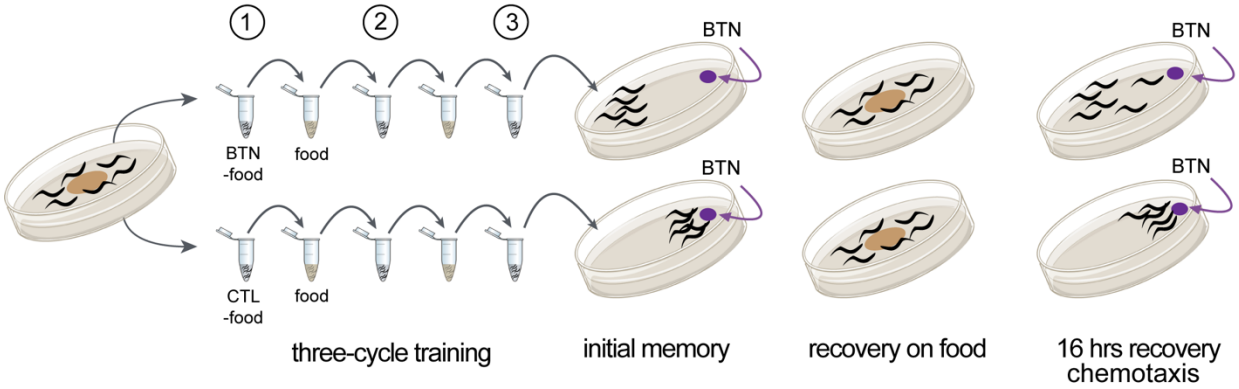


Fig 3.S2 Spaced-training paradigm. Schematic of associative olfactory memory assay to induce long-term odor memory. Age-synchronized, one-day old adult animals are washed from plates with food into tubes and are conditioned to either buffer (CTL) or butanone (BTN) for 80 minutes per cycle in the absence of food for three cycles total, with a 30-minute recovery on food (OP50, OD = 10) in between cycles. After spaced-training, animals are either tested in a butanone chemotaxis assay or are recovered on OP50-seeded NGM plates and then tested.

