TITLE

Mitochondrial calcium uniporter regulates odor learning and memory by controlling neuropeptide release

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SUMMARY

Despite growing understanding of the various roles mitochondria play in neurons, how they contribute to higher brain functions such as learning and memory remains underexplored. Here, using *C. elegans,* we found that expression level and activity of the mitochondrial calcium uniporter (MCU) pore forming unit MCU-1 impacts learning and memory for odors sensed by a specific sensory neuron, AWC^{ON}. Genetic and pharmacological manipulation showed that MCU-1 was required in the sensory neuron during conditioning. For aversive odor learning, MCU-1 mediates learning to 60-min conditioning by facilitating the selective release of NLP-1 neuropeptide through mitochondrial ROS production. MCU activation and neuropeptide release likely coincide with the AWC calcium peak following odor removal. We propose that mitochondria contribute to learning and memory by acting as a duration sensor to control select neuropeptide release in response to prolonged stimuli.

KEYWORDS

Caenorhabditis elegans, mitochondrial calcium uniporter, redox signaling, learning, memory, forgetting, behavioral plasticity

INTRODUCTION

Learning and memory allows past experience of an organism to adjust and shape their behavior, thereby increasing its fitness and chance of survival. This behavioral plasticity is modulated by multiple factors such as the length and frequency of the experience, or concurrent external and internal sensory inputs. Such set of factors ensures that the learning and strength of the memory is commensurate to the type of experience and its context.

The cellular and molecular substrate for learning and memory is the change in synaptic activity strength. Among the numerous factors involved in modulating synaptic activity, mitochondria are reported to play a role by supplying ATP through energy metabolism[1-4], buffering calcium[5-8], and producing localized ROS[9,10]. Consistent with this, a few studies have shown instances where mitochondria are involved in learning and memory[1,11,12], and mitochondrial dysfunction in the brain has been linked to synaptic dysfunction and decline in cognitive abilities[13]. Further studies of mitochondria's role in neuronal function will help us understand the various ways in which metabolic activities and their dysfunction are interconnected with higher brain functions such as learning and memory.

The mitochondrial calcium uniporter (MCU) complex is the main route of calcium entry into mitochondria. The complex consists of MCU channel protein and several regulatory subunit proteins that play modulatory roles. In *C. elegans*, the complex consists of the channel protein MCU-1, along with two regulatory subunit proteins MICU-1 and EMRE-1. With the molecular identity revealed a little more than a decade ago[14,15], studies are beginning to uncover its various roles in different tissues during normal and pathological conditions[16,17]. Similarly, their function in neurons is only beginning to be understood.

The nematode *C. elegans* displays complex behavior that can be modified through past experience. Its simple nervous system consisting of 302 neurons, its genetic tractability, and the array of tools available, offer the chance to investigate the detailed molecular underpinnings of learning and memory. *C. elegans* can learn to increase or decrease its attraction to odors based on past experience and can control the degree of these modifications depending on the context and length of the experience[18-20].

In this study, we show that MCU is involved in controlling neuropeptide release to facilitate odor learning in *C. elegans*. We found that *mcu-1* mutants show defects in both aversive and positive associative learning of AWC^{ON}-sensed odors. Moreover, MCU-1 expression or activity levels correlated with long-term memory: over-expression of MCU or pharmacological activation caused long-term memory retention, whereas *mcu-1* mutants were unable to form long-term memory even after multiple training sessions. We found that aversive odor learning after 60-min conditioning required MCU-1 in the AWC sensory neurons. MCU was required during the time of learning and led to mtROS production, and subsequent secretion of NLP-1. Blocking MCU-1 at different time points during odor conditioning revealed that MCU-1 function was needed towards the end of conditioning for NLP-1 secretion and odor learning, which is when AWC calcium transient appears in response to odor removal. Lastly, we show that not all neuropeptides require MCU-1 for secretion, demonstrating specificity in how different neuropeptides are controlled. Taken together, our study shows that mitochondria contribute to learning and memory by acting as a duration sensor to control select neuropeptide release.

RESULTS

mcu-1 mutants display AWC^{ON} specific aversive learning defect

To study the role of MCU in neurons, we tested a null mutant strain of *mcu-1* for any behavioral defects, including odor behaviors (Figure 1A). *C. elegans* detects volatile attractive odors mainly through two pairs of sensory neurons, AWC and AWA. The AWC neuron pair is asymmetric, and expresses different sets of receptors[21]. With this in

mind, we selected odorants that represented the function of each sensory neurons: butanone, pentanedione, benzaldehyde, and diacetyl, which are mainly sensed by AWC^{ON}, AWC^{OFF}, both AWCs, and AWA, respectively. Mutant worms showed normal chemotaxis for all odorants tested, indicating that worms can sense and move toward attractive odors in the absence of MCU-1 (Figure 1B).

We next tested *mcu-1* mutants for odor learning behaviors. In the aversive odor learning paradigm, previous exposure to odors without food causes worms to ignore or avoid what used to be an attractive odor. After 60 min of pre-exposure, *mcu-1* mutants failed to display aversive learning to benzaldehyde and butanone, whereas learning for other odors remained normal (Figure 1C). Since butanone is sensed by AWC^{ON}, and benzaldehyde is sensed by both AWC neurons, this suggested that learning was specifically defective for AWC^{ON}. Consistent with this, pentanedione, which is sensed by the AWC_{OFF} neuron, showed normal aversive learning. Thus, *mcu-1* mutants fail to learn odors sensed by the AWC^{ON} neuron.

Aversive learning defect in *mcu-1* is specific to 60 min pre-exposure

In aversive odor learning, chemotaxis index (CI) to an odor progressively decreases with longer pre-exposure time[19]. Some of these steps are molecularly distinct: 30 min pre-exposure requires *cng-3*[22], and further decrease after 60 min or more requires EGL-4 translocation into the nucleus[19].

To further characterize the learning defect of *mcu-1* mutants, we tested different pre-exposure times ranging from 30 to 120 min. At 30 min pre-exposure, both wild type and *mcu-1* mutant strain showed a modest decrease in attraction (Figure 1D,E). At 60 min pre-exposure, wild type showed further decrease in CI, whereas *mcu-1* mutants remained at the 30 min level. However, after 90 min and beyond, both wild type and *mcu-1* worms similarly displayed a much lower CI. The pattern was observed for both butanone and benzaldehyde. Although 90 min exposure to benzaldehyde resulted in slightly lower CI in *mcu-1* mutants than in wild type, in other trials this difference was not

as pronounced (Figure 2B). Thus, to our surprise, the learning defect was specific to 60 min pre-exposure.

MCU-1 is required in the AWC sensory neuron for aversive odor learning

We next sought to find where MCU-1 was needed for odor learning. We hypothesized that MCU-1 is likely needed in the neurons. To test this, we generated a transgenic strain that expressed MCU-1 under a neuron-specific promoter (*rab-3p*). As expected, neuronal *mcu-1* rescue strain showed normal aversive learning to both butanone and benzaldehyde (Figure 1F,G). To further narrow down the neuron responsible, we next expressed MCU-1 under the AWC-specific promoter (*ceh-36p*). Expressing MCU-1 in the AWC neuron was also sufficient to restore aversive learning (Figure 1F,G). Thus, MCU-1 is needed in the sensory neuron AWC to mediate odor learning.

MCU-1 expression in adults is sufficient for odor learning

To determine when MCU-1 is needed for learning, we generated a transgenic strain that expressed MCU-1 under a heat-inducible promoter. Inducing MCU-1 expression at different larval stages revealed that aversive learning occurred only when MCU-1 was induced at the adult stage, which was only a few hours before the aversive learning assay (Figure 2A). This shows that MCU-1 is required post-developmentally, and likely close to the time of learning.

A previous study using *Drosophila* had shown that the absence of MCU during development causes morphological defects in the mushroom body neurons[23]. To see whether similar defects occur in *C. elegans*, we imaged fluorescently labeled AWC neurons in wild type and *mcu-1* mutant to assess their morphology. We found no obvious difference in morphology in the two strains (Figure S1), adding to the evidence that the role of MCU-1 in odor learning is post-developmental. It is also noteworthy that inducing MCU-1 at earlier time points was not effective (Figure 2A), which could suggest high turnover of MCU-1 and the need for continuous supply of newly synthesized proteins.

