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Off-response in ASH neurons evoked by $CuSO_4$ requires the TRP channel OSM-9 in *Caenorhabditis elegans*

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ABSTRACT

The off-response of ASH neurons had been overlooked until the microfluidic devices were introduced for *in vivo* imaging of neuronal activity in *Caenorhabditis elegans*. The mechanisms of ASH off-response were completely unknown. Here we monitored ASH off-response to CuSO4 stimulation by use of microfluidic device and genetically encoded calcium indicator (GECI) - Case12. We found ASH neurons exhibited a multiphasic response to 10 mM and 50 mM $CuSO_4$ of 30-s stimulation duration. ASH off-responding to Cu^{2+} had been dramatically reduced in *goa-1*, *mod-5*, *trpa-1* and *egl*-8 mutants. Moreover, in *osm-9* mutants ASH off-response was completely eliminated. Neuron-specific rescue of *osm-9* in ASH neurons restored the off-response and the normal avoidance behavior in worms.

1. Introduction

The ability to detect aversive sensory stimuli in the environment allows animals to avoid noxious chemicals and dangerous conditions and is essential for their health and life. Multicellular animals sense aversive chemicals using specialized cells in the nervous systems. The small soil nematode *Caenorhabditis elegans* is sensitive to numerous chemical stimuli in environments and avoid acid pH, copper ions, sodium dodecyl sulfate, high osmotic strength, D-Tryptophan and some volatile organic molecules [1] [2], [3] [4], and [5]. This relatively simple animal has only 302 neurons in the nervous system of the adult hermaphrodite. The anatomies and morphological connections of all neurons were well described [6] [7], and [8]. Three types of sensory organs, consisting of 16 types of sensory neurons, in *C. elegans* hermaphrodite appear to be chemosensory [5]. The general functions of all amphid sensory neurons have been defined through genetic and cell-killing studies. Different *C. elegans* chemosensory neurons function in different chemical responses [3] and [5]. ASH, ADL, ASK and ASE sensory neurons are reported to be responsible for the detection of chemical repellents [3] [9], and [10]. Particularly, ASH neurons act as polymodal sensory neurons which play a central role in mediating avoidance behavior. Previous studies have demonstrated that ASH neurons respond to a variety of noxious stimuli including quinine, denatonium, detergents, heavy metals, both hyper- and hypo-osmotic shock, and nose touch [11] and [12], and are required for behavioral avoidance of water-soluble and volatile chemical repellents, high osmotic strength and light touch to the tip of the worm's nose [3] [9], [10] [13] [14], and [15].

In *C. elegans*, some types of sensory neurons respond not only to the present of but also the removal of stimuli. With precise addition and removal of high-osmolality stimulus, the activity of ASH neurons exhibited a biphasic pattern. This means ASH neurons show transient increases of intracellular calcium upon both addition and removal of the stimulus [12]. However, the mechanism of ASH neuron off-response (neuron exhibits activity upon removal of the sensory stimuli) is still unknown. To answer this open question, we used Ca^{2+} imaging in ASH neurons combined with microfluidic devices. The microfluidic *in vivo* calcium imaging is an effective way to monitor neuronal activity because it is possible to engineer microenvironments to deliver specific stimuli and record neuronal activities *in vivo* in high tempo-spacial resolution.

Our results showed under 30-s stimulus duration, ASH neurons had a multiphasic response to 10 mM and 50 mM $CuSO_4$. The off-response of ASH neurons was dramatically reduced in *goa-1*, *mod-5*, *trpa-1*, and *egl-8* mutants, and completely eliminated in *osm-9* mutants. Neuron-specific rescue of *osm-9* in ASH neurons restored normal off-response in the neurons and normal worms'

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avoidance behavior, suggesting that OMS-9 plays an essential role in ASH off-response and worm avoidance behavior.