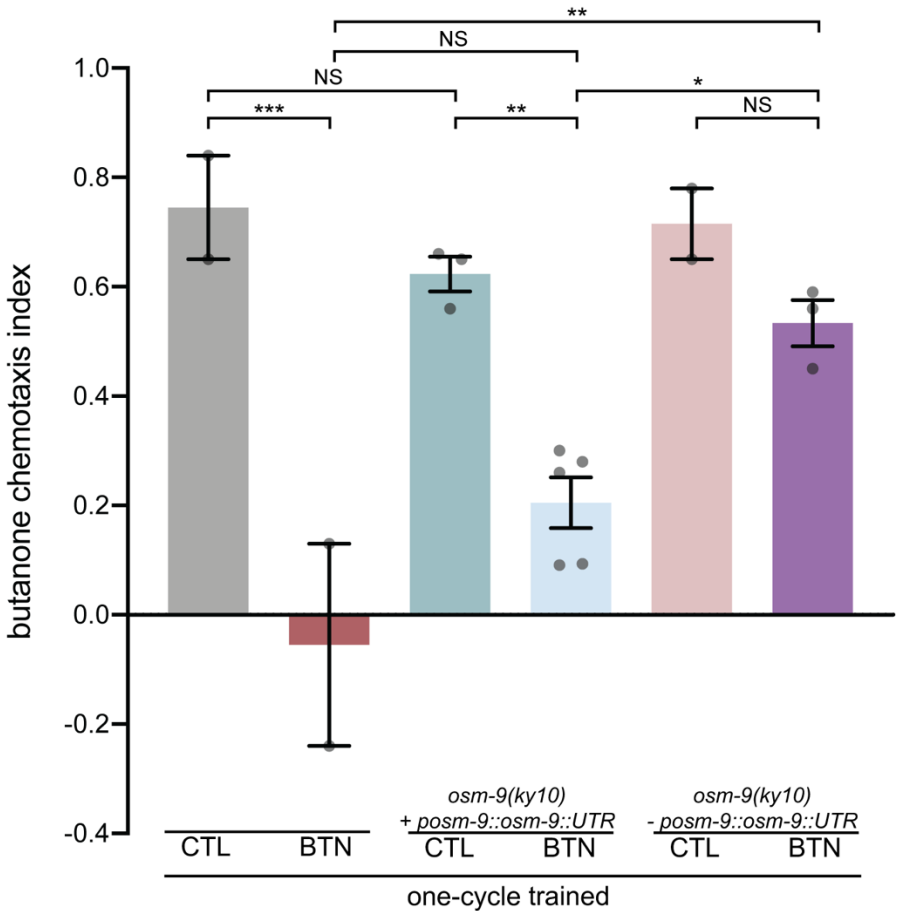


Fig 3.S3. Expressing *osm-9* gDNA under its upstream and downstream endogenous promoters in the *ky10* background rescues short-term memory defects. Chemotaxis indices of one-cycle trained animals. *KBC56/$_p$osm-9::osm-9::UTR* is the rescue transgene, where *$_p$osm-9* is the 1.6 kb upstream promoter (Fig 7A), *osm-9* is the full-length gDNA (6342 bp), and UTR is the 3.2 kb downstream promoter (Fig 7A). Teal and blue bars are *osm-9(ky10)* animals expressing *$_p$osm-9::osm-9::UTR* and pink and purple bars are the *osm-9(ky10)* non-transgenic siblings. Uneven number of grey dots because only datasets with N>50 were counted in the graph and analysis. One-way ANOVA was performed, followed by Bonferroni correction for CTL vs BTN, *osm-9(ky10)* + *$_p$osm-9::osm-9::UTR* CTL vs *osm-9(ky10)* + *$_p$osm-9::osm-9::UTR* BTN, *osm-9(ky10)* - *$_p$osm-9::osm-9::UTR* CTL vs *osm-9(ky10)* - *$_p$osm-9::osm-9::UTR* BTN, BTN vs *osm-9(ky10)* + *$_p$osm-9::osm-9::UTR* BTN, *osm-9(ky10)* + *$_p$osm-9::osm-9::UTR* BTN vs *osm-9(ky10)* - *$_p$osm-9::osm-9::UTR* BTN, CTL vs *osm-9(ky10)* + *$_p$osm-9::osm-9::UTR* CTL, and BTN vs *osm-9(ky10)* - *$_p$osm-9::osm-9::UTR* BTN.

