Positive interaction between ASH and ASK sensory neurons accelerates nociception and inhibits behavioral adaptation

Highlights
- ASK accelerates ASH Ca\textsuperscript{2+} responses by transferring cGMP
- ASH promotes ASK by a disinhibitory circuit consisting of ASH, AIA, and ASK
- The fast-rising phase of ASH Ca\textsuperscript{2+} responses inhibits behavioral adaptation
- The positive feedback circuit of ASH/AIA/ASK downregulates behavioral adaptation
Positive interaction between ASH and ASK sensory neurons accelerates nociception and inhibits behavioral adaptation

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SUMMARY
Central and peripheral sensory neurons tightly regulate nociception and avoidance behavior. The peripheral modulation of nociception provides more veridical and instantaneous information for animals to achieve rapid, more fine-tuned and concentrated behavioral responses. In this study, we find that positive interaction between ASH and ASK sensory neurons is essential for the fast-rising phase of ASH Ca\textsuperscript{2+} responses to noxious copper ions and inhibits the adaptation of avoiding Cu\textsuperscript{2+}. We reveal the underlying neuronal circuit mechanism. ASK accelerates the ASH Ca\textsuperscript{2+} responses by transferring cGMP through gap junctions. ASH excites ASK via a disinhibitory neuronal circuit composed of ASH, AIA, and ASK. Avoidance adaptation depends on the slope rate of the rising phase of ASH Ca\textsuperscript{2+} responses. Thus, in addition to amplitude, sensory kinetics is significant for sensations and behaviors, especially for sensory and behavioral adaptations.

INTRODUCTION
Nociception, the sensation of injurious stimuli with actual or potential tissue injuries, produces a diverse set of sensations, pain perception, emotions, and actions, including behavioral responses. This vital sensation serves as an essential protective function and is crucial for animal health and survival. It is not generated by an immutable and hardwired system but results from the involvement of highly plastic molecules and circuits. It is fine-tuned and tightly controlled by central and peripheral sensory neurons (Baliki and Apkarian, 2015; Basbaum et al., 2009; Peirs and Seal, 2016). Primary nociception and its modulations at the level of sensory neurons or the initial chain of sensory pathways provide more veridical and instantaneous information for animals to achieve rapid, more fine-tuned, and concentrated behavioral responses (Baliki and Apkarian, 2015; Guo et al., 2015). Peripheral circuitry modulations of nociception include that nociceptive ASI neurons reciprocally inhibit nociceptive ASH (Guo et al., 2015) and secondary nociceptive ASK neurons inhibit ASH nociceptive signal transduction by providing cGMP through gap junctions (Voelker et al., 2019) in nematode Caenorhabditis elegans (C. elegans). The molecular and neuronal circuitual mechanisms of nociception, especially pain perception, have been extensively studied. However, they are still poorly understood, and related studies are critically important.

C. elegans senses and avoids a variety of noxious stimuli. Such as hyperosmotic and hypoosmotic stress, mechanical forces, electric stimulation, light, and odor cues, which are associated with danger or predators. In C. elegans, paired ASH sensory neurons are the main nociceptor, whereas ADL, ASK, ASE, ASI, and AWB play minor roles in the detection of certain chemical repellents (Bargmann, 2006; Bargmann...
et al., 1993; deBono and Maricq, 2005; Ferkey et al., 2021; Filipowicz et al., 2021; Guo et al., 2015; Hilliard et al., 2002, 2004; Kaplan and Horvitz, 1993; Khan et al., 2022; Pradel et al., 2007; Sambongi et al., 1999; Troemel et al., 1997; Yang et al., 2022). ASH senses and triggers avoidance responses to multimodal noxious or aversive stimuli. Upon noxious stimulation, it displays robust ON (increases in cytoplasmic calcium presumably because of depolarization that occurs when the concentration of the chemical cue increases) and relatively more minor OFF (increases in cytoplasmic calcium that occur when the concentration of the chemical cue decreases) Ca\textsuperscript{2+} responses (Ferkey et al., 2021). Among the nociceptor neurons, ASH displays the strongest, fast, and almost non-delayed rising phase followed by a slow decayed phase (sustained phase) of ON response and a smaller OFF response in response to stimulation of noxious copper ions (Cu\textsuperscript{2+}) for about 30 s. The sustained phase is inhibited by Cu\textsuperscript{2+}-sensitive ASI neurons (Guo et al., 2015), while the OFF response depends on OSM-9, a transient receptor potential (TRP) ion channel V homologous to capsaicin receptors (Wang et al., 2015). The sustained phase and OFF response are involved in animal Cu\textsuperscript{2+} avoidance (Guo et al., 2015; Wang et al., 2015). It remains unknown whether the kinetics of the ASH rising phase is vital for nociception and animal avoidance behavior. ASH neurons are glutamatergic and neuropeptidergic. They express the vesicular glutamate transporter (VGlu) EAT-4, which is required for ASH nociceptive responses (Berger et al., 1998; Lee et al., 1999). ASH releases glutamate in a graded manner to elicit and modulate different models of nociceptive escape responses (deBono and Maricq, 2005) and to regulate eating (Zou et al., 2018) and egg laying (Wen et al., 2020). ASH receives elaborate modulations from central and peripheral neurons by various signals of monoamines and peptides (Ezcurra et al., 2016; Ghosh et al., 2016; Guo et al., 2015; Hapiak et al., 2013; Harris et al., 2010; Mills et al., 2012; Voelker et al., 2019; Zahratka et al., 2015). Such as central excitatory feedback from interneuron RIM via humoral tyraminergic signaling (Ghosh et al., 2016), reciprocal inhibition from ASI via SER-5 signaling through the relay of ADF sensory neurons (Guo et al., 2015), inhibition of signal transduction by cGMP from ASK (Voelker et al., 2019), and inhibition from AUA interneurons via NPR-1 signaling when food availability is low (Ezcurra et al., 2016).

The functions of sensory systems lie in detecting stimuli of varied models, intensities, and positions and detecting contrasts and changes in temporal and spatial patterns of stimulations. If a stimulus persists unchanged for several minutes without a change in position or amplitude, the neural response of the receptor and corresponding sensation diminishes, a condition called receptor adaptation. Receptor adaptation is an essential neural basis of sensory or perceptual adaptation. Sensory adaptation enables animals to be sensitive to a new challenge of stimulations and is necessary for animals’ survival. The rate of sensory adaptation is different among various modalities of sensations: fast and slow adaptation. Nociception is usually slowly adapted. The dynamic Ca\textsuperscript{2+} responses of ASH may contribute to nociceptive and behavioral adaption in C. elegans.

Paired ASKs are polymodal sensory neurons that detect attractive and aversive stimuli (Ferkey et al., 2021). ASK plays minor roles that are only evident when ASH is missing in avoidance behavior from protons, detergents, and alkaloids such as bitter tastant quinine (Hilliard et al., 2002, 2004; Sambongi et al., 1999). ASK forms multiple electrical synapses or gap junctions with ASH (Cook et al., 2019; White et al., 1986). ASK and ASH neurons express gap junction components INX-4 (or CHE-7, in neurons including ASH), INX-18 (in neurons including ASK), and INX-19 (in neurons including ASH and ASK) (Altun et al., 2009; Bhattacharya et al., 2019; Chuang et al., 2007). ASK negatively regulates ASH Ca\textsuperscript{2+} signaling in response to quinine by transferring the small molecule cGMP to ASH through gap junctions (Voelker et al., 2019). However, the neuronal circuit mechanisms of ASK’s effect on ASH need to be investigated.