MCU function is required at the time of conditioning

To further narrow down when MCU-1 function is needed, we used ruthenium red and RU360, known pharmacological blockers of MCU[24]. When added to the odor solution during pre-exposure, we found that they exactly mimicked the *mcu-1* mutant phenotype: treatment during 60 min pre-exposure inhibited odor learning, but treatment during 90 min pre-exposure yielded normal odor learning indistinguishable from wild type (Figure 2B-E). Thus, MCU-1 is needed at the time of conditioning for the 60-min odor learning.

***mcu-1* mutants show weaker positive associative odor learning**

Next, we wondered whether the odor learning defect of MCU-1 was limited to aversive learning, or whether other forms of odor learning were also affected. Positive associative memory paradigm tests CI after exposing worms to a neutral odor with food, causing the worm to form a positive association with the odor and develop attraction[20] (Figure 3A). As the attractant butanone becomes neutral then repulsive to worms at progressively higher concentrations, a higher concentration of butanone is used as the neutral odor stimulus[20].

When worms were exposed to butanone with food, both wild type and *mcu-1* mutants showed increased attraction after conditioning, although the increase in *mcu-1* mutants was not as strong as wild type (Figure 3B). When allowed to recover by returning worms to food without butanone, both wild type and *mcu-1* worms lose the attraction in 1-2 hrs. Thus, *mcu-1* mutants are capable of positive odor learning albeit not as strong as wild type.

Overexpression of MCU-1 inhibits forgetting

To test whether positive odor learning was also mediated by MCU-1 in the AWC sensory neuron, we used the transgenic strain expressing MCU-1 in the AWC neuron to see if the strong attraction was restored. Transgenic worms developed strong attraction after conditioning, resulting in CI that is indistinguishable from wild type. Thus, as with aversive learning, MCU-1 in the AWC neurons was sufficient for strong positive odor learning (Figure 3A).

Unexpectedly, when worms were allowed to recover, we found that the transgenic strain sustained the attraction even after 2 hours, rather than progressively losing the attraction (Figure 3B). Such long-term retention of memory is only seen in wild type after multiple training sessions[20]. To explain why the sensory neuron rescue would add a long-term memory retention phenotype, we hypothesized that MCU-1 expression level in the transgenic strain is higher than normal endogenous levels. This was a plausible scenario, since DNA constructs injected into the worms form large extrachromosomal DNA arrays, which contain numerous copies of the gene[25].

Testing whether the transgenic strains overexpress MCU-1 requires isolating the AWC neuron to quantify mRNA or protein expression. Since testing gene expression levels specifically in a single neuron is technically challenging in *C. elegans*, we decided instead to treat wild type worms with kaempferol, a MCU activator, to mimic MCU-1 overexpression. Consistent with our hypothesis, when kaempferol was added during odor conditioning, worms maintained a high CI even after 2 hrs, exactly mimicking the AWC rescue strain phenotype (Figure 3C). This demonstrates that the prolonged memory retention in the transgenic strain is likely due to overexpression of MCU-1. Moreover, it shows that the prolonged memory observed is due to increased MCU activity during memory acquisition, and not memory recall.

To test whether prolonged memory retention also occurred for aversive learning, we went back to the aversive learning paradigm. Following a previously established protocol[26], worms were allowed to recover in plates with food after butanone conditioning (Figure 3D). For this, 90 min pre-exposure time was used, so that *mcu-1* mutants could also form the negative association. As previously established[26], attraction is quickly restored in wild type worms after 30 min recovery (Figure 3E). Likewise, *mcu-1* mutants also showed quick recovery (Figure 3E). In contrast, neuronal and AWC rescue strains maintained the decreased attraction even after 2 hr (Figure 3E). The same was seen for wild type worms treated with kaempferol during pre-exposure (Figure 3F). Thus, longer memory retention in the neuronal or AWC rescue strain is

seen for both aversive and positive odor learning. Taken together, our data suggest that high MCU-1 activity or expression level inhibits forgetting, causing premature formation of long-term memory.

MCU is required for long term memory formation

Since overexpression of MCU-1 resulted in premature long-term memory, where long-term memory was formed after only a single training session, we wondered whether the opposite would occur as well; whether the lack of MCU-1 would prevent long-term memory formation. To test this, we subjected *mcu-1* mutants to 7 rounds of repeated positive associative training ("spaced training"), previously shown to result in memory that persists even after 24 hours[20] (Figure 4A). Consistent with previous studies, we also observed that spaced training in wild-type worms resulted in sustained high CI at 1, 2, 16, and 24 hrs post-recovery (Figure 4B). In *mcu-1* mutants, spaced training resulted in strong attraction for the first 2 hrs. However, learned attraction was progressively lost after 16 hrs, and CI returned to pre-training levels after 24 hrs (Figure 4B). Thus, MCU-1 levels correlate with AWC^{ON}-mediated long-term memory – high MCU-1 expression results in premature long-term memory formation, and lack of MCU-1 results in defective long-term memory formation.

NLP-1 neuropeptide secretion from AWC requires MCU-1

While searching the literature, we found that a similar learning defect had been described previously for two other mutants. Chalasani *et al.* had shown that both *nlp-1* mutant and its G-protein coupled receptor (GPCR) *npr-11* mutant showed defective aversive learning after 60 min pre-exposure, but showed normal learning after 90 min pre-exposure[27]. NLP-1 is expressed in many neurons, including AWC[28,29]. Interestingly, the study also showed that NLP-1 released from AWC is required for lowering AWC calcium response to odors after 60 min conditioning[27]. Conducting the same aversive learning experiment on *nlp-1* mutants confirmed their results (Figure 5A). Since MCU-1 has previously been implicated in neuropeptide secretion[10], this suggested the possibility that MCU-1 may mediate odor learning through regulating neuropeptide secretion. In addition, another study reported that genetically increasing neuropeptide

secretion was sufficient to produce premature long-term memory[30], which is the same phenotype to that of MCU-1 overexpressing strains (Figure 3B). Based on these evidences in the literature, we hypothesized that MCU-1 is involved in neuropeptide secretion.

To test this possibility, we generated a transgenic strain that expressed Venus-tagged NLP-1 in the AWC neurons. The fluorescently tagged NLP-1 is not functional, but it is secreted and accumulates in the coelomocytes, whose fluorescence can be used as a proxy for neuropeptide secretion[31]. To test whether prolonged odor exposure causes NLP-1 release, we exposed worms to 1 hr butanone and observed whether there was any change in coelomocyte fluorescence. Consistent with our hypothesis, 60 min of butanone conditioning was sufficient to increase fluorescence in the coelomocyte in the wild-type background compared to worms exposed to buffer alone (Figure 5B,C). On the other hand, *mcu-1* mutant worms showed no change in coelomocyte fluorescence in response to butanone conditioning (Figure 5B,C). Thus, 60 min butanone conditioning causes secretion of NLP-1 from AWC, and this requires MCU-1.

We next asked whether MCU activation alone is sufficient to increase NLP-1 secretion. We treated worms with the MCU activator kaempferol[32], as well as juglone, an oxidative stressor previously shown to cause calcium influx through MCU[10]. After 10 min treatment of either kaempferol or juglone, there was a significant increase in fluorescent signal in the coelomocytes compared to DMSO treated worms (Figure 5D,E). When *mcu-1* mutants were treated with juglone, however, there was no increase in fluorescence, showing that MCU-1, and thus calcium influx into the mitochondria, is required for NLP-1 release (Figure 5D,E). We note that no difference in fluorescence was observed between DMSO treated wild type and *mcu-1* mutants, suggesting that the basal level secretion of NLP-1 is unaffected in *mcu-1* mutants (Figure 5D,E).

mtROS is required for NLP-1 secretion

How are MCU-1 and calcium influx mediating NLP-1 secretion? Several studies show that calcium influx through MCU results in mitochondrial ROS production[31,33].

Superoxide dismutase converts the superoxide anion produced by the mitochondria into H_2O_2, which can serve signaling functions by oxidizing the cysteine residues of various effector proteins[10]. In addition, it was previously shown in AIY neurons that exogenous H_2O_2 treatment, as well as optogenetically inducing ROS production at the mitochondria, resulted in FMRFamide-like neuropeptide FLP-1 neuropeptide secretion[10]. We reasoned that if NLP-1 was secreted through a similar mechanism, exogenous H_2O_2 treatment should be sufficient to cause NLP-1 release in the *mcu-1* mutant background, which was previously unresponsive to juglone. As hypothesized, *mcu-1* mutant worms treated with 5mM H_2O_2 for 10 min showed increased NLP-1 secretion (Figure 5D,E).

mtROS is required for NLP-1-mediated odor learning

To directly verify that 60 min butanone conditioning results in mtROS production, we generated a transgenic strain that expressed the fluorescent ROS sensor mito-roGFP in the AWC neuron. We first treated the AWCp::mito-roGFP transgenic strain with exogenous H_2O_2 or the antioxidant N-acetyl Cysteine (NAC) to verify roGFP function, and to observe the range of redox ratios we could expect to see. We found that the redox ratio shifted accordingly when worms were treated with H_2O_2 or NAC (Figure S2A,B). However, when worms were exposed to butanone for 1 hr, no change in redox ratio was observed (Figure S2A,B).