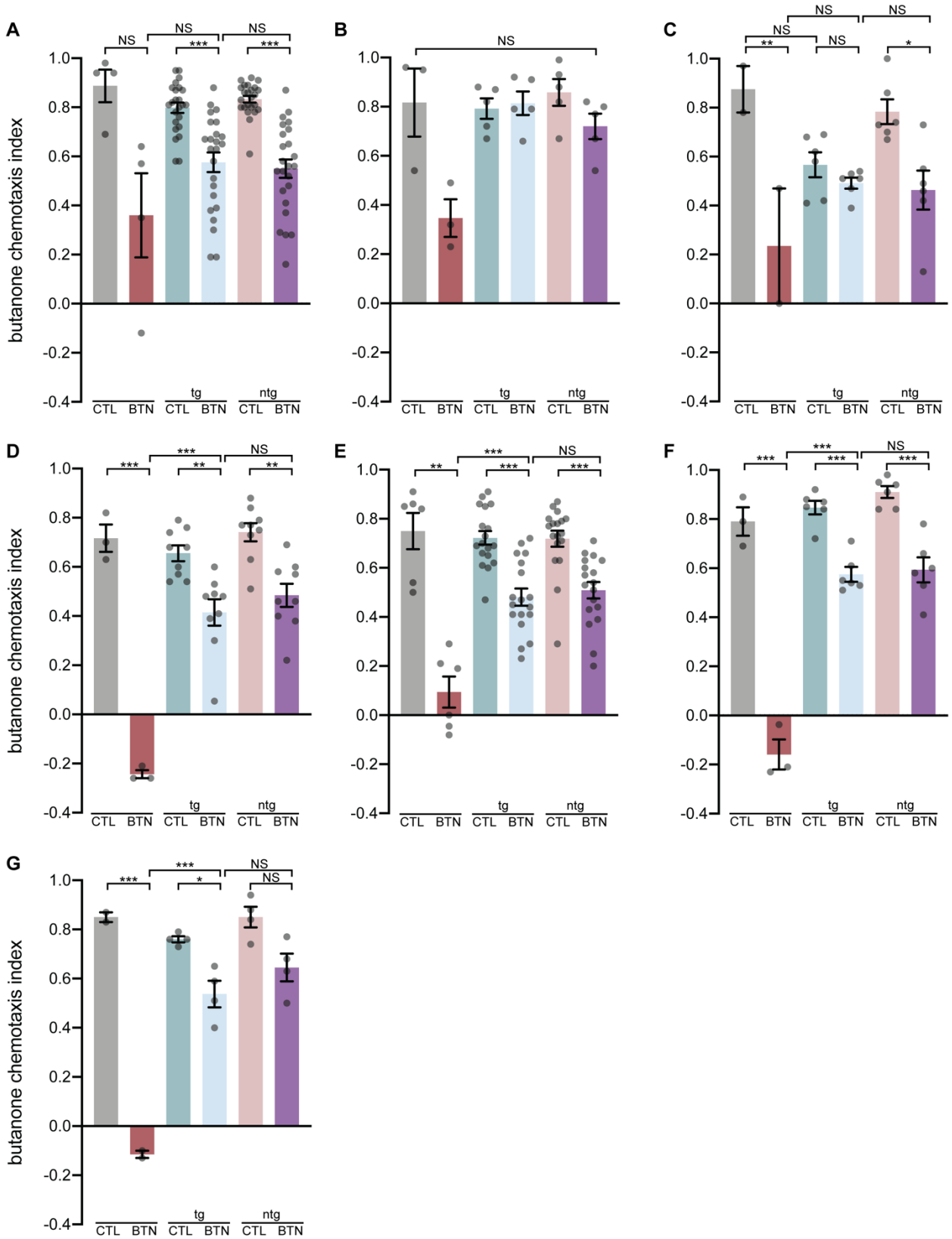


Fig 3.S4. Various *osm-9* expression transgenes are not sufficient to achieve rescue of *osm-9(ky10)* one-cycle butanone conditioning defects. (A) Chemotaxis