Here, we revealed that the second messenger cGMP, produced in ASK and likely passes through gap junctions, is essential for the fast-rising phase of ASH Ca\textsuperscript{2+} responses to Cu\textsuperscript{2+} stimulation. The fast kinetics of ASH Ca\textsuperscript{2+} responses negatively regulates adaptation of animal avoidance from noxious Cu\textsuperscript{2+}. Thus, modulation of sensation kinetics endows animals to fine-tune their sensory, physiological, and behavioral responses. We identify the underlying neural circuit mechanism. ASK connects with ASH by gap junctions containing INX-4. cGMP produced by ODR-1 in ASK diffuses possibly through gap junctions into ASH. It acts through EGL-4 (cGMP-dependent protein kinase in C. elegans, encoded by egg-laying defective gene 4, egl-4) signaling to accelerate the rising phase of ASH nociceptive response to Cu\textsuperscript{2+}. ASH promotes ASK activity via disinhibitory ASH/AIA/ASK circuitry. ASH inhibits interneuron AIA via glutamate/GLC-3 signaling, while AIA inhibits ASK by releasing neuropeptide FLP-1 and acting on the NPR-5 receptor. The activation of ASH removes AIA inhibition on ASK, and thus ASH excites ASK.
Figure 1. Interaction between ASK and ASH sensory neurons is required for normal animals to avoid the toxic Cu²⁺ ions in the Cu²⁺ trap test and proper ASK Cu²⁺-evoked Ca²⁺ response

(A) Illustration of Cu²⁺-trap assay.

(B) Exit time in wild-type N2 (WT, as a control) and transgenic animals in the Cu²⁺-trap test.

(C) The merged image shows co-localization of fluorescence of GCaMP6s and R-GECO1 encoded by sra-9p:GCaMP6s and srg-8p:R-GECO1 in ASK neurons. Scale bar, 20 μm.

(D–I) The somal calcium signals in response to the application of 3 mM CuSO₄ of paired ASK (D–H) and ASH (I) sensory neurons in WT, mutant, and transgenic animals of indicated genotypes (D–I) or M13 buffer (D, as a control). Left panels, curves of Ca²⁺ transients presented as means (solid traces) ± SEM (gray shading); right panels, boxplots of the area under the curve (AUC) of Ca²⁺ signals of the ON response during 30 s stimulation, with each dot representing the data from each individual tested worm. Heat maps of Ca²⁺ signals are shown in Figure S2.

$$\Delta F = F - F_0$$, where $F_0$ is the average background signal-subtracted fluorescence intensity of each ASK or ASH soma within the initial 5 s before application of the solution. Statistical significance of differences was analyzed by one-way ANOVA analysis with the post hoc test of Tukey’s multiple comparison correction or unpaired t-test (1D and 1I), and indicated as follows: ns = not significant, *p< 0.05, **p< 0.01, ***p< 0.001 and ****p< 0.0001, and in different colors for varied comparisons. Black, a comparison of tested animals with the WT control; red, the gene-rescued with related mutants or as indicated. The genotypes of mutant or transgenic animals are as follows: WT N2, hkdEx2086[sra-9p:GCaMP6s; srg-8p:R-GECO1]; unc-13(e1091), hkdEx2087[sra-9p:GCaMP6s; lin-44p:GFP]; osm-9(tm5418), hkdEx2091[sra-9p:osm-9; sl2e:TagRFP-t; sra-9p:GCaMP6s unc-54; lin-44p:GFP], sra-9p:GCaMP6s:unc-54, hkdEx2092[gpa-11p:osm-9; sl2e:TagRFP-t].
RESULTS

Interaction between ASK and ASH augments animal avoidance from Cu\(^{2+}\)

In this study, we used a Cu\(^{2+}\) trap test to assay animal behavior (Figure 1A). In the test, ten animals without pre-treatment of food deprivation or starvation were transferred to a central region surrounded by an annulus of 10 mM CuSO\(_4\) solution as a trap on a test NGM (nematode growth media) plate without food. Their movement was recorded until all animals exited the region. During the test, the food-deprived animals explored bigger and bigger areas to forage for food driven by the need for nutrients and attempted to cross the Cu\(^{2+}\) bar several times (about 12–27 times in wild-type (WT) N2 animals) and finally escaped the Cu\(^{2+}\) trap. The time for all animals to exit the trap, exit time (about 10 min–40 min), was used for each test. A longer exit time indicates more robust Cu\(^{2+}\) avoidance and vice versa. The exit time mainly depends on the animal’s nociception of Cu\(^{2+}\). Because pre-treatment of food deprivation had no significant effect on the exit time (Figures 1B and S1A), animals un-trapped by Cu\(^{2+}\) annulus showed similar exit time (Figure S1B), and locomotion speed had only a weak effect on the exit time (Figure S1C).

ASK modulates the ASH sensation of quinine and down-regulates animals’ avoidance from this bitter tastant (Krzyzanowski et al., 2016; Voelker et al., 2019). For the testing effect of functional interaction between ASK and ASH on Cu\(^{2+}\) avoidance, we first used neuronal manipulation of genetically inhibiting neurotransmission from other neurons (neurons: osm-6; unc-54; sl2e: sra-9p: TagRFP-t; sra-9p: osm-6; unc-54; unc-13; unc-31; unc-31 gene encodes UNC-31 protein, which is required for synaptic vesicle fusion with the presynaptic membrane and is thus essential for neurotransmitter release. The unc-31 gene encodes UNC-31 protein, an ortholog of human CAPS (calcium-dependent secretion activator), which is necessary for exocytosis of dense cored vesicles and thus release of neuropeptides (Richardson et al., 1999; Speese et al., 2007; Tokumaru and Augustine, 1999). The ASK Ca\(^{2+}\) response in unc-13 and unc-31 mutant animals decreased significantly compared to those in WT N2 (Figures 1E and S2A). This result suggests that the ASK sensation of Cu\(^{2+}\) is partially cell-autonomous at most and may be enhanced by signals from other neurons.

Because ASK is involved in regulating animals’ Cu\(^{2+}\) avoidance, it should display responses to Cu\(^{2+}\). To examine whether ASK senses Cu\(^{2+}\), we employed fluorescent Ca\(^{2+}\) imaging with GCaMP6s (Chen et al., 2013) and combined it with microfluidic control of stimulation and animal movement (Chronis et al., 2007; Ge et al., 2020; Guo et al., 2015; Liu et al., 2019; Wang et al., 2016; Wen et al., 2020). ASK sensory neurons in WT N2 worm displayed a robust Ca\(^{2+}\) response to 3 mM CuSO\(_4\) stimulation (Figure 1D). The change in Ca\(^{2+}\) signals may result from neurotransmission from other neurons. We then used unc-13(e1091) and unc-31(e928) mutant animals to assay the cell autonomy of ASK Cu\(^{2+}\) sensation. unc-13 (UN-Coordinated) encodes syntaxin-1 binding protein UNC-13, which is required for synaptic vesicle fusion with the presynaptic membrane and is thus essential for neurotransmitter release. The unc-31 gene encodes UNC-31 protein, an ortholog of human CAPS (calcium-dependent secretion activator), which is necessary for exocytosis of dense cored vesicles and thus release of neuropeptides (Richardson et al., 1999; Speese et al., 2007; Tokumaru and Augustine, 1999). The ASK Ca\(^{2+}\) response in unc-13 and unc-31 mutant animals decreased significantly compared to those in WT N2 (Figures 1E and S2A). This result suggests that the ASK sensation of Cu\(^{2+}\) is partially cell-autonomous at most and may be enhanced by signals from other neurons.
ASH senses Cu²⁺ via OSM-9 (encoded by osm-9, osmotic avoidance abnormal gene 9)/OCR-2 (encoded by ocr-2, osm-9/capsaicin receptor-related gene 2) signaling (Tobin et al., 2002). OSM-9 but not OCR-2 is expressed in ASK. Is OSM-9 required for ASK sensation of Cu²⁺? As shown in Figures 1F and S2B, ASK Ca²⁺ responses to the administration of 3 mM CuSO₄ in osm-9(tm5418) mutant animals were reduced and partially but significantly rescued by osm-9 reconstitution in ASK. Interestingly, osm-9 reconstitution in ASH by cell-selective promoters gpa-11p (3.3 kb, in ASH and ADL) and sra-6p (3.8 kb, in ASH and ASI) also partially but significantly restored the ASK Ca²⁺ response to Cu²⁺. The reconstitution of osm-9 driven by gpa-11p or sra-6p showed a very similar effect on the ASK Ca²⁺ signals, and the overlap of these two promoters is ASH. This effect of genetic rescue may be attributed to its role in ASH. Interestingly, osm-9 reconstitution in neurons, including ASK and ASH, driven by gpa-14p (3.2 kb in ASH, ASK, ASI, and ASJ) fully restored WT Ca²⁺ response in the transgenic animals (Figures 1F and S2B). This result supports that OSM-9 functions in ASK and ASH neurons and is involved in ASK sensory responses to Cu²⁺, even without its interactant OCR-2.