2. Materials and methods

2.1. Molecular biology

To construct *sra-6p::Case12*, sra-6 promoter (3.8 kb) was amplified from N2 wild-type genomic lysates and cloned into the vector pPD95.75 (a gift from A. Fire) via XbaI and SmaI; *Case12* was cloned into the same vector via SmaI and KpnI. For the construct of *unc-122p::tagRFP-t*, *unc-122* promoter (4 kb) was amplified from N2 wild-type genomic lysates and cloned into the vector pPD95.67 (a gift from A. Fire) via SphI and BamHI; *tagRFP-t* was cloned into the same vector via BamHI and AgeI. For *sra-6p::osm-9*, promoter, coding sequence, and *unc-54* 3' UTR were separately cloned into pDONR vectors and then combined into the pDEST R4-R3 vector of MultiSite Gateway™ system (Invitrogen). The *osm-9* cDNA was amplified by RT-PCR from *C. elegans* mixed stage RNA.

2.2. Stain and culture

All strains were grown at 20 °C on nematode growth medium (NGM) under standard condition [16]. Strains were obtained from *Caenorhabditis* Genetic Center (http://www.cbs.umn.edu/CGC/). Transgenic strains were generated using standard microinjection techniques and integration of extrachromosomal arrays by UV irradiation [17] [18], and [19]. Plasmids were injected at 50 ng μl^{-1} together with marker *unc-122p::TagRFP-t* (5 ng μl^{-1}).

2.3. Calcium imaging

Neuronal calcium responses in soma were measured by detecting changes in the fluorescence intensity of Case12, a genetically encoded Ca^{2+} indicators [20]. A home-made microfluidic device was used for calcium imaging. Briefly, a worm was immobilized by entrapment in a micro-channel of the microfluidic chip. Subsequently the head of the worm was exposed to a buffer or $CuSO_4$ solution. The flows of the buffer, $CuSO_4$ and dye solution were delivered using a programmable automatic drug-feeding apparatus (MPS-2, InBio Life Science Instrument Co. LTD, Wuhan, China). Case12 was excited by 460–470 nm light emitted by an Osram Diamond Dragon LBW5AP light-emitting diode (LED) model (Osram, Marcel-Breuer-Straße 6, Munich, Germany) constructed in a multi-LED light source (MLS102, InBio Life Science Instrument Co. LTD), and filtered with a Semrock 520/35 emission filter (Semrock, Inc., Rochester, NY, USA), under an Olympus IX-70 inverted microscope (Olympus, Tokyo, Japan) equipped with a 40 × objective lens (numerical aperture (NA) = 1.3, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Fluorescence images were captured with an Andor iXon^{EM} + DU885 K EMCCD camera (Andor Technology plc., Springvale Business Park, Belfast, United Kingdom) with 100 ms exposure time and 256 × 256 pixels at 10 frames per second. The averaged fluorescence intensity (AFI) in the region of interest (ROI) of the soma was captured and analyzed by user of Image-Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA). The AFI within the initial 5 s before stimulation was taken as basal signal F_0. The change of fluorescence intensity relative to the initial intensity F_0, $\Delta F = (F-F_0)/F_0$, was plotted as a function of time for all curves. The mean values of Ca^{2+} signals and the SEM were plotted in various colors as indicated and in light gray, respectively, using IGOR Pro 6.10 (Wavemetrics, Portland, OR, USA). For statistical analysis of off-responding, the average fluorescent intensity in the 5 s before stimulation removal was taken as basal signal F_0. Asterisks denote statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

2.4. Behavioral analysis

Chemotaxis behavior in wild-type and transgenic worms toward 100 μM $CuSO_4$ were examined. The behavioral test was modified from previous work [9]. About 22 ml buffered agar was poured into a 9 cm diameter petri dish with central area blocked using a 3.5 cm diameter petri dish. After the agar cooled and solidified, the dish was removed and origin area was filled with buffered agar containing 100 μM $CuSO_4$. The solidified gel was turned bottom side up to ensure that the agar surface is smooth. After drying for 40 min at room temperature, the plates were stored and used on the same day. 100 to 200 worms were washed and transferred onto the central area. The worms were allowed to move freely for 45 min and the number of worms in each area was counted. Avoidance indexes were defined as $(c-t)/(c+t)$, where c and t were the number of worms in the control and test areas, respectively. Results are presented as mean values ± s.e.m. and the number of test repetition is indicated in the figure legends. Statistical significance was evaluated using the *Student's t*-test or *Mann–Whitney Rank Sum* -test depending on the normality of the data distribution. Asterisks denote statistical significance: $^{***}P < 0.001$.