indices of wild type versus *osm-9(ky10),* with or without the transgene *KBC8/$_p$ceh-36::osm-9SS::GFP::osm-9.* The *pceh-36* is a promoter driving expression in both of the AWC olfactory sensory neurons. The *osm-9SS* (signal sequence) cDNA was 5' of the GFP and the rest of the cDNA was 3' of the GFP. Data shown from six combined transgenic lines. The Kruskal-Wallis test was performed. Transgenic is tg and non-transgenic is ntg. The u-test was used for CTL vs BTN and ntg CTL vs ntg BTN. The t-test was performed for tg CTL vs tg BTN, BTN vs tg BTN, and tg BTN vs ntg BTN.
(B) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene *pJK21/$_p$odr-3::GFP::osm-9.* The *$_p$odr-3* promoter drives expression in all three of the pairs of olfactory sensory neurons, including AWA, AWB, and AWC. The *$_p$odr-3* used was the short promoter, 2653 bp in length. The *osm-9* used in the same partial cDNA as in panel A. Data shown from two combined transgenic lines. The Kruskal-Wallis test was used and p>0.05.
(C) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene *KBC24/$_p$odr-3::GFP::osm-9.* The *$_p$odr-3* promoter drives expression in all three of the pairs of olfactory sensory neurons, including AWA, AWB, and AWC. The *$_p$odr-3* used was the long promoter, 2686 bp in length. The *osm-9* used in the same partial cDNA as in panel A. Data shown from three combined transgenic lines. One-way ANOVA was performed, followed by Bonferroni correction for CTL vs BTN, tg CTL vs tg BTN, ntg CTL vs ntg BTN, BTN vs tg BTN, tg BTN vs ntg BTN, CTL vs tg CTL, and tg CTL vs ntg CTL.
(D) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene *osm-9::GFP5* (Colbert et al., 1997), which includes the *osm-9* partial cDNA (2497 bp, missing the last 317 bp) driven by the upstream ~1.6 kb promoter and downstream ~3.2 kb promoter, and GFP, cloned into the pBS parent vector. Data shown from three combined transgenic lines. One-way ANOVA was performed, followed by Bonferroni correction for CTL vs BTN, tg CTL vs tg BTN, ntg CTL vs ntg BTN, BTN vs tg BTN, and tg BTN vs ntg BTN.
(E) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene *KBC42/$_p$osm-9::osm-9::UTR*, where *posm-9* is the upstream ~1.6 kb promoter, *osm-9* is the full-length cDNA (2814 bp), and *UTR* is the ~3.2 kb downstream promoter. Data shown from three combined transgenic lines. The Kruskal-Wallis test was performed. The u-test was used for CTL vs BTN and ntg CTL vs ntg BTN. The t-test was performed for tg CTL vs tg BTN, BTN vs tg BTN, and BTN tg vs ntg BTN.
(F) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene WRM065bE12, a ~32 kb fosmid containing the entire *osm-9* gene its upstream and downstream UTR regions. Data shown from two combined transgenic lines. One-way ANOVA was performed, followed by Bonferroni correction for CTL vs BTN, tg CTL vs tg BTN, ntg CTL vs ntg BTN, BTN vs tg BTN, and tg BTN vs ntg BTN.
(G) Chemotaxis indices of wild type versus *osm-9(ky10),* with or without the transgene *KBC38/$_p$nphp-4::osm-9::GFP.* The *$_p$nphp-4* promoter drives expression in all of the ciliated neurons (~60) in the adult worm. The *osm-9* transgene was the full-length cDNA. Data shown from two combined transgenic lines. One-way ANOVA was performed, followed by Bonferroni correction for CTL vs BTN, tg CTL vs tg BTN, ntg CTL vs ntg BTN, BTN vs tg BTN, and tg BTN vs ntg BTN.