The osm-6 gene is required for developing sensory cilia in sensory neurons such as ASK and ASH (Tobin et al., 2002) and is thus essential for nociception in neurons. We then measured the Cu²⁺-evoked Ca²⁺ signals of ASK in osm-6(p811) and the gene-rescue transgenic animals. As shown in Figures 1G and S2C, ASK Ca²⁺ transients were similar to those in osm-9(tm5418) and the gene-rescued transgenic animals. The reduced ASK Ca²⁺ levels caused by the osm-6 loss-of-function (lof) mutation were partially and fully restored to WT levels by rescue expression of osm-6 in ASK or ASH alone and neurons including both ASK and ASH, respectively.

Next, we employed ASH:TeTx, and ASK:TeTx transgenic animals to assay ASK and ASH Ca²⁺ responses to stimulation of 3 mM CuSO₄ solution. TeTx expression in ASH (driven by promoters of gpa-11 or sra-6) significantly decreased ASK Ca²⁺ responses (Figures 1H and S2D). However, the ASH Ca²⁺ responses to Cu²⁺ stimulation, measured by fluorescence imaging with G-CaMP3 (Tian et al., 2009), did not change obviously in the ASK:TeTx transgenic animals compared with those in WT N2 worm (Figures 1I and S2E), suggesting that neurotransmission from ASK is unlikely to alter the excitability of ASH upon Cu²⁺ stimulation. This result is expected. Because although there are chemical synapses between ASK and ASH, ASK is postsynaptic but not presynaptic to ASH.

In summary, the above results suggest that ASK and ASH sense Cu²⁺, two types of neurons act synergistically in Cu²⁺ avoidance in the Cu²⁺ trap test but not in the wet drop test, and ASH augments ASK response to Cu²⁺.

ASK accelerates ASH Ca²⁺ responses to Cu²⁺ by sending cGMP to ASH, possibly via gap junctions containing INX-4, and cGMP functions through cGMP-EGL-4 signaling

What is the mechanism underlying the role of interaction between ASK and ASH in Cu²⁺ avoidance in the Cu²⁺ trap test but not in the wet drop test? ASK connects with ASH via electric synapses, sends cGMP to ASH, and negatively regulates the ASH sensation of quinine (Krzyzanowski et al., 2016; Voelker et al., 2019). ASK may use a similar mechanism to regulate ASH’s role in avoiding Cu²⁺. First, we used the odr-1 mutant and gene-rescued animals to assay the avoidance behavior in the Cu²⁺ trap and the wet drop tests. The odr-1 gene encodes a transmembrane guanylyl cyclase and is involved in multimodal sensations (Bargmann et al., 1993; L’Etoile and Bargmann, 2000). The lof mutant animal of odr-1(n1936) displayed discrepant behavior phenotypes in two models of tests: remarkably reduced and WT Cu²⁺ avoidance in the Cu²⁺ trap test (Figure 2A) and the wet drop test (Figures S2A and S3B), respectively. The behavioral phenotype was restored fully to the WT by reconstitution of the gene in its expressing neurons (AWB, AWC, ASI, ASJ, and ASK, driven by 3.1 kb odr-1p), ASK specifically (directed by 4 kb sra-9p), but not in AWB, AWC, ASI, and ASJ alone. ASH is known as an odr-1-nonexpressing neuron. We introduced odr-1 into ASH of odr-1(n1936) animal (ectopic expression) using the promoters of both gpa-11 (3.3 kb, in ASH and ADL) and sra-6 (3.8 kb, in ASH and ASI) and assayed Cu²⁺ avoidance behavior. Interestingly, the transgenic animal displayed a WT behavior (Figure 2A). To ask whether the catalytic activity of ODR-1 was required for its function in animal Cu²⁺ avoidance, a mutant protein with a point mutation within the catalytic domain lacking a GTP binding site (ODR-1(E874A)) was used as previously reported (L’Etoile and Bargmann, 2000). The introduction of fusion protein GFP-ODR-1(E874A) in ASK or ASH in the odr-1(n1936) animal had no significant impact on the Cu²⁺ avoidance (Figure S3C), suggesting that the function of ODR-1 requires cyclase activity and cGMP production. These results suggest that cGMP is necessary and sufficient for ASH’s role in Cu²⁺ avoidance behavior in the Cu²⁺ trap test.
Figure 2. cGMP synthesized by ODR-1 is required for normal animals’ avoidance from Cu⁡⁺ in the Cu⁡⁺ trap test, normal ASK Cu⁡⁺-evoked Ca⁡⁺ responses, and the fast-rising phase of ASH ON Ca⁡⁺ responses to Cu⁡⁺

(A) Exit time in wildtype (WT, as a control), mutant, and the transgenic animals of genotype as indicated in the Cu⁡⁺-trap test. (B) Ca⁡⁺ transients in ASK in response to 3 mM Cu⁡⁺ in animals of indicated genotypes, presented as means ± SEM (solid traces ± gray shading, left panel) and the area under the curve (AUC) of the ON response during 30 s stimulation (boxplots, right panel). (C) Ca⁡⁺ transients in ASH in response to 3 mM Cu⁡⁺, presented as means ± SEM (gray shading, left panel), the AUC of Ca⁡⁺ signals of the ON response (boxplots, medial panel), and the slope rate of the ON response rising phase (boxplots, right panel). Heat maps of Ca⁡⁺ signals are shown in Figure S4. ∆F = F – F₀, F₀, the average fluorescence intensity of each ASK or ASH soma within the initial 5 s before application of the solution. Data are displayed as means (solid traces) ± SEM (gray shading) or boxplots with each dot representing the data from each individual tested worm. Statistical significance of difference was analyzed by one-way ANOVA analysis with the post hoc test of Tukey’s multiple comparison correction and indicated as follows: ns = not significant; *p < 0.05, **p < 0.01, ***p < 0.001, and in different colors for varied comparisons. Black, a comparison of tested animals with the WT control; red, the gene-rescued with related mutants. The genotypes of mutant or transgenic animals are as follows: WT N2, odr-1(n1936); hkdEx2082[odr-1::GCaMP6s; lin-44p:GFP], odr-1(n1936); hkdEx2081[odr-1::GCaMP6s; lin-44p:GFP], odr-1(n1936); hkdEx2083[odr-1::GCaMP6s; lin-44p:GFP], odr-1(n1936); hkdEx2084[odr-1::GCaMP6s; lin-44p:GFP].

As odr-1 is involved in Cu⁡⁺ avoidance, it should impact sensory responses to Cu⁡⁺ in ASK and ASH. Next, we assayed Ca⁡⁺ responses to Cu⁡⁺ stimulation in these neurons, using the odr-1 mutant and the gene rescued animals. The Cu⁡⁺-evoked Ca⁡⁺ transients in ASK soma in odr-1(n1936) displayed a noticeable reduction. They were fully and partially restored to the WT by the gene rescue in ASK and its ectopic expression in ASH, respectively (Figures 2B and S4A). In contrast, the Ca⁡⁺ responses in ASH soma showed only a change in kinetics, an apparent slower rising phase indicated by the slope rate, but not in the amplitude of Ca⁡⁺ signals indicated by the area under the curve (AUC) of ON responses. The odr-1 reconstitution in ASK or its ectopic expression in ASH restored the Cu⁡⁺-evoked Ca⁡⁺ signals in ASH (Figures 2C and S4B). These results showed that cGMP (synthesized by ODR-1) in either ASH or ASK significantly augments ASK Ca⁡⁺ signals in response to Cu⁡⁺ and has only a weak effect on the amplitude of ASH ON responses of Ca⁡⁺.
Figure 3. cGMP generated in ASK passing through INX-4 contained gap junctions is required for proper animals’ avoidance from Cu2+ in the Cu2+ trap test and the fast kinetics of ASH nociceptive response

(A) Exit time in animals of indicated genotypes in the Cu2+-trap test.
(B) Ca2+ transients in ASH in response to 3 mM Cu2+, presented as means ± SEM (solid traces, gray shading, left panel), the area under the curve (AUC) of the ON response of Ca2+ signals during 30 s stimulation, and the slope rate of the ON response rising phase (boxplots, medial and right panels). Heat maps of Ca2+ signals are shown in Figure S4.