We reasoned that mtROS produced from 1 hr butanone conditioning was likely too subtle to be observed. To get around this, we reasoned that if 1 hr of butanone conditioning produces mtROS, neutralizing mtROS during odor exposure would prevent odor learning. We treated worms with NAC, a general antioxidant, or mitoTEMPO, which specifically scavenges mitochondrial ROS. Consistent with our hypothesis, both NAC and mitoTEMPO were effective in preventing odor learning at 60 min (Figure 6A). On the other hand, antioxidant treatment did not prevent learning in worms conditioned for 90 min, although their chemotaxis index was slightly higher than untreated control. Taken together, this shows that mtROS is produced by 60-min odor conditioning, and that it is required for NLP-1-mediated 60 min aversive odor learning.

MCU activation accelerates odor learning

Since 10 min treatment of kaempferol caused NLP-1 secretion from AWC, we asked whether activating MCU during a much shorter odor exposure time would cause faster decrease in attraction. Therefore, we tested whether activating MCU during 10 min odor conditioning could cause the worms to display CI similar to 60-min learning. Normally, 10 min of butanone pre-exposure results in only a slight decrease in CI (Figure 6B). Treating worms with kaempferol alone for 10 min did not alter their CI, and worms showed high CI comparable to naïve worms. This shows that NLP-1 secretion alone, without the odor stimulus, does not produce odor learning. However, when kaempferol was added together with butanone for 10 min, worms exhibited a far lower CI that is typical of 60-min pre-conditioning (Figure 6B). Thus, when odor is paired with NLP-1 secretion, it causes faster aversive odor learning.

MCU-1 function is required at the end of conditioning

Our results so far suggest that during conditioning, calcium enters the mitochondria through MCU, causing mtROS production, which then triggers NLP-1 secretion. Although the requirement for NLP-1 signaling for odor learning has been known[27,34], at what point it is released has not been examined. The AWC sensory neuron is considered an OFF neuron in that odor stimulus causes a dip in cytoplasmic calcium rather than a surge, followed by a fast spike upon subsequent odor removal[35]. Similarly, glutamate is tonically released from the AWC synapse during basal conditions, progressively decreases during the length of the odor stimulus, then quickly returns to baseline upon odor removal[36].

How does NLP-1 release compare to that of glutamate neurotransmitters? Since MCU activity is required for NLP-1 release, blocking MCU at specific time points during odor pre-conditioning allows us to estimate the time of calcium influx into MCU, as well as NLP-1 release (Figure 6C). Adding and removing MCU inhibitor RU360 in the first or last half of pre-exposure showed that MCU function was needed during the second half of odor conditioning (Figure 6C). Furthermore, blocking MCU function during the last 15

min was sufficient to prevent odor learning. This suggested that MCU function was required at the end of odor exposure, which roughly coincides with the timing of the calcium transient that appears upon odor removal. When RU360 was added during the washing step immediately after odor exposure, it had no effect on odor learning (Figure 6C).

To see whether MCU inhibition towards the end of odor exposure prevented NLP-1 release, we conducted the same procedure in NLP-1::Venus expressing transgenic strains, and added RU360 during the last 15 min of butanone exposure. When we imaged the coelomocytes, we found that, consistent with the behavior, inhibiting MCU function towards the end of 60 min butanone exposure prevented NLP-1 release (Figure 6D). We noted that RU360 treatment resulted in slightly subdued fluorescence in the coelomocytes compared to buffer-treated control, but it was not statistically significant. Taken together, we conclude that MCU-mediated calcium influx, as well as NLP-1 secretion, takes place at the end of odor conditioning, likely at the time of AWC calcium transient upon odor removal. This is consistent with the observations made so far in neurons that mitochondrial calcium increase occurs synchronously with cytoplasmic calcium increase[37].

Not all neuropeptides are regulated by MCU

Neurons of *C. elegans* express multiple neuropeptide genes[29]. Whether these are co-released or whether a mechanism exists to differentially regulate the release of each neuropeptide is not well understood. Having found that NLP-1 release is regulated by MCU-1, we asked whether MCU-1 was required for all neuropeptide release from AWC. We selected two more neuropeptides that were known to be highly expressed in AWC, NLP-47 and NLP-3[29]. Venus-tagged neuropeptides were expressed under the AWC-specific promoter to assess their accumulation in the coelomocytes. Fluorescent signal was observed in the coelomocytes, showing that they are secreted. However, contrary to NLP-1, treatment with juglone did not result in change in fluorescence in the coelomocytes (Figure 6E,F). This shows that among the neuropeptides tested, only NLP-1 is regulated by MCU-1. Such selectivity is consistent with what has been

observed previously in the AIY neuron[10], which showed juglone or H_2O_2 treatment only affected FLP-1 secretion, and not FLP-18. This demonstrates selectivity in the neuropeptides controlled by MCU-1.

DISCUSSION

Here, we showed that MCU-1, and thus mitochondrial calcium, contributes to neuronal and behavioral plasticity in *C. elegans* by regulating neuropeptide secretion from the sensory neuron. We characterized the odor learning and memory phenotypes mediated by MCU-1, whose expression level in the AWC neuron broadly correlates to the worms' ability to learn and remember odors. Mutants are defective for 60 min aversive odor learning, and correct learning requires MCU-1 expression and activity at the time of learning. MCU activity results in the production of mtROS, which then causes secretion of the NLP-1 neuropeptide, resulting in decreased attraction. Whereas *mcu-1* mutants fail to form long-term memory even after repeated training, increased MCU-1 expression or MCU activity causes premature long-term memory formation. Whether defective or premature long-term memory is also mediated by NLP-1 remains to be seen, but since neuropeptide signaling was previously identified as an integral component of long-term memory of odors[30], *mcu-1* is expected to contribute in a similar mechanism. Finally, we show that there is selectivity in MCU-controlled neuropeptides, as the release of other highly expressed neuropeptides were not affected by MCU activation.

A previous study in Drosophila had found odor memory defects in MCU mutant flies[23]. However, MCU was required during development, and silencing MCU after pupation had no effect on memory. On the other hand, in *C. elegans*, we show through larval-stage-specific induction and pharmacological manipulation that MCU-1 is required at the time of learning, through its control of specific neuropeptide release. Such differences observed between the two organisms highlight the benefit of studying gene function in multiple model organisms.

MCU as a stimulus duration sensor

Based on our data and what is known in the literature, we predict that prolonged odor stimulation in the AWC sensory neuron causes either a stronger influx or a switching on of influx of calcium into the mitochondria, which causes the release of NLP-1.

Our results show that mitochondrial calcium influx in the AWC neuron is the determinant for the difference between 30 min and 60 min aversive odor learning. This suggests that mitochondrial calcium entry may act as a duration sensor for sensory stimulation. Simultaneous imaging of cytoplasmic and mitochondrial calcium in neurons show mitochondrial calcium influx occurs concurrently with cytoplasmic calcium increase[37]. Concurrent cytoplasmic and mitochondrial calcium elevation is the norm for most tissues, as there is a minimum threshold of surrounding calcium that needs to be reached for the MCU pore to open[38]. The pharmacological treatment used in our study does not provide sufficient temporal resolution to definitively show the same is occurring in the AWC neuron: treatment with the MCU blocker RU360 was effective when added towards the end of odor exposure but was not effective when added immediately after. We argue that this is due to the time it takes for the pharmacological blocker to diffuse into the worm's body, which is likely slower than the time worms respond to odor removal with a calcium transient. Likewise, when the blocker is introduced during odor conditioning then removed during washing, it would also take time for the blocker to wash off to restore MCU function. This delay would leave MCU still blocked at the moment of the calcium transient. Therefore, we expect that mitochondrial calcium influx in the AWC sensory neuron also occurs synchronously with the cytoplasmic calcium transient at the time of odor removal.