MATERIALS AND METHODS

Strains and genetics

Strains were maintained by standard methods (Brenner, 1974) and were grown on either 5.5 cm or 10 cm Petri dishes with NGM media seeded with OP50 *E. coli*. All strains were grown at and assayed at 20°C. The wild-type strain is the *C. elegans* N2 Bristol variant. Mutants used in the study include *osm-9(ky10) IV* (Colbert and Bargmann, 1995)*, osm-9(ok1677) IV,* and *osm-9(yz6) IV.* All rescue experiments (**Fig 3.1, Fig 3.S2, Fig 3.S4**) were performed in the *osm-9(ky10) IV* background. The transgenic lines used for the rescue experiments are JZ1967 and JZ1968 [*KBC41/$_p$odr-3::osm-9::UTR* (50 ng/µl), *$_p$unc-122::mCherry* (20 ng/µl)], (**Fig 3.1**); JZ2034, JZ2035, and JZ2036 [*KBC56/$_p$osm-9::osm-9::UTR* (50 ng/µl), *pCFJ90/pmyo-2::mCherry* (2.5 ng/µl), *pSMdelta* (100 ng/µl)], (**Fig 3.S2**); JZ1655 and JZ1656 [*KBC8/$_p$ceh-36::GFP::osm-9* (100 ng/µl), *$_p$unc-122::GFP* (20 ng/µl)], (**Fig 3.S4A**); JZ1621 and JZ1622 [*pJK21/$_p$odr-3::GFP::osm-9* (50 ng/µl), *$_p$unc-122::mCherry* (20ng/µl), ScaI-digested N2 DNA (30 ng/µl)], (**Fig 3.S4B**); JZ1819 and JZ1874 [*KBC24/$_p$odr-3::osm-9* (50 ng/µl), *pCFJ104/$_p$myo-3::mCherry* (5 ng/µl)], (**Fig 3.S4C**); JZ1940 and JZ1941 [fosmid WRM065bE12 (from Source BioScience, 5 ng/µl), *$_p$unc-122::GFP* (20 ng/µl), *$_p$str-2::GCaMP3::mCherry* (25 ng/µl)], (**Fig 3.S4D**); lines containing *osm-9::GFP5* (25 ng/µl), *$_p$unc-122::mCherry* (20 ng/µl), (**Fig 3.S4E**); lines containing *KBC42/$_p$osm-9::osm-9::UTR* (50 ng/µl), *$_p$unc-122::mCherry* (20 ng/µl) or *KBC42/$_p$osm-9::osm-9::UTR* (10 ng/µl), *pCFJ90/$_p$myo-2::mcherry* (2.5 ng/µl), *pSMdelta* (140 ng/µl), (**Fig 3.S4F**); JZ1905 and JZ1906 [*KBC38/$_p$nphp-4::osm-9::GFP* (5 ng/µl), *$_p$unc-122::mCherry* (20 ng/µl); *osm-*

9(ky10) IV gDNA (8 ng/µl); *$_p$ceh-36::mCherry* (5 ng/µl)], (**Fig 3.S4G**). ScaI-digested N2 DNA, *osm-9(ky10) IV* gDNA, or *pSMdelta* (empty vector) were used to bring up the total injection concentration to ensure transgenesis (Evans, 2006). JZ designations are L'Etoile lab strain names.

Molecular Biology

Constructs were made using standard molecular techniques. The construct *pJK21/$_p$odr-3::osm-9SS::GFP::osm-9cDNA* was made by replacing the *$_p$str-2* promoter in *pJK17/$_p$str-2::osm-9SS::GFP::osm-9cDNA*, digested with NotI and AscI restriction enzymes, with the subcloned *$_p$odr-3* fragment from *pJK15/$_p$odr-3::osm-9SS::GFP::osm-9cDNA*, using NotI and AscI. *KBC8/$_p$ceh-36::osm-9SS::GFP::osm-9cDNA* was made by amplifying *$_p$ceh-36* with primers KLB30 (TAACGGCCGGCCTCATCAGCCTACACGTCATC) and KLB31 (ACATAGGCGCGCCCGCAAATGGGCGGAGGGT) to add FseI and AscI sites on and digesting *pAW1/$_p$odr-3::osm-9SS::GFP::osm-9cDNA* with FseI and AscI to remove *$_p$odr-3* and subclone the *$_p$ceh-36* fragment in. *KBC41/$_p$odr-3::osm-9cDNA::UTR* was made by digesting *KBC24/$_p$odr-3::osm-9cDNA* with NheI and NcoI and subcloning in the *osm-9* downstream promoter (~3.2kb), amplified off of phenol-extracted followed by ethanol precipitated whole-worm gDNA (Phenol pH 8 from Sigma Aldrich), using the KLB161 (AGACGCTAGCGAACTTTTTTCTTCTAATTTTTTGA) and KLB162 (AACTCCATGGTTAGGTACATTTAAGGTCGATC) primers, adding on the NheI and NcoI sites. *KBC24/$_p$odr-3::osm-9cDNA* was made by digesting *KBC23/$_p$odr-3* with KpnI and NheI and subcloning in *osm-9* cDNA, amplified from whole-worm cDNA (a kind gift