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\Delta F/F = F - F_0
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\(F_0\), the average fluorescence intensity of the region of interest (ROI) of an ASH soma in each frame; \(F\), the average background signal-subtracted fluorescence intensity of each ASH soma within the initial 5 s before application of the solution.

(C–G) Exit time in animals of indicated genotypes in the Cu2+-trap test. Data are displayed as means (solid traces) ± SEM (gray shading) or boxplots with each dot representing the data from each individual tested worm. Statistical significance of difference was analyzed by one-way ANOVA or two-way ANOVA analysis (only in D) analysis with the post hoc test of Tukey’s multiple comparison correction and indicated as follows: ns = not significant, **p < 0.01, ****p < 0.0001, and in different colors for varied comparisons. In D: black, a comparison of indicated mutant and transgenic animals with WT animals under non-irradiated (no light); blue, that of indicated mutant and transgenic animals vs. WT animals under blue-light irradiation; red, that of blue-light irradiated animals vs. non-irradiated animals of the same genotype. The genotypes of mutant or transgenic animals are as follows: WT N2, inx-18(ok2425), inx-19(tm1898), inx-4(e1128), odr-1(n1936), inx-4(e1128), tax-2(tk91), tax-3(tp71), tax-4(tk28), tax-4(tax-4), tax-4.

Inx-18, a transgenic allele that affects Cu2+ handling, and inx-19, a null allele that affects Cu2+ handling, are required for proper avoidance of Cu2+ in the Cu2+ trap test. Additionally, the axonal transport of INX-4 is required for proper avoidance of Cu2+. These results suggest that INX-4 plays a crucial role in the avoidance behavior of C. elegans in response to Cu2+.
to Cu²⁺, however, it substantially accelerates ASH Ca²⁺ responses. Combing with the result of the wet drop test of Cu²⁺ that odr-1(n1936) displayed WT Cu²⁺ avoidance (Figures S3A and S3B), it is reasonable to conclude that the kinetics of ASH Ca²⁺ responses is vital for avoidance behavior in the Cu²⁺ trap test in that animals are challenged with a relatively short-term stimulation, however, not of significance in the wet drop test in that animals are exposed to more prolonged noxious stimulation.

We used genetic analyses to test the function of gap junction proteins in animal Cu²⁺ avoidance and ASH Ca²⁺ responses to Cu²⁺ stimulation. Different from the results of quinine avoidance (Krzyzanowski et al., 2010). Under normal culture conditions and no light irradiation, animals of the single transgene of inx-4(e1128); odr-1(n1936); inx-4(e1128) showed a slower rising phase but similar AUC of ON responses. The ASH Ca²⁺ signals were restored to the WT by the inx-4 rescue in ASH but not ASK (Figure 3B and S4C). We then employed a double-gene analysis of both odr-1 and inx-4. Single mutant animals of genotypes odr-1(n1936) and inx-4(e1128) and the double knockout (DKo) animal with a genotype of odr-1(n1936); inx-4(e1128) showed similar behavioral phenotype, significantly decreased exit time. Neither single gene rescue of odr-1 in ASK nor that of inx-4 in ASH recovered WT behavior. Only double reconstitution of both odr-1 in ASK and inx-4 in ASH fully restored the WT behavioral phenotype in the transgenic animal (Figure 3C). These genetic analyses support that cGMP synthesized by ODR-1 in ASK may pass through gap junctions containing INX-4 and enters ASH to promote ASH’s function in animal Cu²⁺ avoidance. To further confirm the role of odr-1 and inx-4 in the regulation of the behavior in adult animals, we performed optogenetic manipulation of cGMP levels in ASH and ASK in odr-1(n1936); inx-4(e1128) DKo and gene-rescued transgenic animals, using the blue light activation of guanylate cyclase BlgC (Ryu et al., 2010). Under normal culture conditions and no light irradiation, animals of the single transgene of odr-1 or inx-4 or double transgene of both genes in the odr-1; inx-4 DKo displayed similar defects in Cu²⁺ avoidance behavior compared with the WT N2. Under blue light irradiation, which resulted in elevated cGMP levels in BlgC transgenic animals, the WT N2 displayed no difference in the exit time compared with the animal under standard culture conditions, as expected. Animals of the DKo and ASK:BlgC; DKo still showed remarkably reduced exit time, while the ASH:BlgC; DKo and ASK:BlgC; ASH:inx-4; DKo transgenic animals restored WT exit time (Figure 3D). In other words, the photogenetic elevation of intracellular cGMP by BlgC photoactivation in ASH and ASK in animals with functional gap junctions between ASK and ASH made animals restore WT Cu²⁺ avoidance. We tried fluorescence imaging using FlincG (Nausch et al., 2008) and Green cGull (Matsuda et al., 2017) to visualize the diffusion of cGMP from ASK to ASH but failed. All these results show that cGMP and functional gap junctions are essential for ASH enhancement of ASH’s role in animal avoiding Cu²⁺ in the Cu²⁺ trap test.

What is the signaling pathway by that cGMP augments ASH function in Cu²⁺ avoidance in the Cu²⁺ trap test? Is the pathway of cGMP-TAX-2/TAX-4 complex or cGMP-EGL-4? tax-2 and tax-4 genes encoded a cyclc nucleotide-gated channel complex which is essential for the functions of many sensory neurons (Coburn and Bargmann, 1996). Mutant animals of tax-2(ks31) and tax-2(p671) showed WT behavior. Although the tax-4(ks28) mutant worm displayed an apparent reduced exit time, the behavioral defect could not be eliminated by reconstitution of tax-4 (Figure 3E). This result suggests that cGMP unlikely functions through the cGMP-TAX-2/TAX-4 complex pathway. EGL-4, a β isofom of cGMP-dependent protein kinase 1 (PKG-1), is a more important mediator of cGMP functions. cGMP-EGL-4 signaling negatively regulates avoidance from quinine but not from Cu²⁺, primarque, and detergent SDS in the wet drop test (Krzyzanoski et al., 2013). EGL-4 is required for olfactory adaptation after prolonged exposure to odor detected by AWC neurons in C. elegans (L’Etoile et al., 2002). This protein kinase may also function in ASH’s role in avoidance behavior in the Cu²⁺ trap test. Here, our results show that the egl-4(ks79) mutant and ASH egl-4 knockdown transgenic animals displayed a significant increase in the exit time or stronger behavioral sensitivity of Cu²⁺ in the Cu²⁺ trap test (Figures 3F and 3G) but no noticeable impact on Cu²⁺ avoidance in the
Figure 4. ASH excites ASK by acting on AIA interneurons through the glutamatergic signaling pathway