How would mitochondria distinguish between different durations of odor stimulation? A careful characterization of AWC calcium response has previously shown that progressively longer odor exposure time from 10 s, 1 min, to 3 min, resulted in a progressively faster and higher calcium spike at the synaptic terminal upon odor removal[36]. This shows that cytoplasmic calcium can reflect the duration of odor stimulation. Whether this will also apply to 30 min and 60 min odor exposure remains to

be seen, but it is one possible way that different durations of odor stimulation could be signaled in the cell.

Secondly, the emerging view of MCU-mediated calcium influx is that of selective influx in response to more significant changes in cellular activity. Multiple studies show that mitochondrial calcium influx is usually coupled to stronger electrical stimulation of the cell, localized cytoplasmic calcium increase, or two temporally coincidental stimuli[37-43]. Gating of MCU is regulated by the auxiliary subunits of the MCU complex – MICU and EMRE. MICU can sense calcium through its EF-hand domain, causing MCU to open in response to cytoplasmic calcium changes[44]. In addition to MICU and EMRE, CAMKII, MCUR1, or mitochondrially localized IGFR were also found to regulate MCU in mice[37,40,45]. As detailed mechanisms of MCU regulation is only beginning to be revealed, there may be yet unidentified factors that allow MCU to respond to the duration of odor stimulation in *C. elegans*.

mtROS and neuropeptide release

Mitochondrial calcium influx is a prerequisite for hormone secretion in endocrine cells, most notably insulin from pancreatic β cells[46]. In β cells, glucose uptake and subsequent metabolic processes trigger mitochondrial calcium influx, as part of a process known as metabolism-secretion coupling[47,48]. Mitochondrial calcium influx produces “coupling factors” that together with other signals contribute to insulin release, among which include mtROS and H_2O_2[49,50]. Similar to our study, treatment with antioxidants prevented insulin release, showing that hormone secretion and neuropeptide secretion share similar mechanisms.

In *C. elegans* neurons, it was previously shown that H_2O_2 produced from mtROS modifies a cysteine residue of protein kinase C, PKC-1, to trigger the release of FLP-1 from the AIY interneuron[10]. Whether the same happens in AWC will need to be determined. However, as expressing the gain-of-function *pkc-1* allele in the AWC neuron showed premature formation of long-term odor memory[30], it is a highly promising candidate.

Selectivity in neuropeptide release

Although previously recognized, single-cell sequencing technology is beginning to reveal the prevalence of the existence of multiple neuropeptides per single neurons[51,52]. Drawing from what is known about neurotransmitters and neuromodulators expressed in the same neurons[53,54], it is possible that some neuropeptides are co-released while some are separately regulated. This raises the question of what kinds of mechanisms exist that would allow for separate regulation of neuropeptide release from the same neuron[55]. Our study suggests one possible mechanism of differential release. We show that among NLP-1, NLP-3, and NLP-47, all highly expressed in AWC, only NLP-1 is regulated by MCU activation. Similar selectivity in neuropeptide release was also observed in the AIY interneuron of *C. elegans*, where mtROS caused the release of the FMRFamide-like neuropeptide FLP-1 but not FLP-18[10]. What determines whether a neuropeptide is controlled by the mitochondria will be a topic with important implications.

Mitochondria are involved in a range of functions, from energy production, calcium buffering, lipid biosynthesis, ROS signaling, to cell death. In cells with extreme structural polarity such as neurons, these range of functions contribute to many compartment-specific roles. As mitochondrial dysfunction often accompanies metabolic disorders, neurodegenerative disorders, and aging, understanding these specific functions will lead to a deeper understanding of the neuronal pathology associated with these conditions.

MATERIALS AND METHODS

Worm cultivation

C. elegans were maintained at 20°C on plates containing nematode growth media (NGM) with OP50 bacteria. Unless otherwise specified, day 1 adults were used for behavioral experiments. All strains used in this study are listed in Table S1.

Construction of plasmids and transgenic strains

All primers used in this study are listed in Table S2. Whole worm RNA was isolated using Direct-zol RNA Microprep kit (Zymo Research). cDNA was prepared using SuperScript IV First Strand Synthesis System (Invitrogen). *mcu-1* sequence was amplified from worm cDNA using PrimeSTAR MAX DNA Polymerase (Takara) and cloned into pCR-Blunt II-TOPO vector (Invitrogen).

Chemotaxis assay

Assay was performed as described previously[18]. For assay plates, 10 ml of agar media (1.6% Difco Granulated Agar (Beckton Dickinson, Franklin Lakes, NJ, USA), 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KPO_4) was poured into 10 cm petri dish and were left out to dry for at least 1 day. 100-200 well-fed day 1 adult worms were used for all assays. Odors were diluted as follows unless otherwise stated: 1 µl Benzaldehyde (Sigma) in 200 µl ethanol, 1 µl 2-Butanone (Sigma) in 1,000 µl ethanol, 1 µl Diacetyl (Waco) in 5,000 µl, and 1 µl 2,3-Pentanedione (Alfa aesar) in 10,000 µl ethanol.

Aversive associative learning assay

Day 1 adults were collected from plates and washed three times with S-basal buffer. For conditioning, worms were placed in 1 ml of S-basal buffer with or without each odorant with the following concentrations: Benzaldehyde 1:10,000, 2-Butanone 1:10,000, Diacetyl 1:5,000, 2,3-Pentanedione 1:100,000. The microcentrifuge tubes were rotated on a rotator for conditioning, then washed twice with s-basal buffer and once with 3DW before conducting the chemotaxis test. For heat-shock-inducible *mcu-1* mutant worms, synchronized worms at the L1 stage were transferred to seeded NGM plates to grow until ready for heat shock. At each developmental stage, plates of worms were sealed with parafilm and immersed into a 32°C water bath for 1 hr. Plates were moved back to 20°C until day 1 stage when the aversive learning assay was conducted. Worms heat-shocked at the adult stage were placed at 20°C for 2 hr until used for the assay.

Positive associative memory training

Assays were performed as previously described[20,56]. Well-fed day 1 adult worms place in M9 buffer and were starved for 1 hr. Starved worms were then transferred to seeded NGM plates with an agar plug on the lid containing 2 µl of 10% butanone in ethanol and trained for 1 hr. 100-200 worms from the trained population were tested for butanone chemotaxis either immediately (0 hr), or after being moved to seeded NGM plates at specific times before testing (30 min to 2 hr). To test long-term memory, the worms were trained with the odor for 30 min and was returned to regular plates for 30 min. After this process was conducted 7 times, worms were transferred to holding plates for the indicated time frame (1 to 24 hr), then tested for chemotaxis.

Forgetting assay

Forgetting assays were performed as previously described[26]. Day 1 adult worms are washed three times with S-basal buffer and conditioned with 2-Butanone (dilute 1:10,000 in S-basal buffer) for 90 minutes. The worms were later transferred to seeded NGM plates without the odor, and recovered for 30 minutes to 2 hours to test chemotaxis.

Pharmacological treatment

All pharmacological substances used in this study, except for juglone and H_2O_2, necessitated permeabilizing the cuticle. This was accomplished by growing worms in *dpy-8* RNAi bacteria at the last molting stage, from L4 to adult stage. *dpy-8* RNAi bacteria clone was obtained from the Ahringer RNAi library[57] (Source BioScience). RNAi plates were prepared as previously described[58]. Permeabilization of cuticle did not affect their behavior (data not shown). Following are the stock solutions used: 6.358 mM Ruthenium Red in water (Millipore), 0.5 mg/mL RU360 (Millipore) in deoxygenated water, 50 mM of Kaempferol (Santa Cruz Biotechnology) in EtOH, 50 mM of Juglone (Sigma-Aldrich) in DMSO, 100 mM of N-Acetyl-L-Cysteine (BioBasic) in water, 18.938 mM of mitoTEMPO (Santa Cruz Biotechnology) in DMSO, and 30% H_2O_2 (Sigma-Aldrich). For behavior assay, about 1000 adults worms were transferred into a 1.5 mL Eppendorf tube with S-basal, and washed three times. Pharmacological treatments were added to washed worms at final concentration of 100 μM for Ruthenium Red, 0.5 μM for RU360, 100 μM for Kaempferol, 300 μM for Juglone, 0.5 mM for N-Acetyl-L-Cysteine, 10 μM for mitoTEMPO, and 5 mM H_2O_2.