3. Results

To monitor the activity of ASH sensory neurons in response to $CuSO_4$ stimulation, we used a microfluidic device (Fig. 1A) modified from previous studies [12] and [21]. The microfluidic device incorporates a worm trap with a microfluidic solution delivery system. The worm trap channel was designed for young adult worms, sizes with 40 μm wide × 1200 μm long × 40 μm high. Young adult worms can be entirely immobilized in the worm trap channel. When the worm is loaded, the tip of the nose sticks out of the trap into a flowing laminar flow that contains the buffer or stimulus solution (Fig. 1B).

The microfluidic flow system delivers the stimulus (channel 3) and the control (channel 1) solutions to the nose of the worm. The dye-solution stream (channel 2) was used in a test stage before each calcium imaging trial to ensure the fluidity of the device.

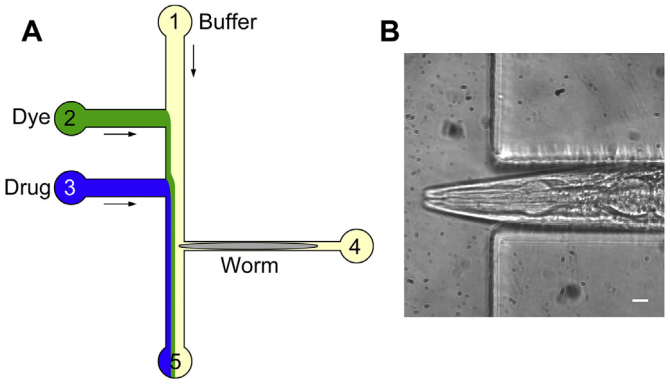

Fig. 1. The diagram of the microfluidic chip. (A) The microfluidic biochip consists of a worm trap and a 4-flow microfluidic network (labeled as 1, 2, 3, 4 and 5) for stimulus delivery. In the test state, the buffer (channel 1), dye (channel 2) and $CuSO_4$ solution (channel 3) were opened at the same time. Three fluid flows could be visualized by fluorescent imaging (A). Channel 4 is the worm trap and channel 5 is for effluent. The figure is not drawn to scale. (B) Higher magnification photographs of the chip with a trapped worm. As shown, the nose of the worm is exposed to the buffer stream. Scale bar, 10 μm.

When three channels open simultaneously, three fluid flows can be visualized by fluorescent imaging with the help of the dye-solution laminar flow (Fig. 1A). During experiments, channel switches alternately between buffer (channel 1) and $CuSO_4$ solutions (channel 3).

To examine the activity of ASHs, we constructed a transgenic strain expressing Case12 in ASH neurons (ZXW206 *hkdEx206[sra-6p::Case12; unc-122p::TagRFP-t]*). Case12 is a genetically encoded calcium sensor protein which has a high dynamic range of 12 folds [20].