(A) Exit time in animals of indicated genotypes in the Cu^{2+}-trap test.
(B–D) Ca^{2+} transients in ASK in response to 3 mM Cu^{2+} in animals of indicated genotypes, presented as means ± SEM (solid traces ± gray shading) and the area under the curve (AUC) of Ca^{2+} signals of the ON response during 30 s stimulation (boxplots).
(E) Exit time in animals of indicated genotypes in the Cu^{2+}-trap test.
(F–J) Ca^{2+} transients in AIA and ASK (only G) in response to 3 mM Cu^{2+} in animals of indicated genotypes, presented as means ± SEM (solid traces ± gray shading) and the AUC of Ca^{2+} signals of the ON response (boxplots). Heat maps of Ca^{2+} signals are shown in Figures S5 and S6. ΔF = F – F₀, the average fluorescence intensity of the region of interest (ROI) of an ASK or AIA soma in each frame; F₀, the average background signal-subtracted fluorescence.
wet drop test (Figures S3A and S3B). The behavioral alteration in the egl-4(n479) animal was eliminated by re-expression of egl-4 in ASH driven by the sra-6 promoter but not by that directed by egl-4p or gpa-11 (Figure 3F). EGL-4 dampens quinine sensitivity via phosphorylation and activation of the regulator of G protein signaling (RGS) proteins RGS-2 and RGS-3 (Krzysznowski et al., 2013) and triggers late forms of olfactory adaptation via regulation of gene expression (L’Etoile et al., 2002). What is the mechanism of EGL-4 regulating Cu²⁺ avoidance behavior in the Cu²⁺ trap test: via the cytoplasmic or nuclear pathway, or both pathways? To answer this issue, we introduced the following egl-4 genes into ASH of the egl-4(n479) animal: GFP-egl-4, GFP-egl-4(d4NLs) mutant gene of which the nuclear localization sequence (NLS) was truncated, and NSL-GFP-egl-4 (Krzysznowski et al., 2013; L’Etoile et al., 2002). Without NLS, GFP-EGL-4(d4NLs) fusion protein could not translocate into the nucleus and show no effect on animal Cu²⁺ avoidance. In contrast, the fusion proteins with NLS could translocate into the nuclear and play roles in the Cu²⁺ avoidance behavior (Figures 3F and S4D). This result shows that EGL-4 regulates animal Cu²⁺ adaptation through nuclear pathways, regulating gene transcription.

The above results show that ASK enhances ASH function in animals avoiding Cu²⁺ in the Cu²⁺ trap test by sending cGMP to ASH, possibly via gap junctions containing INX-4, and that cGMP acts through the EGL-4 signaling pathway. Why this mechanism plays a distinct role in avoidance behavior in different models of behavioral tests and avoidance from varied stimuli in the same test remains unknown.

**ASH promotes ASK by a disinhibitory circuit consisting of ASH, AIA, and ASK**

ASK augments ASH function in animals avoiding Cu²⁺ in the Cu²⁺ trap test by sending cGMP to ASH through electrical synapses. What is the neural circuit mechanism underlying the ASH promotion of ASK activities? ASH is a glutamatergic and neuropeptidergic neuron. It is known to release glutamate and several neuropeptides, FLP-21, INS-1, NLP-3, and NLP-15, for neurotransmission. EAT-4, a vesicular L-glutamate transporter, is essential for filling glutamate into synaptic vesicles and, thus, glutamatergic neurotransmission (Avery, 1993; Lee et al., 1999, 2008). We then used eat-4 and neuropeptide mutant animals to test the function of these neurotransmitters in Cu²⁺ avoidance behavior. Our test results showed that only the eat-4(ad819), but none of the neuropeptide mutant animals, displayed a noticeable behavior change. The change was eliminated by eat-4 rescue driven by eat-4 promoter but not by ASH expression promoters, gpa-11p and sra-6p (Figure 4A). However, the mutant animal of eat-4(ad819) displayed WT Cu²⁺ avoidance in the wet drop test (Figures S3A and S3B). eat-4 is widely expressed in the *C. elegans* nervous system, including sensory neurons, interneurons, and motor neurons (https://wormbase.org). It sounds reasonable that eat-4 reconstitution in ASH alone cannot restore WT Cu²⁺ avoidance behavior. However, our previous study shows that eat-4 reconstitution in ASH alone is sufficient to restore the WT phenotype of egg laying (Wen et al., 2020). We next checked the Cu²⁺-evoked Ca²⁺ transients of ASH soma in the eat-4 mutant, gene rescued, and knocked down transgenic animals. The mutant and ASH
eat-4 knocked down transgenic animals displayed significantly reduced ASK Ca\(^{2+}\) responses to the stimulation of 3 mM CuSO\(_4\), while the ASH eat-4-rescued animal showed WT Ca\(^{2+}\) levels. Under exogenous administration of 50 \(\mu\)M glutamate, the eat-4(ad81f9) also exhibited WT ASK Ca\(^{2+}\) responses to Cu\(^{2+}\) (Figures 4B and SS A). This result was also supported by the observation in ASH: TeTx (driven by gpa-17p or sra-6p) transgenic animals. The animals showed reduced Ca\(^{2+}\) transients and WT Ca\(^{2+}\) signals in ASK under conditions without and with exogenous glutamate treatment, respectively (Figures 4C and SS B).

The above results suggest that the glutamatergic pathway is involved in the regulation of ASK activities by ASH. ASK is postsynaptic to ASH; however, there is no reported glutamate receptor on ASK (https://www.wormatlas.org). Thus, ASH may promote ASK activities through the relay of an intermediate neuron or neurons. Based on the synaptic connections with ASK and glutamatergic receptors expression, we focused on testing AIA and AIB. AIA is pre- and postsynaptic to ASK. AIA expresses glutamatergic receptors, including GLR-2 (AMPA-type ionotropic glutamate receptor), GLC-3 (an L-glutamate-gated chloride channel subunit), and MGL-1 (metabotropic glutamate receptor 1). AIB is postsynaptic to ASK and expresses GLR-1, GLR-2, possibly GLR-5, and MGL-2 (https://www.wormatlas.org). We examined the effect of genetically inhibiting AIA and AIB interneurons neurotransmission on Cu\(^{2+}\)-evoked Ca\(^{2+}\) transients in ASK. Our results showed that genetic manipulation of AIA: TeTx marvelously enhanced ASK Ca\(^{2+}\) responses to Cu\(^{2+}\) stimulation, hinting that AIA interneurons inhibit ASK activity. However, AIB: TeTx had no effect (Figures 4D and SS C). We then identified what receptor on AIA mediates the ASH glutamatergic signal using receptor mutant animals. Both glr-2(ok2343) and mgl-1(tm1811) animals showed WT behavior. While the glc-3(ok321) displayed significantly decreased exit time or abated Cu\(^{2+}\) avoidance. The behavioral alteration was eliminated by reconstitution of glc-3 in its expressed neurons and AIA alone (Figure 4E). We further used Ca\(^{2+}\) imaging in AIA and ASK to confirm the result of the behavioral test. As expected, the AIA Cu\(^{2+}\)-evoked Ca\(^{2+}\) signals increased in the glc-3(ok321) and returned to WT levels in the AIA: glc-3; glc-3 transgenic animal (Figures 4F and SS D). Whereas ASK Cu\(^{2+}\)-evoked Ca\(^{2+}\) signals in the glc-3(ok321) decreased and returned to the WT in the AIA: glc-3; glc-3 (Figures 4G and SS E). These results suggest that the sensory neuron ASH inhibits AIA interneurons via inhibitory GLC-3 signaling and that AIA suppresses ASK sensory neurons. Thus, ASH excites ASK by a disinhibitory modality of neuronal signal integration via a neuronal circuit consisting of ASH, AIA, and ASK.