Microscopy and analysis

All images were obtained using Zeiss LSM800 confocal microscope with Zeiss C-Apochromat 40x/1.2 W Korr objective and ZEN 2.6 software. For coelomocyte imaging, 30–40 L4 stage animals were collected from NGM plates, washed three times, and exposed to either butanone (1:10,000 in S-basal) for 1 hr or to each pharmacological treatment for 10 min in S-basal. After treatment, worms were briefly washed, then paralyzed using sodium azide and mounted on 2% agarose pads. Coelomocytes at the posterior gonad bend were imaged. Maximum intensity projection was obtained using image stacks and used for analysis. Fluorescence was quantified by manually delineating each coelomocyte using the polygon selection tool and measuring the average pixel intensity in ImageJ.

To image mito-roGFP and calculate reduction/oxidation ratios, worms were imaged at two different wavelengths for excitation, 488/405 for the reduced/oxidized (R/O) ratio[59]. Maximum intensity projection was obtained for each image. After setting the ROI for each mitochondrion in image J, the fluorescence intensity of the reduced mitochondria was measured, and the same ROI is applied to the oxidized mitochondria to obtain the ratio of 2 stacks after subtracting the background value from the two values. To observe the range of roGFP redox ratios, 5 mM H_2O_2 for 10 min was used. To treat worms with NAC, worms were grown in dpy-8 RNAi bacteria from L4 to adult to permeabilize the cuticle, then treated with 5 mM NAC for 10 min.

Statical analysis

All data are expressed as means ± standard error of the mean (SEM). Statistical significance was determined by student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. All experiments were performed in at least 3 independent trials on different dates.

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Author contributions

Conceptualization, H.K.L., K.S.P., and K.-h.Y.; Investigation, H.K.L. and K.-h.Y.; Writing – Original Draft, H.K.L. and K.-h.Y.; Writing – Review & Editing, H.K.L., K.S.P., and K.-h.Y.; Funding Acquisition, K.S.P. and K.-h.Y.; Supervision, K.S.P. and K.-h.Y.

Declaration of interests

The authors declare no competing intrests.

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FIGURE LEGEND

Figure 1. *mcu-1* is required in the AWC^{ON} sensory neuron for aversive odor learning. (A) Chemotaxis assay plate and chemotaxis index (CI) calculation. (B) Chemotaxis index for naïve N2 and *mcu-1(tm6026)* worms. (C) Chemotaxis index in worms after 60 min of odor pre-exposure. (D,E) Chemotaxis index after different pre-exposure times ($n \geq 6$ trials per group). (F,G) Chemotaxis index to in neuron(rab-3p)- and AWC(ceh36p)-specific rescue strains after 60 min conditioning.

Figure 2. MCU-1 is required at the time of training for aversive odor learning. (A) Chemotaxis index of transgenic strains expressing MCU-1 under the heat-shock-inducible promoter (hsp-16.2p). Worms were given heat shock at different larval stages and tested as day 1 adults. (B-E) Mutant worms treated with pharmacological blockers of MCU displayed the same learning defect as *mcu-1* mutants.

Figure 3. Transgenic rescue strains of *mcu-1* are defective in forgetting. (A) Scheme for positive associative odor learning assay. (B) Change in chemotaxis index

after positive odor learning and recovery. (C) Change in chemotaxis index in wild type worms treated with Kaempferol. Values for N2 (gray) and the AWC-specific rescue strain (purple) are identical to (B) ($n \geq 5$ trials per group). (D) Scheme for forgetting assay. (E-F) Change in chemotaxis index after recovery. Values for N2 and AWC transgenic strain are identical in E and F ($n \geq 5$ trials per group).

Figure 4. *mcu-1* mutants are unable to form long term memory after repeated training. (A) Scheme for long-term associative learning. (B-C) Chemotaxis index after different recovery times in N2 and *mcu-1* mutants.

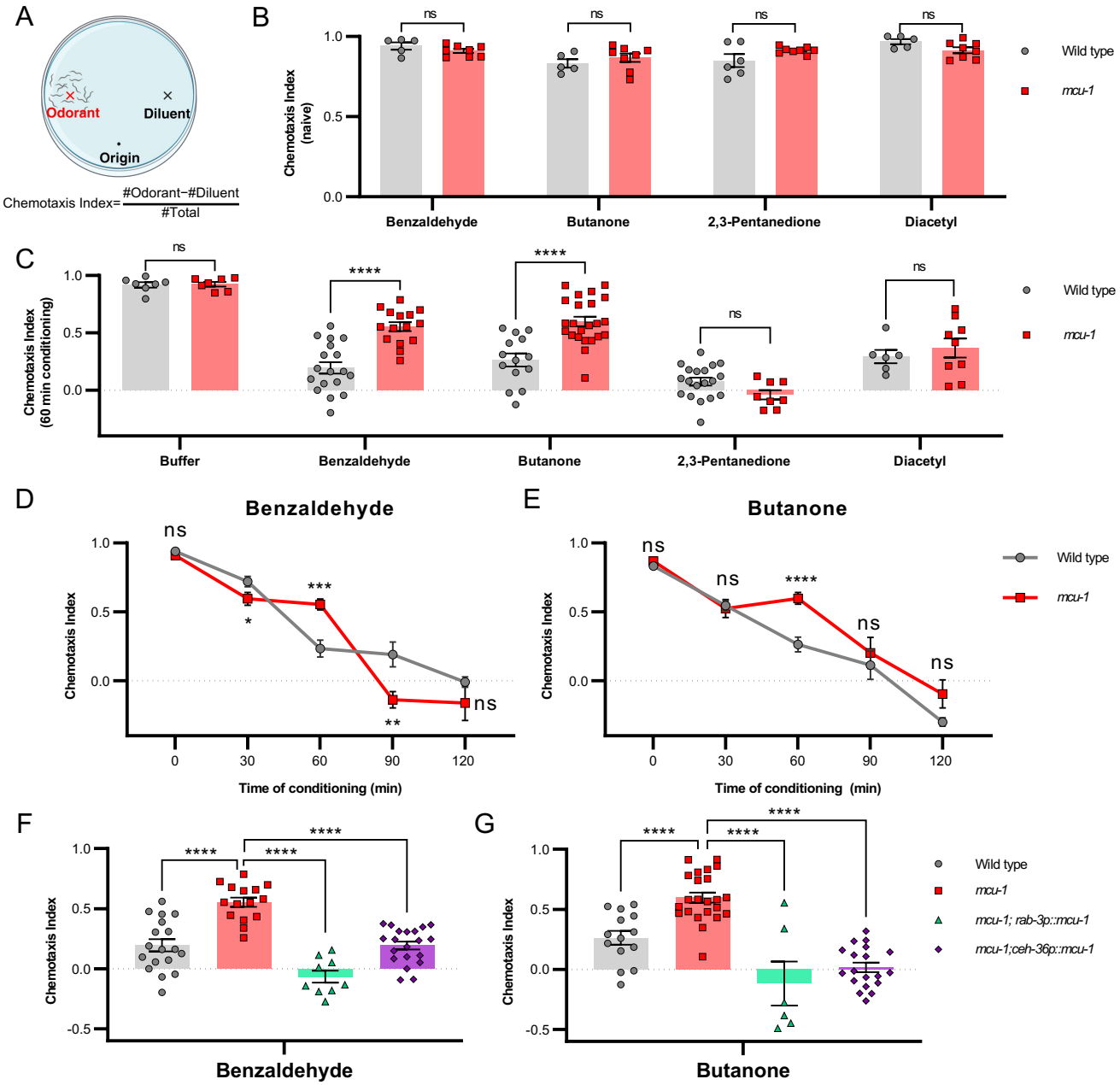
Figure 5. Butanone exposure and pharmacological MCU activation causes NLP-1 secretion from AWC neurons. (A) Chemotaxis index after 60-min and 90-min conditioning in *nlp-1* mutants. (B) NLP-1::Venus fluorescence measured in coelomocytes in response to 60 min exposure to buffer or butanone. (C) NLP-1::Venus fluorescence in coelomocytes in response to kaempferol, juglone, and H_2O_2 treatment in wild type and *mcu-1* mutant.

Figure 6. Coincident mitochondrial ROS (mtROS) production and odor exposure is sufficient for odor learning. (A-B) Antioxidant treatment inhibits 60 min aversive odor learning, but not 90 min learning. (C) Kaempferol treatment during odor exposure causes results in faster learning after 10 min. (D) RU360 treatment at different blocks during 60 min odor conditioning. (E) Chemotaxis index of worms after different treatments. (F) NLP-1 secretion in worms treated with RU360 during the last 15 min of 60 min butanone exposure. (G-H) NLP-3::Venus and NLP-47::Venus secretion does not change in response to kaempferol treatment.