We diluted $CuSO_4$ in M13 buffer and used it as a chemosensory stimulus. In order to find an optimum stimulus condition, different stimulus durations and different concentrations of $CuSO_4$ were tested. We found that ASH neurons responded only to the application of 10 mM $CuSO_4$ of 5-s or 15-s duration with a sustained calcium response (Fig. 2A and B), but no obvious off-response. Whereas ASH neurons exhibited a biphasic pattern upon 30-s Cu^{2+} stimulation (Fig. 2C), in accordance with previous work [12]. In this case, ASH neurons not only responded to the introduction of $CuSO_4$ (on-response), but also responded to the removal of the stimulus (off-response). Next, we tested the ASH neuron response to different concentrations of $CuSO_4$. In order to quantitatively analyze the fluorescent intensity changes, we generated an integration strain (ZXW999 *hkdIs999[sra-6p::Case12; unc-122p::TagRFP-t]*). Since the kinetics remained the same in the integration strain and the microinjection strain (Fig. 2C and D), we used the integration strain in the following calcium imaging experiments. While low concentration of $CuSO_4$ at 1 mM evoked a sustained on-response, high concentration of $CuSO_4$ at 10 mM and 50 mM leaded to both on- and off-responses (Fig. 2D). As 30-s stimulus of 10 mM $CuSO_4$ evoked the biphasic response in ASH neurons, we used this stimulus protocol for the following experiments.

To identify genes that might be involved in ASH biphasic response to $CuSO_4$, we screened mutants related to neurotransmission, signal transduction molecules, and ion channels. We found *mod-5*, *goa-1*, *egl-8* and *trpa-1* mutants displayed notable decrease in off-response (Fig. 3A and B). More interestingly, ASH off-response in *osm-9* mutants was completely eliminated (Fig. 3A and B). Extrachromosomal expression of *osm-9* in ASH neurons restored off-response significantly (Fig. 3C and D). This result suggested *osm-9* played a significant role in off-response to $CuSO_4$ stimulation.

Next, we examined the function of OSM-9 in the behavioral responses of *C. elegans* toward $CuSO_4$. We tested whether worms were able to avoid entering into or escape from the region containing 100 μM $CuSO_4$. (Fig. 4A). In wild-type animals and wild-type animals expressing calcium indicator, more than 85% of the populations did not enter in the area containing 100 μM $CuSO_4$. *osm-9* mutants showed a significant defect in the avoidance behavior. Cell specific extrachromosomal expression of *osm-9* in ASH neurons yielded a dramatic rescuing effect (Fig. 4B). Together with the calcium imaging data, the results suggest off-response is crucial for the avoidance of $CuSO_4$ in *C. elegans*.