AIA interneurons were excited by the application of 3 mM Cu\(^{2+}\), as shown in Figure 4F. However, the above results support that ASH inhibits AIA through glutamate/GLC-3 signaling. What is the source of AIA excitation: ASH or other Cu\(^{2+}\)-sensitive neurons? Our previous report shows that C. elegans is very sensitive to Cu\(^{2+}\), with an EC\(_{50}\) (the concentration for 50% of maximal effect) of 4.68 \(\mu\)M for Cu\(^{2+}\) enhancing egg laying (Wen et al., 2020). The Cu\(^{2+}\) concentration of 3 mM used in this study is relatively high. Intense nociceptive stimulation may trigger the simultaneous release of classical small molecule neurotransmitters and neuropeptides (Horn and Swanson, 2013). ASH expresses glutamatergic and neuropeptidergic receptors. ASH inhibits AIA through GLC-3 signaling. In contrast, ASH or other neurons may excite AIA via neuropeptidergic signaling pathway(s) at high probability or excitatory glutamatergic receptor(s) at less likelihood. We then used mutant animals of glutamate receptor genes and egl-3 to identify the signaling of AIA excitation preliminarily. egl-3 encodes a proprotein convertase essential for the biosynthesis of neuropeptides (Thacker and Rose, 2000). Animals of glr-2(ok2343) and mgl-1(tm1811) showed WT AIA Ca\(^{2+}\) responses, whereas the egl-3(gk238) displayed significantly decreased AIA Ca\(^{2+}\) responses (Figures 4H and S6 A). This result suggests the neuropeptidergic source of AIA excitation. Both primary and secondary Cu\(^{2+}\) nociceptive ASH and ASI are neuropeptidergic. We focused on these two types of sensory neurons using egl-3 knockdown. The egl-3 knockdown by RNAi in ASH and ASI is driven by promoters of gpa-11p (3.3 kb, in ASH and ADL), sra-6p (3.8 kb, in ASH and ADL), and gpa-4p (2.5 kb, in ASL). The egl-3 knockdown in ASH/ADL showed no noticeable effect on AIA excitation. While the egl-3 knockdown in ASH/ASI and ASI almost similarly inhibited the AUC of ASK Ca\(^{2+}\) responses with a reduction of 75 and 65%, respectively (Figures 4I and S6 B). This result suggests that neuropeptides from ASI excite AIA. Interestingly, neurotransmission inhibition by TeTx in ASH/ADL and ASI increased and decreased AIA Ca\(^{2+}\) responses to Cu\(^{2+}\) stimulation, respectively. TeTx inhibition of ASI neurotransmission decreased the AUC of the AIA ON Ca\(^{2+}\) responses with a reduction of 92% (Figures 4J and S6 C). This result, combined with the egl-3 knockdown result, suggests that AIA excitation mainly comes from ASI. This indicates that neurotransmission from ASH and ASI inhibits and excites AIA, respectively. All the above results support that ASH inhibits AIA by glutamatergic signaling, and ASI excites AIA through neuropeptidergic signaling.
AIA inhibits ASK, known from the above results. It is waiting to be revealed what signaling pathway mediates the action. AIA is an acetylcholinergic and neuropeptidergic neuron. It releases FMRFamide-like neuropeptides FLP-1, FLP-2, and insulin-like peptide INS-1. In addition, ASK possesses the neuropeptidergic receptor NPR-5 and no known cholinergic receptor (https://www.wormatlas.org). Thus, we focused our tests on neuropeptidergic signaling. Among mutant animals of \textit{flp-1}, \textit{flp-2}, and \textit{ins-1}, only \textit{flp-1} mutant animals, \textit{flp-1(yn2)} and \textit{flp-1(yn4)}, displayed decreased exit time. In contrast, \textit{npr-5(ok1583)} showed significantly increased exit time, while the gene-rescued animal of ASK: \textit{npr-5} possessed WT exit time (Figure 5A).

We further used AIA-specific knockdown of \textit{flp-1} and \textit{egl-3} (driven by \textit{gcy-28} (\textit{d}) promoter, 2.8 kb) to support our observation of the mutant animals. Out of our expectations, AIA knockdown of \textit{flp-1} or \textit{egl-3} equally increased the exit time (Figure 5B). The extensive functions of FLP-1 may explain the inconsistency between the mutant and knocked down animals. \textit{flp-1} mutant animals displayed numerous defects in sensations and locomotion (Buntschuh et al., 2018). AIA-specific knockdown of \textit{flp-1} highlights the gene function in AIA.

![Figure 5](image-url)
These results strongly suggest that AIA interneurons inhibit ASK activities in Cu²⁺ avoidance in the Cu²⁺ trap test via the FLP-1/NPR-5 signaling pathway. Then we used ASK Ca²⁺ imaging to support our behavioral observation results. As expected, animals of npr-5(ok1583) and AIA-specific knockdown of flp-1 or egl-3 showed remarkably increased Cu²⁺-evoked Ca²⁺ signals in ASK, and the gene-rescued animal of ASK:npr-5; npr-5 restored almost WT Ca²⁺ signals (Figures 5C, 5D, S6D, and S6E). In summary, all the above results support that AIA interneurons mediate ASK excitation by ASH through FLP-1/NPR-5 signaling.

**Neuronal circuit consisting of ASH, AIA, and ASK functions to inhibit nociceptive and behavioral adaption to noxious Cu²⁺**

The neuronal circuit consisting of ASH, AIA, and ASK, and their molecular and cellular components, are involved in Cu²⁺ avoidance in the Cu²⁺ trap test. However, they show no noticeable impact on avoidance from Cu²⁺ (Krzyzanowski et al., 2013; Voelker et al., 2019; this study), primaquine, and SDS (Krzyzanowski et al., 2013; Voelker et al., 2019) in the wet drop tests. This inconsistency may result from different test models and varied stimuli. In the Cu²⁺ trap and wet drop tests, stimulation duration and behavioral models differ. In the wet drop test, animals are usually tested once. However, tested animals made several touching the dried Cu²⁺ bar before they finally exited the trap in the Cu²⁺ trap test. The exit time of test animals may reflect their sensory and behavioral adaption to Cu²⁺. Varied behavioral adaptation may display changes in the numbers of touching the Cu²⁺ bar (Cu²⁺ touch) and the delay time of Cu²⁺ avoidance behavior. First, we examined how many times of Cu²⁺ touch are needed for animals to exit the trap using critical mutant animals tested in the above experiments. As shown in Figure 6A, animals with a fast exit time needed fewer numbers of Cu²⁺ touch. The exit time is positively and linearly correlated with touch numbers (Pearson r = 0.9058, p < 0.0001, Figure 6B). Second, we analyzed the dynamic changes in the delay time of individual Cu²⁺ touch. The delay time becomes longer following the increase of touch numbers. Interestingly, when the delay time reached about 6–8 s, a threshold, all animals of various genotypes used in this test escaped the trap (Figure 6C). As the exit time linearly correlated with the numbers of Cu²⁺ touch. Logically, different numbers of Cu²⁺ touch should be needed for animals of different genotypes and varied behavioral sensitivities of Cu²⁺ to reach the delay time threshold. As shown in Figure 6D, animals with lower sensitivities needed fewer numbers of Cu²⁺ touch and vice versa. This analyses result supports the hypothesis. What mechanism determines animals’ behavioral adaptation? Functional interaction between ASH and ASK not only augments ASK nociceptive response to Cu²⁺ but also accelerates the rising phase of the ASH ON response to Cu²⁺. ASK nociceptive response is involved in behavioral adaption (Figure 1). Does the kinetics of the rising phase of the ASH ON response function in behavioral adaptation? To answer this question, we analyzed the correlation of the slope rate of the rising phase of the ASH ON responses with the number of Cu²⁺ touches. As expected, the slope rates positively and linearly correlated with the numbers of Cu²⁺ touch (Pearson r = 0.9086, p < 0.0001, Figure 6E). This suggests that the Cu²⁺ touch number depends on the kinetics of the rising phase of the ASH ON response. We tried fluorescence calcium imaging in ASH to assay sensory adaptation. However, the Ca²⁺ responses to Cu²⁺ in fixed animals in the microfluidic chips did not acquit itself well as the avoidance behavior in free-moving animals (data not shown). It was tough to get enough numbers of Ca²⁺ responses in response to the repeated stimulation. Calcium imaging in freely moving animals is needed to solve this problem.

All the above results demonstrate that positive interaction between ASH and ASK via ASH/AIA/ASK circuit functions to inhibit behavioral adaption to the noxious Cu²⁺ stimulus. The fast-rising phase of ASH Ca²⁺ responses is required for normal behavior adaptation.