Figure S1. AWC morphology in wild type and mcu-1 mutant worms.

Figure S2. Redox ratio in mitochondria using mito-roGFP in AWC axons. (A) Redox ratio for all mitochondria. (B) Redox ratio for axonal mitochondria.

Figure 1


A
Odorant
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Chemotaxis Index= #Odorant−#Diluent / #Total
B
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ns
Benzaldehyde
Butanone
2,3-Pentanedione
Diacetyl
Wild type
mcu-1
C
Chemotaxis Index (60 min conditioning)
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ns
Buffer
Benzaldehyde
Butanone
2,3-Pentanedione
Diacetyl
Wild type
mcu-1
D
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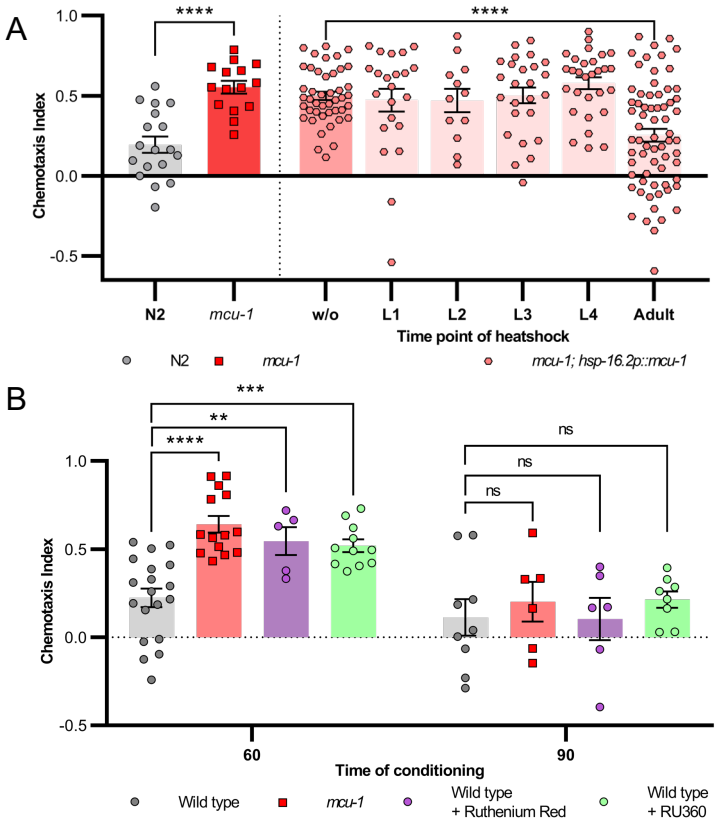
Time of conditioning (min)
E
Butanone
Chemotaxis Index
Time of conditioning (min)
Wild type
mcu-1
F
Chemotaxis Index
Benzaldehyde
G
Chemotaxis Index
Butanone
Wild type
mcu-1
mcu-1; rab-3p::mcu-1
mcu-1;ceh-36p::mcu-1

Figure 2


A
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1.0
0.5
0.0
-0.5
Chemotaxis Index
N2
mcu-1
w/o
L1
L2
L3
L4
Adult
Time point of heatshock
N2
mcu-1
mcu-1; hsp-16.2p::mcu-1
B
***
**
****
ns
ns
ns
1.0
0.5
0.0
-0.5
Chemotaxis Index
60
90
Time of conditioning
Wild type
mcu-1
Wild type
+ Ruthenium Red
Wild type
+ RU360

Figure 3

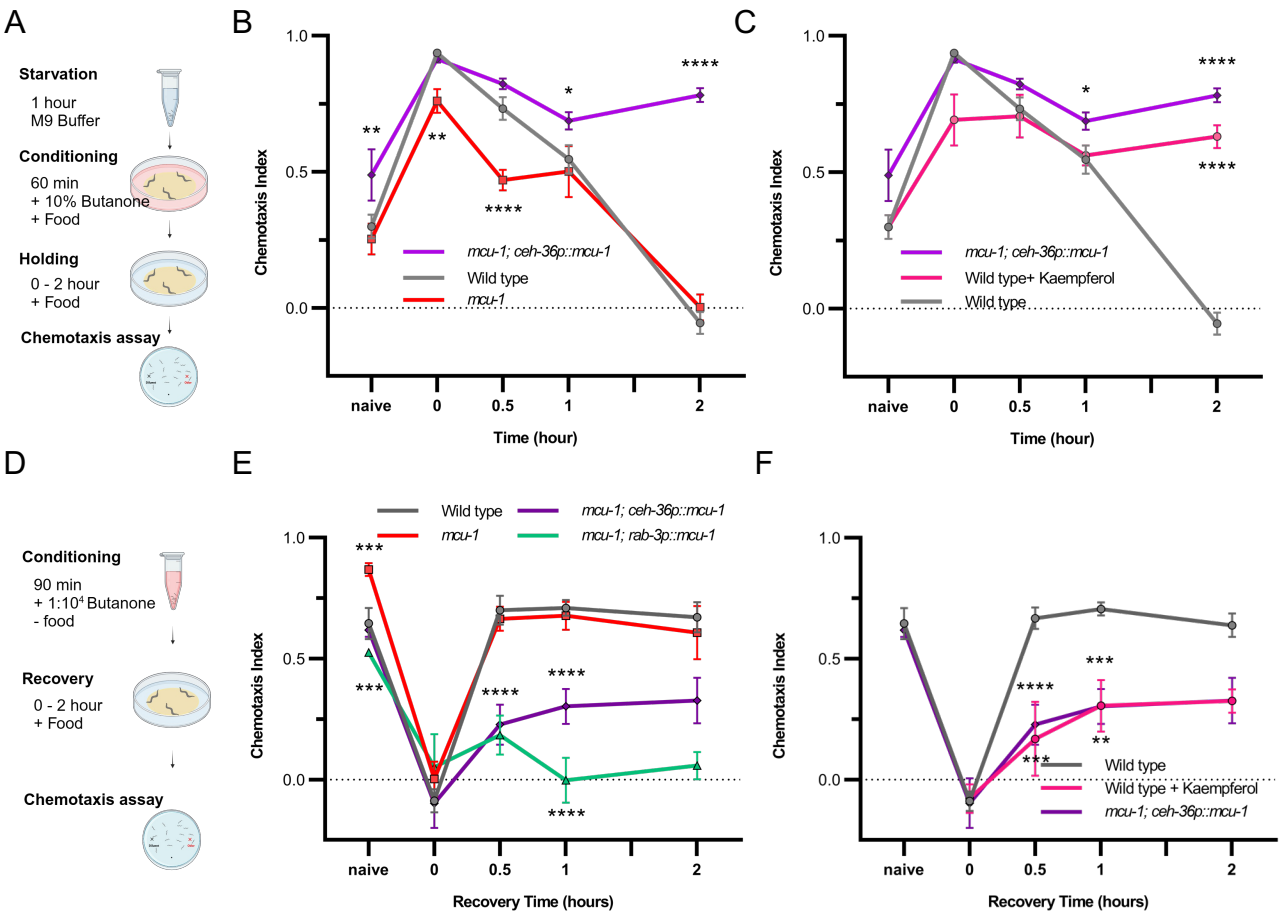
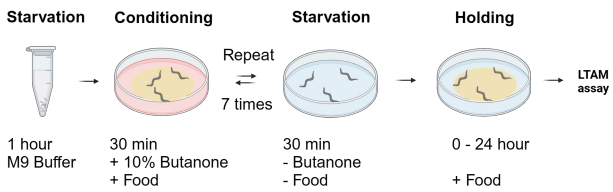

A
Starvation
1 hour
M9 Buffer
Conditioning
60 min
+ 10% Butanone
+ Food
Holding
0 - 2 hour
+ Food
Chemotaxis assay
B
Chemotaxis Index
Time (hour)
naive
0
0.5
1
2
mcu-1; ceh-36p::mcu-1
Wild type
mcu-1
C
Chemotaxis Index
Time (hour)
mcu-1; ceh-36p::mcu-1
Wild type+ Kaempferol
Wild type
D
Conditioning
90 min
+ 1:10⁴ Butanone
- food
Recovery
0 - 2 hour
+ Food
Chemotaxis assay
E
Wild type
mcu-1
mcu-1; ceh-36p::mcu-1
mcu-1; rab-3p::mcu-1
Chemotaxis Index
Recovery Time (hours)
F
Chemotaxis Index
Recovery Time (hours)
Wild type
Wild type + Kaempferol
mcu-1; ceh-36p::mcu-1