from Maria Gallegos) using primers KLB25 (AACTGGTACCTCATTCGCTTTTGTCATTTGTC) and KLB90 (AGACGCTAGCATGGGCGGTGGAAGTTCG), which add on the KpnI and NheI sites. *KBC42/$_p$osm-9::osm-9cDNA::UTR* was made by digesting *KBC41/$_p$odr-3::osm-9cDNA::UTR* with NotI and XmaI and subcloning in the upstream *osm-9* promoter (~1.6 kb), amplified off of phenol-extracted followed by ethanol precipitated whole-worm gDNA (Phenol pH 8 from Sigma Aldrich), using the primers KLB163 (AGACGCGGCCGCCGCGGGAGTACTTTACGGG) and KLB164 (AACTCCCGGGGTTTGGTTTCTGAAAAAATTGG), adding on NotI and XmaI sites. *KBC38/$_p$nphp-4::GFP::osm-9* was made by digesting *KBC26/$_p$odr-3::osm-9cDNA::GFP* with NotI and NruI and subcloning in the *nphp-4* promoter amplified from *pAK59/$_p$nphp-4::GFP::npp-19* (a kind gift from Piali Sengupta) with primer KLB127 (AGACGCGGCCGCCAACATTATTAATCACTGCAAC) and KLB128 (AACTTCGCGAACTTCCACCGCCCATCTCATTTTTCGAGACTTTGTTA), adding on NotI and NruI sites. KBC56 was made by the following instructions: osm-9 gDNA was amplified with the primers KLB289 (GTTGTTTACCTTTTATGTTCATCCG) and KLB290 (AAATTTTCTACTGCCTGGTATCAAA) off of phenol-extracted followed by ethanol precipitated whole-worm gDNA (Phenol pH 8 from Sigma Aldrich). The *osm-9* gDNA fragment plus extra upstream and downstream homologous sequence was amplified off of the gDNA amplified with KLB289 and KLB290, using the primers KLB298 (GTTGTTTACCTTTTATGTTCAT) and KLB299 (AAAATGATCCACATAAAATTTTCTACTGCCTGGTAT). The *osm-9* upstream partial fragment (~1.2 kb) was amplified using the primers KLB296

(TGATTACGCCAAGCTTCGCGGGAGTACTTTACGG) and KLB297 (TAAAAGGTAAACAACTAGTTTTTAGTACATGAAATAATT) off of the template KBC42/ $_p$*osm-9::osm-9cDNA::UTR*, and the downstream promoter partial fragment was amplified with KLB300 (TATGTGGATCATTTTTGTCTC) and KLB301 (CCGCGCATGCAAGCTTTTAGGTACATTTAAGGTCGAT) off of KBC42/ $_p$*osm-9::osm-9cDNA::UTR*. The three fragments were then recombined in the *pSM* plasmid (linearized with HindIII) using the Takara Infusion HD Cloning kit. The *pSM* plasmid was from Steve McCarroll.

Chemotaxis assay

We utilized the chemotaxis assay from Bargmann et al., 1993. About 4-5 Larval stage 4 (L4) animals were put onto NGM plates seeded with OP50 *E. coli* for 5 days at 20°C, or until populations are one-day old adults. Any plates with fungal or bacterial contamination were not included in the assays. Animals were then washed off the plates using S. basal buffer and into microfuge tubes (sterile, but not autoclaved), where they were conditioned to either S. basal buffer (0.1M NaCl, 0.05M K_3PO_4, pH 6.0), or 2-butanone (Sigma) diluted in S. basal buffer, 1:10,000 (1.23 mM) concentration. Animals were incubated for 80 minutes on a rotating microfuge tube stand. Next, animals were washed two times with S. basal, where the worms were allowed to pellet (about two to three minutes) without spinning them down. Next, the worms were washed with ddH_2O to ensure all salts were removed, and then placed onto a 10 cm Petri dish chemotaxis plate. The chemotaxis plate media was made by adding 100 mL ddH_2O to 1.6 g of Difco bacto agar, then boiled, and then 500 µl of 1M K_3PO_4, 100 µl 1M $CaCl_2$ and 100 µl 1M