**DISCUSSION**

Nociception is tightly regulated by central and peripheral nervous systems. In *C. elegans*, primary nociceptor ASH neurons are modulated by secondary nociceptive neurons ASI (Guo et al., 2015) and ASK (Krzyzanowski et al., 2016; Voelker et al., 2019; and this study). In this study, we find that positive interaction between ASH and ASK is essential for the fast-rising phase of ASH ON responses to copper ions and functions to inhibit adaptation of avoiding Cu²⁺. The underlying molecular and circuitual mechanisms are illustrated by a working model shown in Figure 7. ASK transfers the second messenger cGMP to ASH, possibly through gap junctions with inx-4, as a component and accelerates ASH nociception via the cGMP-EGL-4 signaling pathway. ASH excites ASK via a disinhibitory neuronal circuitry composed of ASH, AIA and ASK.

In this neuronal circuit, AIA plays a pivotal role. AIA pair is the first layer amphid interneuron that sums inputs from various chemosensory neurons, including ADL, ASE, ASG, ASH, ASI, ASK, AWC, etc., before passing...
Neuronal signal integration is the basis of neuronal circuit functions. The modalities of neural information integration are evolutionarily conserved in animals and humans. Disinhibition or unmasking processes (Craig and Bushnell, 1994; Craig et al., 1996; Lee et al., 2013; Liu et al., 2019; Piggott et al., 2011) are among a few neuronal signal integration modalities, such as excitatory and inhibitory connections, reciprocal inhibition (Drew and Kiehn, 2021; Guo et al., 2015; Li et al., 2012), gate control (Basbaum, 2021; Melzack and Wall, 1965), and disexcitation (Liu et al., 2019).
ASK responds to Cu²⁺ stimulation. The response is not cell-autonomous and requires OSM-9 in ASK and ASH (Figure 1). Besides, osm-9 expression in both neurons is essential for the normal adaptation of Cu²⁺ (Figure 6). ASH expresses both OSM-9 and OCR-2, whereas ASK expresses OSM-9 only. OCR channels are typically co-expressed with OSM-9 but not vice versa (Jose et al., 2007; Tobin et al., 2002). OSM-9 and OCR-2 depend on each other for cilia localization rather than the cell body. In neurons that normally express the osm-9 gene without any ocr gene, OSM-9 protein is found in the cell body. Neurons that use OSM-9 in the regulation of adaptation express only osm-9, whereas neurons in which OSM-9 are primary signal transduction channels express both osm-9 and at least one ocr gene. OSM-9 and OCR-2 possibly form heteromeric channels that function in primary sensations. Besides, OSM-9 channels regulate sensory adaptation after prolonged exposure to an odor or taste. In AWC olfactory neurons and the ASE gustatory neurons, osm-9 is not required for primary sensory signaling but is necessary for sensory adaptation (Colbert and Bargmann, 1995; Jansen et al., 2002; see also reviews: Kahn-Kirby and Bargmann, 2006; Montell, 2005; Xiao and Xu, 2009, 2011). In ASK/AIA/ASK circuit, OSM-9 channels in ASH and ASK are involved in both primary nociception and adaptation of avoidance behavior.

cGMP synthesized by ODR-1 in ASK diffuses to ASH, possibly via gap junctions with INX-4 as a component. INX-4 expression is necessary for the regulatory roles of ASH Ca²⁺ signals and animals’ behavioral adaptation of cGMP synthesized in ASK (Figure 3). However, as previous work did (Voelker et al., 2019), directly visualizing cGMP flow is needed to confirm the cGMP flow through gap junctions containing INX-4. Gap junctions are dense aggregates of intercellular channels between adjacent cells that permit direct diffusion of ions and small molecules, including amino acids, metabolites, and signaling molecules. Each gap junction is formed by head-to-head binding of two hemichannels composed of six subunits on each cell membrane. Gap junctions may be made of the same or different subunits and are found in different types. For example, homotypic, a single protein species on both cell membranes; heterotypic, two different homomeric hemichannels on each membrane; and heteromeric, a mixture of subunit compositions on both membranes. Gap junction subunits belong to two superfamilies of proteins: connexins (found only in vertebrates) and pannexins (found in vertebrates and invertebrates; invertebrates’ pannexins are called innexins). Gap junctions not only function in electric and metabolic coupling between cells but are also involved in various forms of paracrine signaling (Connors and Long, 2004; Goodenough and Paul, 2009).

The innexin family of gap junction proteins contains 25 members in C. elegans (Altun et al., 2009). The expression pattern of most innexins is stable throughout larval and adult stages under non-crowded, well-fed conditions. But displays a striking extent of plasticity of neuron-type-specific expression of innexin genes in response to environmental cues (Bhattacharya et al., 2019). According to the expression of inx genes, the gap junctions between ASH and ASK are heterotypic or heteromeric. The results of this study show that inx-4 expressed in ASH is required for passing cGMP through gap junctions between ASK and ASH neurons and normal behavioral adaptation. This result differs from Bai Laboratory’s that inx-19 is needed in both ASK and ASH for proper quinine sensitivity (Voelker et al., 2019). The difference may
reflect the differential configuration and, thus, function regulation between INX-19 and INX-4 proteins in sensations of varied stimuli and different signaling transduction pathways.

Cyclic guanosine monophosphate (cGMP) in ASH likely functions through EGL-4 signaling. EGL-4, a cGMP-dependent protein kinase and an ortholog of human PRKG1 (protein kinase cGMP-dependent 1), is an essential effector of cGMP in *C. elegans* (Daniels et al., 2000; Hirose et al., 2003). EGL-4 is involved in several processes. Such as regulating egg laying (Trent et al., 1983) and chemotaxis behavior (Daniels et al., 2000), promoting the turning behavior of *C. elegans* males during mating (Rahmani and Tuck, 2021), regulating growth size and lifespan (Fujivara et al., 2002, 2015; Hirose et al., 2003); regulation of signal transduction (Daniels et al., 2000); modulating integration of signals from olfactory neurons in AIL interneurons (Hino et al., 2021), regulating adaptation to prolonged odor exposure (Lee et al., 2010; O’Halloran et al., 2009), and regulating sensory adaptation to odor changes with different rates or adaptive concentration threshold for sensation in AWCON neurons, and animal navigation (Levy and Bargmann, 2020).

In the wet drop test, the cGMP-EGL-4 signaling down regulates *C. elegans* avoidance from quinine (Krzyzanowski et al., 2013; Voelker et al., 2019) and amodiaquine (Krzyzanowski et al., 2013), but not in that from copper ion (Krzyzanowski et al., 2013; Voelker et al., 2019; this study), primaquine and SDS (Krzyzanowski et al., 2013; Voelker et al., 2019). The mechanism underlying is that a G-protein coupled receptor senses quinine, and EGL-4 inhibits G protein and thus the signal transduction via phosphorylation of RGS-2 and RGS-3 (Krzyzanowski et al., 2013, 2016). Proper adaptation of nociceptive responses is necessary for organisms’ physiological homeostasis and survival. Of note, the cGMP-EGL-4 signaling displays different roles in varied signal transduction pathways. EGL-4, *C. elegans* homolog of protein kinase G, acts on a broad spectrum of effector proteins and plays multiple functions in development, sensations, behaviors, etc. Varied effector molecules may mediate the distinct roles of EGL-4 signaling.

EGL-4 upregulates animal adaptation of Cu²⁺ through nuclear signaling pathways or regulation of gene expressions because its nuclear translocation is essential for its roles. However, its target genes or molecules remain unknown. The fast-rising phase of ASH nociceptive response is required for the slow Cu²⁺ adaption. The genes encoding molecules needed for the ASH fast-rising, such as ion channels and their interactants, ion channel gating regulators, and even regulators of ion channel transportation or localization, may be targets of EGL-4. OSM-9 in ASK and ASH downregulates the Cu²⁺ adaption. So, its gene may be a target of EGL-4. The mechanisms of EGL-4 regulating sensory adaptation and the molecular basis for the fast kinetics of ASH sensory responses need further studies.