Figure 4

A


Starvation
Conditioning
Starvation
Holding
Repeat
7 times
LTAM assay
1 hour
M9 Buffer
30 min
+ 10% Butanone
+ Food
30 min
- Butanone
- Food
0 - 24 hour
+ Food

B

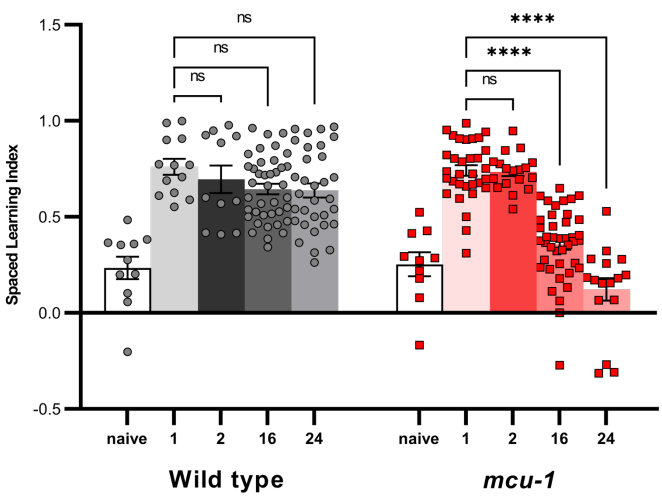

Spaced Learning Index
1.5
1.0
0.5
0.0
-0.5
ns
ns
ns
****
****
ns
naive
1
2
16
24
naive
1
2
16
24
Wild type
mcu-1

Figure 5

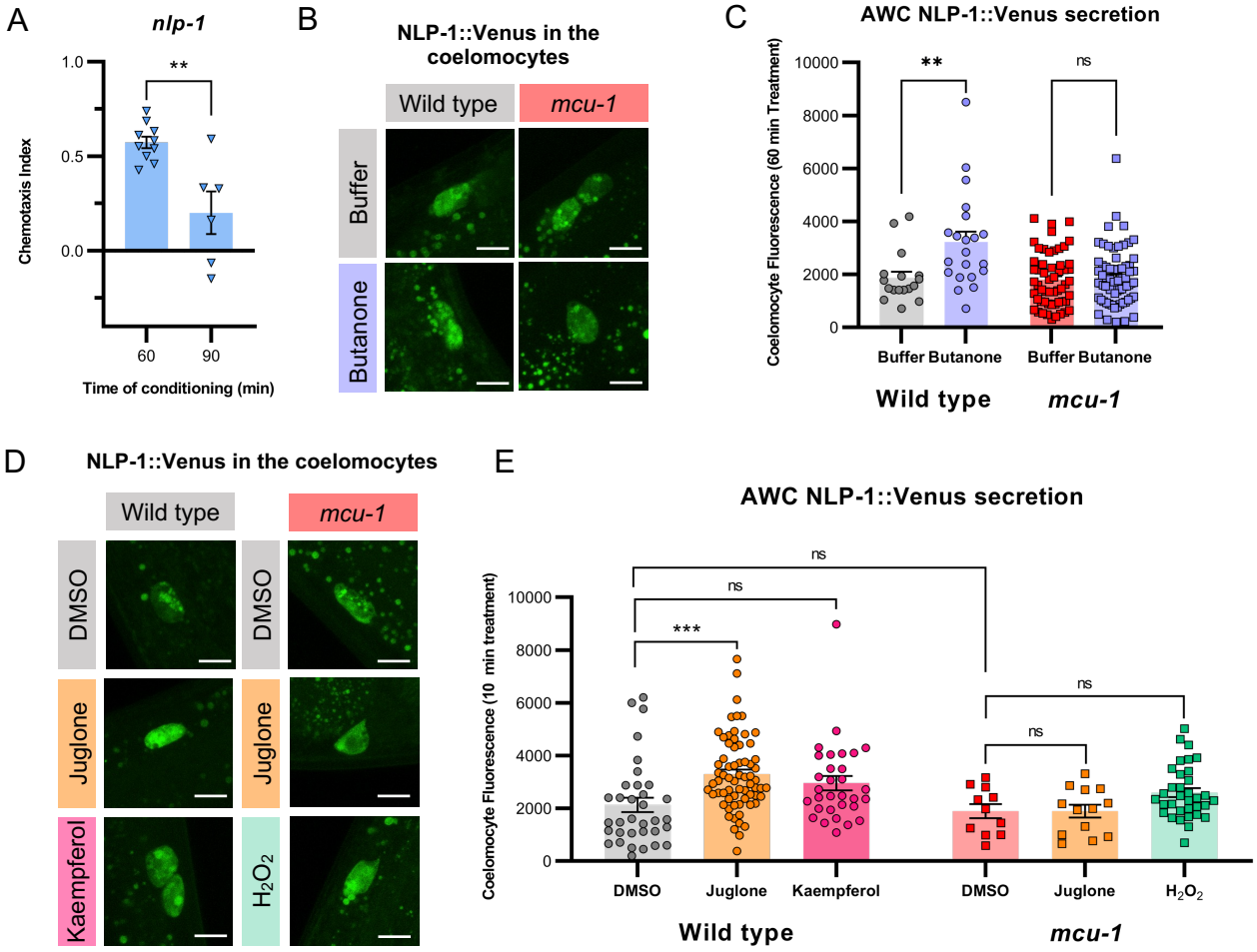
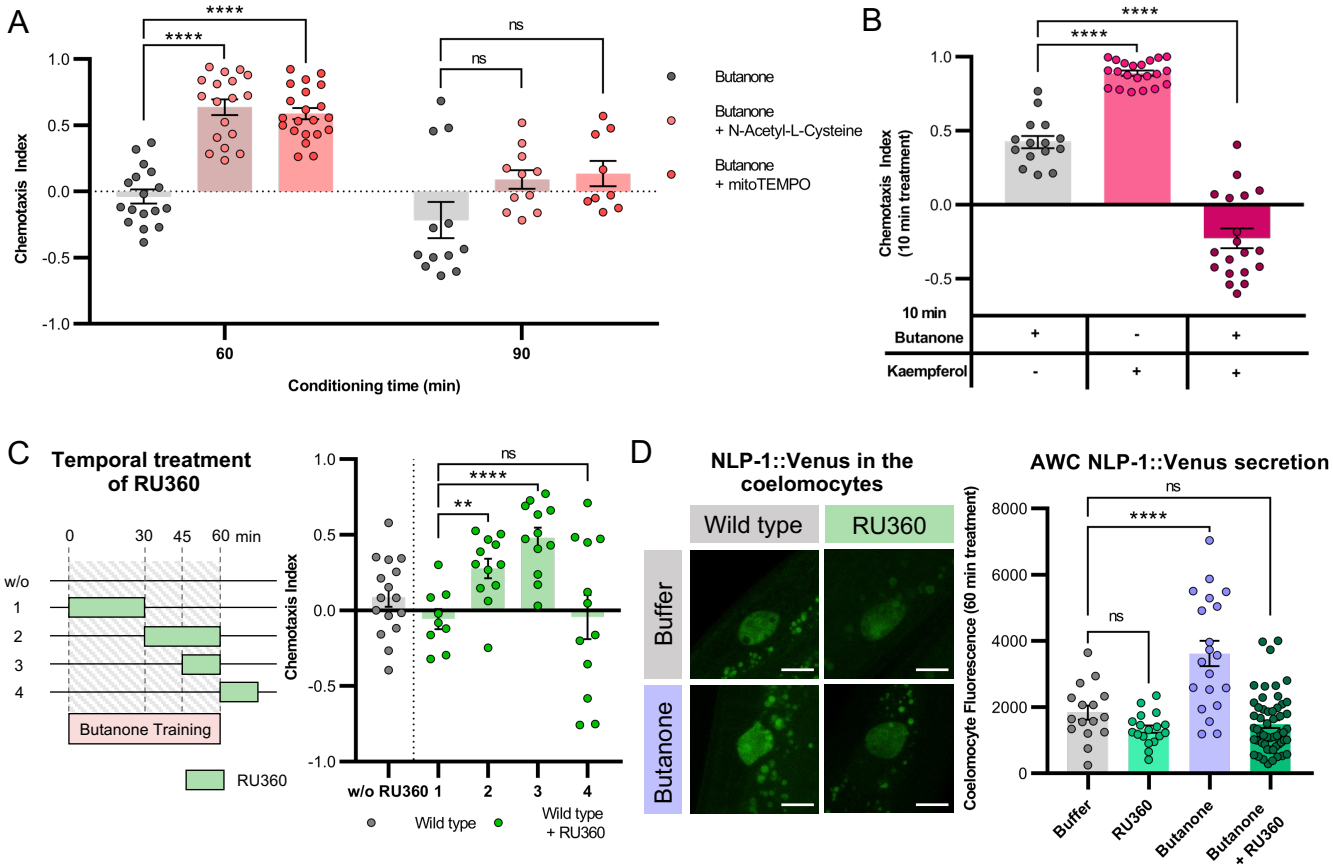