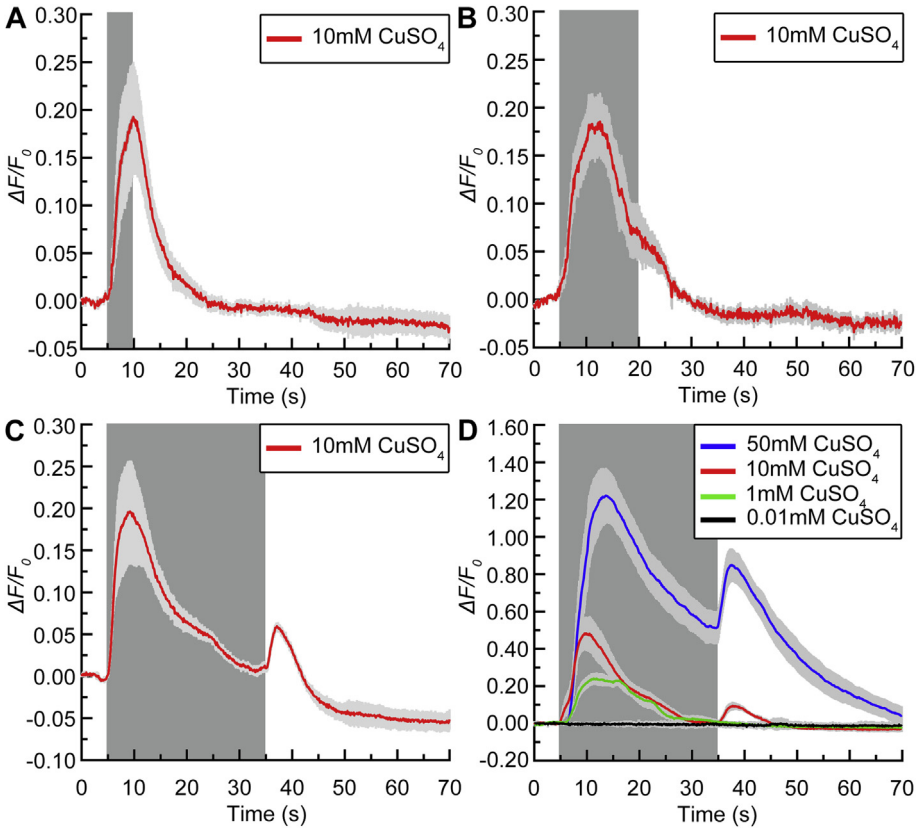

Fig. 2. Calcium transients in ASH neurons in response to different stimulus durations and different concentrations of $CuSO_4$. (A–C) Intensity changes in the Case12 signal upon 10 Mm $CuSO_4$ addition and removal of 5-s (n = 5), 15-s (n = 5) and 30-s (n = 18) stimulus. (D) Intensity changes in the Case12 signal upon different concentrations of $CuSO_4$ addition and removal of 30-s stimulus (n = 6 for 0.01 mM $CuSO_4$, n = 12 for 1 mM $CuSO_4$, n = 8 for 10 mM $CuSO_4$, n = 12 for 50 mM $CuSO_4$). The average fluorescent intensity within the initial 5 s before stimulation was taken as basal signal F_0. Dark grey shading indicates presence of $CuSO_4$ solution. Data are shown as mean ± s.e.m. indicated by solid traces in color and light grey respectively.

4. Discussion

The properties of off-response (neuron exhibits activity upon removal of sensory stimuli) have been described in AWC, ASER and ASK neurons in *C. elegans*. These three types of neurons exhibit off-responses upon stimulations and regulate turnings and backward movements [22] [23], and [24]. ASH is an essential noxious sensory neurons, but their off-responses was overlooked in the past, due to the confounding effects of mechanical fluid flow in the glued worm preparation and imprecise removal of stimulus [11] and [12]. By using the microfluidic device, Chronis and colleagues demonstrated that ASH neurons exhibited both on-response (neuron exhibits activity upon presentation of sensory stimuli) and off-response in response to high-osmolarity stimulus [12]. Here, we showed that ASH neurons also displayed a similar biphasic calcium responses to 10 mM and 50 mM $CuSO_4$ solutions of 30-s under condition of precise application and removal of $CuSO_4$ solutions by use of microfluidic devices.

Through reverse genetic screening, gene rescue and calcium imaging, we found that the ASH off-response to Cu^{2+} is mediated mainly by OSM-9, a TRPV channel homologous to capsaicin receptors which is involved in sensory responses to chemical, osmotic and mechanical stimuli, and in adaptation to salts and volatile substances [25] [26], and [27]. Perplexedly, cell specific rescue of *osm-9* in ASH neurons only restores the off-response but the amplitude of on-response remained the same with *osm-9* mutants. But interestingly, worm avoidance to nociceptive Cu^{2+} is fully rescued. These results hint that off-response in ASHs alone is enough to trigger avoidance behavior. The results suggest that, in ASH neurons the cellular level of off-response might corresponding to avoidance behavior generating turning and backward movements at behavioral level, similar to the off-response in AWC [22], ASER [23] and ASK neurons [24].

In addition, the genes of *goa-1*, *mod-5*, *trpa-1* and *egl-8* possibly play roles in the off-response. *trpa-1* encodes a transient receptor potential (TRP) ion channel orthologous to the vertebrate and *Drosophila* TRPA1 channels [28]. *mod-5* encodes the only serotonin reuptake transporter which is required for serotonin uptake by neurosecretory motor neurons in *C. elegans* [29]. *goa-1* encodes an ortholog of the heterotrimeric G protein alpha subunit Go [30]. *egl-8* encodes a phospholipase C beta (PLCβ) [31]. So, besides the functions of TRP channels in off-response, serotonin signaling pathway also likely regulates the off-response.