$MgSO_4$ were added, and then the media was pipetted at 10 ml per 10 cm plastic petri dish and let cool to solidify, and then square odor arenas and an origin were drawn (S1 Fig). The chemotaxis plate was made with two point sources – one arena with 1 ul of 200-proof ethanol, and the other arena with 1 µl of 1:1000 butanone in ethanol. Each point source also contained 1 µl of 1M sodium azide (Fisher Scientific) to paralyze worms once they reached the arenas (S1 Fig). Animals were placed at the origin, the liquid was removed with a Kim Wipe, and then animals were allowed to roam for at least two hours. Then, the chemotaxis index or learning index was calculated (Fig 1 legend). For reference, most untrained or buffer-trained wild-type animals have a butanone chemotaxis index from 0.6 to 0.9. If pairwise comparisons between the chemotaxis indices of the buffer-trained and butanone-trained populations of the same genotype were not significantly different from each other, then we deemed them memory defective.

LTM chemotaxis assay

The assay is performed as written above in the “Chemotaxis assay” section, but for three cycles instead of just one, with recovery periods on food in between. Animals are incubated for 80 minutes in either buffer or butanone diluted in buffer. Animals are washed, and then incubated for 30 minutes in OP50 diluted in S. basal buffer (OD = 10). Animals are washed as before, but then conditioned for another 80 minutes in either buffer or diluted butanone, making this the second odor-treatment cycle. Animals are then washed and incubated with OP50 for another 30 minutes, washed, and then subjected to a third buffer or odor conditioning cycle. Animals are then washed as

before, and plated onto chemotaxis plates (this is the 0' recovery, three-cycle trained worms), or recovered on 5.5 cm NGM plates seeded with OP50 for either 30', 120', or 16 hours, where they are then subjected to the chemotaxis assay.

WorMotel assay

The WorMotel (Churgin et al., 2017) was used according to previously published methods, as well as this link with more detailed methods (http://fangyenlab.seas.upenn.edu/links.html). In brief, a 48-well PDMS chip called the WorMotel was filled with media and seeded with OP50 (OD = 10), and one worm was placed into each individual well. Behavior was recorded by video using the Multiple Worm Tracker software (https://sourceforge.net/projects/mwt/) and movement was analyzed with Matlab software (KS_analysis_CFY_Jan2019.m and MC_QuiescenceActivity_v1202.fig, both at https://github.com/cfangyen/LEtoile_WorMotel). Excel sheets with total quiescence for each worm is generated from the Matlab code. The mean total quiescence was then taken for statistical analysis.

Statistical Analysis

All data included in the same graph were subjected to the Shapiro-Wilk normality test. If all of the datasets were normally distributed, then one-way ANOVA was performed, followed by Bonferroni correction for pairwise comparisons. If any datasets were non-normally distributed, then the Kruskal-Wallis test was performed. If $p>0.05$, then no further analysis was performed. If $p<0.05$, then the test was followed up by either the

Mann-Whitney u-test for non-parametric data pairwise comparisons or the Student's unpaired t-test for parametric data pairwise comparisons. All p-values included in the same graph were then adjusted using the Hochberg test to remove any type I statistical error, which prevents incorrect rejection of the null hypothesis. ***$p<0.001$, **$p<0.01$, *$p<0.05$, NS indicates $p>0.05$. All graphs show S.E.M. Graphpad Prism and R studio were used for all of the statistical tests. Each data point (represented by grey dots) on the graphs indicates one population of $400>N>50$, run on independent days.

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