A
nlp-1
Chemotaxis Index
1.0
0.5
0.0
**
60
90
Time of conditioning (min)
B
NLP-1::Venus in the coelomocytes
Wild type
mcu-1
Buffer
Butanone
C
AWC NLP-1::Venus secretion
Coelomocyte Fluorescence (60 min Treatment)
10000
8000
6000
4000
2000
0
**
ns
Buffer Butanone
Buffer Butanone
Wild type
mcu-1
D
NLP-1::Venus in the coelomocytes
Wild type
mcu-1
DMSO
Juglone
Kaempferol
DMSO
Juglone
H2O2
E
AWC NLP-1::Venus secretion
Coelomocyte Fluorescence (10 min treatment)
10000
8000
6000
4000
2000
0
ns
ns
***
ns
ns
DMSO
Juglone
Kaempferol
DMSO
Juglone
H2O2
Wild type
mcu-1

Figure 6


A
Chemotaxis Index
Conditioning time (min)
60
90
****
****
ns
ns
Butanone
Butanone
+ N-Acetyl-L-Cysteine
Butanone
+ mitoTEMPO
B
Chemotaxis Index
(10 min treatment)
10 min
Butanone
Kaempferol
C
Temporal treatment
of RU360
0
30
45
60 min
w/o
1
2
3
4
Butanone Training
RU360
Chemotaxis Index
w/o RU360 1
2
3
4
Wild type
Wild type
+ RU360
D
NLP-1::Venus in the
coelomocytes
Wild type
RU360
Buffer
Butanone
AWC NLP-1::Venus secretion
Coelomocyte Fluorescence (60 min treatment)
Buffer
RU360
Butanone
Butanone
+ RU360

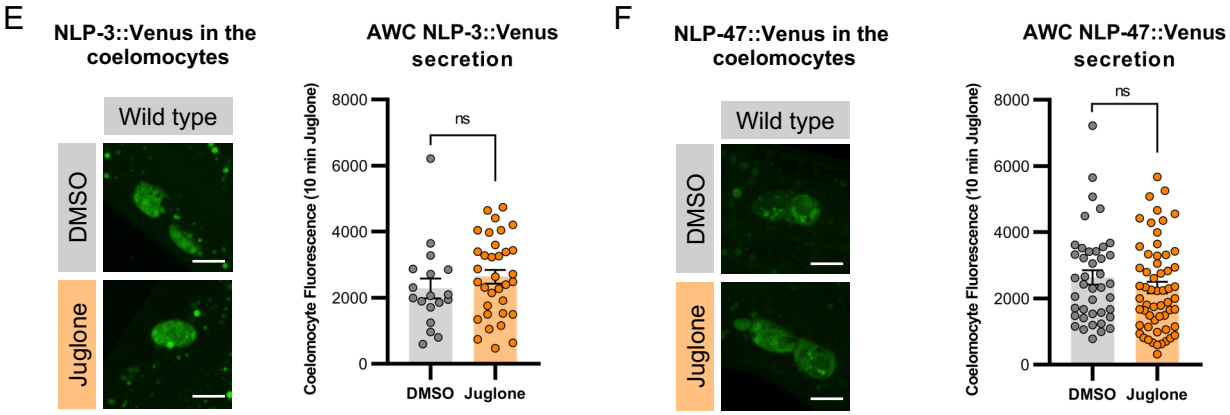

E
NLP-3::Venus in the
coelomocytes
Wild type
DMSO
Juglone
AWC NLP-3::Venus
secretion
Coelomocyte Fluorescence (10 min Juglone)
DMSO
Juglone
F
NLP-47::Venus in the
coelomocytes
Wild type
DMSO
Juglone
AWC NLP-47::Venus
secretion
Coelomocyte Fluorescence (10 min Juglone)
DMSO
Juglone

Figure S1

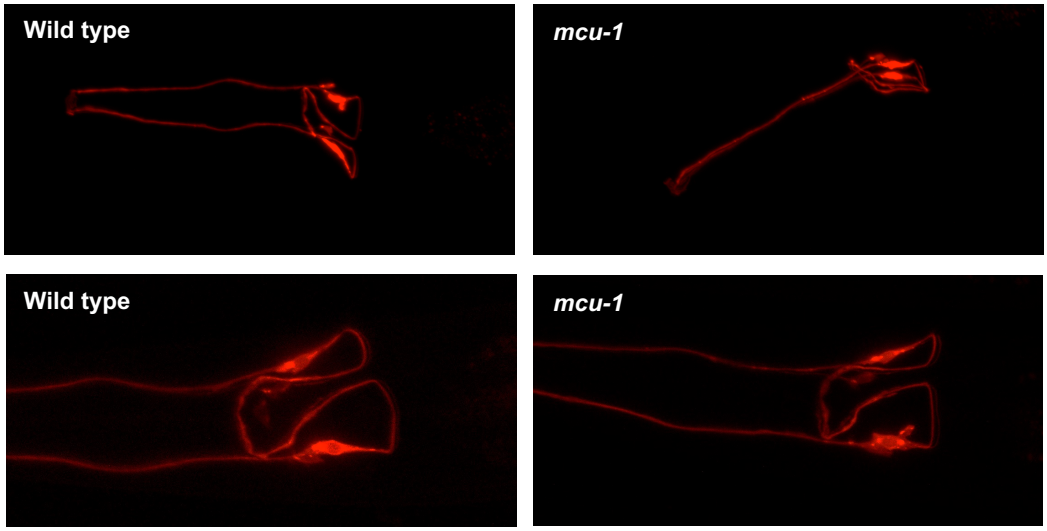
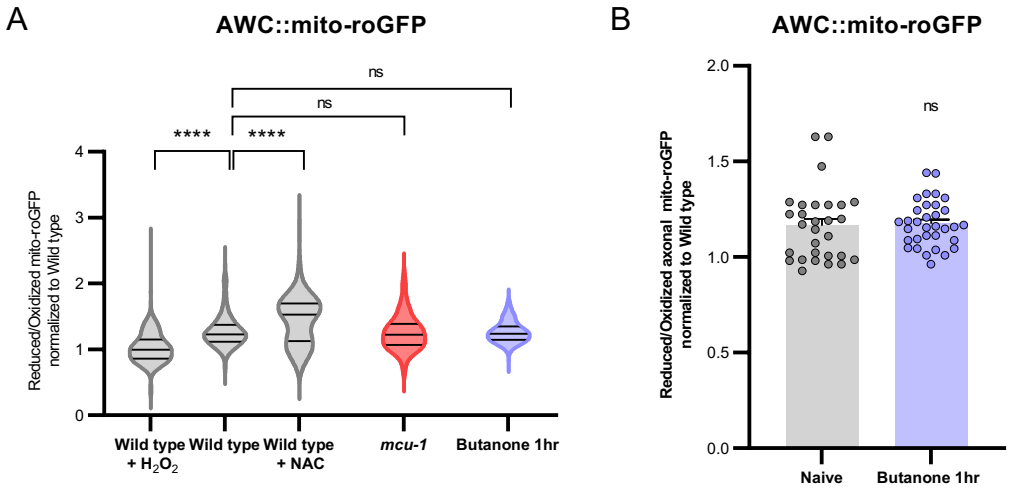


Figure S2


A
AWC::mito-roGFP
Reduced/Oxidized mito-roGFP normalized to Wild type
0
1
2
3
4
****
****
ns
ns
Wild type + H_2O_2
Wild type
Wild type + NAC
mcu-1
Butanone 1hr
B
AWC::mito-roGFP
Reduced/Oxidized axonal mito-roGFP normalized to Wild type
0.0
0.5
1.0
1.5
2.0
ns
Naive
Butanone 1hr