The possible signal transduction pathway of off-response in ASH neurons is still needed to be identified. OSM-9 is related to the G-protein-regulated sensory transduction channel, the TRP/TRPL

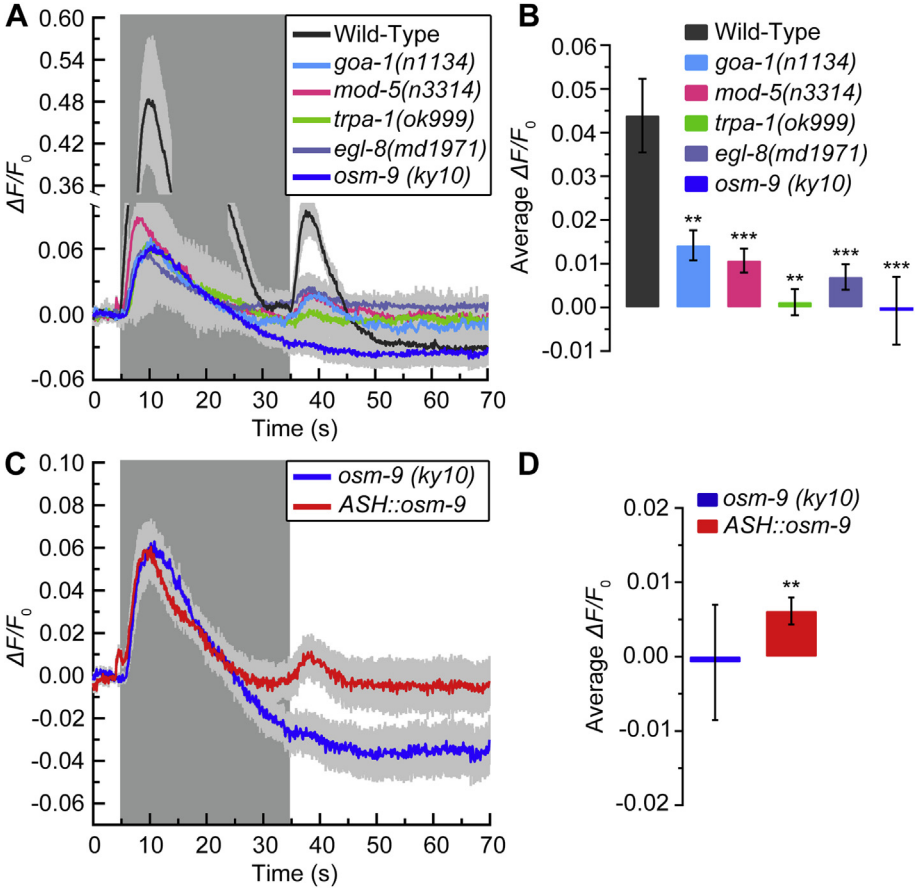

Fig. 3. Response in ASH to $CuSO_4$ of animals of different genetic backgrounds. (A) Calcium transients of ASH neurons in wild-type (n = 8), *goa-1* (n = 9), *mod-5* (n = 9), *trpa-1* (n = 5), *egl-8* (n = 11) and *osm-9* (n = 10) mutants. The average fluorescent intensity within the initial 5 s before stimulation was taken as basal signal F_0. Dark grey shading indicates presence of $CuSO_4$ solution. Data are shown as mean ± s.e.m. indicated by solid traces in color and light grey respectively. (B) Average intensity change during the 10 s after $CuSO_4$ removal. The average fluorescent intensity in 5 s before stimulation removal was taken as basal signal F_0. (C) Calcium transients of ASH neurons in *osm-9* mutants (n = 10) and ASH specific rescue strains (n = 12). The average fluorescent intensity within the initial 5 s before stimulation was taken as basal signal F_0. Dark grey shading indicates presence of $CuSO_4$ solution. Data are shown as mean ± s.e.m. indicated by solid traces in color and light grey respectively. (D) Average percentage change during the 10 s after $CuSO_4$ removal. The average fluorescent intensity in 5 s before stimulation removal was taken as basal signal F_0. Statistics: $^*P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with wild-type control or with *osm-9* mutant (*Student's t*-test or *Mann–Whitney Rank Sum*-test depending on the normality of the data distribution). Error bar indicates s.e.m.

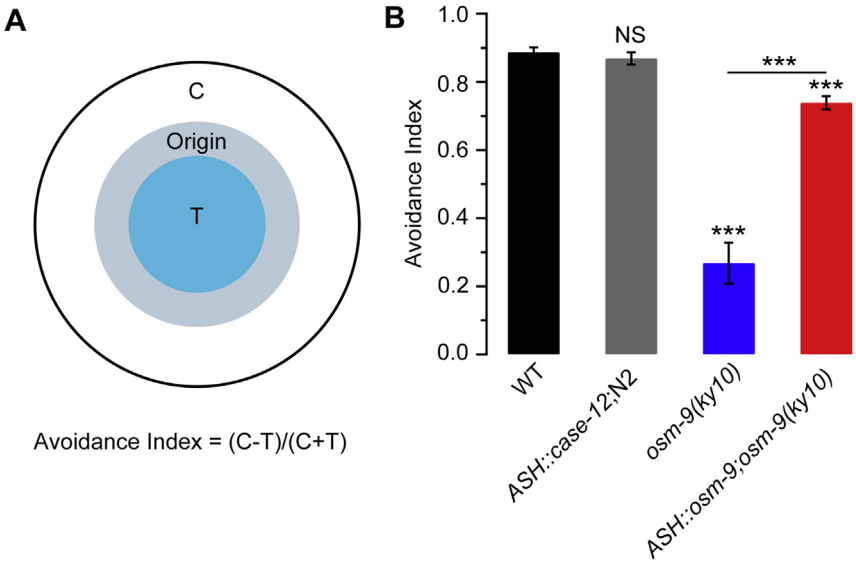

Fig. 4. Chemotaxis behavior of *osm-9* mutants and ASH specific rescue strains. (A) Schema of $CuSO_4$ chemotaxis. The central blue area (area T) was filled with buffered agar containing 100 μM $CuSO_4$, the origin area and area C was filled with buffered agar. Washed worms were transferred into the origin area, 45 min later the avoidance index was calculated as shown. (B) Avoidance index of *osm-9* mutants and ASH specific rescue strains. [n = 24 wild-type, n = 17 *ASH::Case-12*;N2, n = 20 *osm-9(ky10)*, n = 28 *ASH::Case-12;osm-9(ky10)*] Statistics: $^{***}P \leq 0.001$ compared with wild-type control or as indicated (*Student's t*-test or *Mann–Whitney Rank Sum*-test depending on the normality of the data distribution). Error bar indicates s.e.m. WT, wild-type.

channel, which mediates the light-activated conductance in the *Drosophila* visual system [26]. During phototransduction, activation of the G-protein-coupled receptor rhodopsin leads to stimulation of phospholipase C and an increase in inositol triphosphate (IP_3) [26] and [32]. It is possible that *goa-1* and *egl-8* might function as G protein and phospholipase C together with OSM-9 in the $CuSO_4$ sensory transduction pathway although further study should provide insight to verify this assumption. Serotonin signaling allows *C. elegans* to respond to changes in its environment by modulating locomotion behavior [33]. The defective phenotype of *mod-5* mutants suggested serotonin was involved in ASH in response to $CuSO_4$. It is possible that ASH might be regulated by other neurons which reuptake serotonin via MOD-5. *trpa-1* mutants have defects in reversal response to nose-touch, but are normal for ASH-mediated osmosensory and chemosensory avoidance behaviors [28]. However, we found *trpa-1* also defected in the transient response to $CuSO_4$ stimuli in ASH neurons. Previous studies also demonstrated TRPA-1 can be activated downstream of the G protein-coupled receptor as well as mechanosensory stimuli [28]. It is possible TRPA-1 does not mediate sensory transduction directly, but plays a modulatory role in the ASH neurons.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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