Interestingly, ASH and ASK sensory neurons and AIA interneurons form a positive feedback circuit. This positive feedback is necessary for normal kinetics, a fast-rising phase of the ASH ON nociceptive response, and inhibition of avoidance adaptation. However, this feedback is not required for animal avoidance from a drop of CuSO₄ solution in the wet drop test (Figure S1). Behavior adaptation can reasonably explain the behavioral inconsistency in these two test models. In the wet drop test, animals are tested once. Thus, behavioral adaptation does not display and not be examined. Whereas in the Cu²⁺ trap test, free-moving animals touch the dried Cu²⁺ bar multiple times before they finally escape the trap. The exit time correlates with the numbers of touching the Cu²⁺ bar positively and linearly. The Cu²⁺ touch numbers depend on the rate of increase of Cu²⁺ avoidance delay time. The delay time increases with the number of Cu²⁺ touches, from 2 s to about 7 s. There is a window or threshold of the delay time of about 6–7 s for animals to cross the Cu²⁺ bar and finally escape the trap. This delay time is determined by the slope rate of the rising phase of the ASH ON nociceptive response (Figure 6). On these facts, we conclude that the fast-rising phase of the ASH ON nociceptive response is required for a slow adaptation of nociception and avoidance behavior. The slow adaptation is significant for animals’ avoiding the noxious surroundings and thus the organism’s survival and wellbeing. Our previous reporters show that the kinetics of the sustained phase of ASH nociceptive ON response (Guo et al., 2015) and the OFF response (Wang et al., 2015) are essential for proper animals’ Cu²⁺ avoidance. Thus, in addition to the amplitude, the kinetics of nociception or other sensations of various modalities is necessary for sensory information and organism responses.

Limitations of the study

The results in the study support that ASK accelerates ASH Ca²⁺ responses to Cu²⁺ by sending cGMP to ASH via gap junctions containing INX-4. However, directly visualizing cGMP flow is needed to validate the conclusion.
STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105287.

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AUTHOR CONTRIBUTIONS
Z.X.W. supervised the study; JJ.W., S.W.Y., and H.L. performed a major part of the experiments, analyzed the data, and created the figures; R.L., J.H.H., J.L.Z., and P.P.W. performed the minor part of the experiments; J.J.W. and Z.X.W. wrote the article. All authors participated in discussions and data interpretation.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *Escherichia coli* OP50 | CGC | WB Cat# WBStrain00041969 |
| **Experimental models: Organisms/strains** | | |
| N2 | CGC | WB Cat# WBStrain00000001 |
| CX2065 odr-1(n1936) | CGC | WB Cat# WBStrain00005220 |
| CB1128 inx-4(e1128) | CGC | WB Cat# WBStrain00004251 |
| MT1074 egl-4(n479) | CGC | WB Cat# WBStrain00026780 |
| DA819 eat-4(ad819) | CGC | WB Cat# WBStrain00005518 |
| ZXW2079 odr-1(n1936); inx-4(e1128) | This study | N/A |
| ZXW2130 hkdEx2130[ora-6p::GCaMP3::unc-54; lin-44p::GFP]; odr-1(n1936) | This study | N/A |
| ZXW2140 hkdEx2140[ora-6p::GCaMP3::unc-54; lin-44p::GFP]; inx-4(e1128) | This study | N/A |
| **Oligonucleotides** | | |
| sra-9 promoter PCR forward primer 5'-GAAAAAGTT GCTCCAAGCTTACAAGGGGCCAGCTGCAA-3' | This study | N/A |
| sra-9 promoter PCR reverse primer 5'-AAGACTTG CACGACTATGGAATCTTGAANATGAAA-3' | This study | N/A |
| gpa-11 promoter PCR forward primer 5'-GAAAAAGTT GCTCCAAGCTTACGCCCAGCCAAAATA-3' | This study | N/A |
| gpa-11 promoter PCR reverse primer 5'-CAAACCTTGACGACTAGTCAATTGTGAGCTTCG-3' | This study | N/A |
| sra-6 promoter PCR forward primer 5'-GAAAAAGTT GCTCCAAGCTTACTGAGGCCGCCC-3' | This study | N/A |
| sra-6 promoter PCR reverse primer 5'-AAATCCTGACGACTAGTGGAGCAACCTTAAA-3' | This study | N/A |
| gcy-28(d) promoter PCR forward primer 5'-GAAAAA GTTGCTCCAAGCTTACCAATTGTAGGACGCTTCCG-3' | This study | N/A |
| gcy-28(d) promoter PCR reverse primer 5'-CAAACCTTGACGACTAGTGGGACACCTCACCA-3' | This study | N/A |

### RESOURCE AVAILABILITY

#### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zheng-Xing Wu (ibbwuzx@mail.hust.edu.cn).

#### Materials availability
All worm strains and plasmids used in this study are available to the scientific community. Please email the lead contact.

#### Data and code availability
All data reported in this article will be shared by the lead contact on request.

This article does not report original code.
Any additional information required to reanalyze the data reported in this article is available from the lead contact on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals of all C. elegans strains were cultured on nematode growth media (NGM) plates at 20°C using E. coli bacteria OP50 as food by standard procedures (Brenner, 1974). The strains were obtained from the CGC (http://www.cbs.umn.edu/CGC/) or the National Bio-Resources Project (http://www.shigen.nig.ac.jp/c. elegans/index.jsp). Double mutants were generated using standard genetic techniques (Brenner, 1974) and confirmed by PCR or sequencing. All transgenic animals were developed with standard microinjection techniques (Mello et al., 1991). Plasmids were injected at 50 ng/μL together with lin-44p::GFP as a coinjection marker at 10 ng/μL. All strains used in this study are listed in Excel-format Table S1. We used two or three lines of transgenic animals to conduct the experiments and summarized all data for statistical analysis.

METHOD DETAILS

Molecular biology

All expression constructs were generated with a Three-Fragment Multisite Gateway® system (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). Briefly, three entry clones comprising three PCR products (promoter, the gene of interest, sl2::TagRFP-t or 3’ UTR, in the name of slot1, slot2, and slot3, respectively) were recombined into the pDEST™ R4-R3 Vector II or custom-modified destination vectors using attL-attR (LR) recombination reactions to generate expression clones.

We constructed an “A” entry clone containing a sequence of the promoter used in this study by the In-Fusion method. In short, a modified attL4-attR1 entry clone was linearized by PCR. Then, the linearized product and a PCR product of promoter were used to generate the “A” entry clones using the ClonExpress™III OneStep Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). The PCR products of promoters were amplified from C. elegans genomic DNA with primers containing 15–20 bp sequences that carry some sequence of attL4 and attR1 recombination sites. Alternatively, vectors in the C. elegans Promoters Library (Thermo Fisher Scientific, Waltham, MA, USA) were directly used. The length of each promoter used in this study is as follows: odr-1p 3.1 kb, str-1p (in AWB) 4 kb, str-2p (in AWC and ASI) 3.7 kb, gpa-4p (in ASI) 2.5 kb, trx-1p (in ASJ) 1.1 kb, sra-9p (in ASK) 4 kb, srg-8p (in ASK) 2 kb, gpa-11p (in ASH and ADL) 3.3 kb, sra-6p (in ASH and ASI) 3.8 kb, gpa-14p 3.2 kb, gcy-28d (in AII) 2.8 kb, npr-9p (in AIB) 1.9 kb, and glc-3p 2 kb, respectively.

BP recombination reactions were used to generate entry clones B (containing a sequence of a tested gene) and C (containing unc-54 3’ UTR or sl2-GFP). The following genes were used to create the B entry clones. The odr-1, osm-6, npr-5, inx-4, and tax-4 genes were amplified from the genomic DNA of wild-type N2 worms. osm-9, eat-4, gcl-3, and egl-4 cDNA were amplified by reverse transcription-PCR (RT–PCR) from C. elegans mixed stage RNA. Their PCR products flanked by attB1 and attB2 were recombined with the pDONR-221 vector containing attP1 and attP2. To generate entry clone C, BP reaction sites attB2 and attB3 were inserted into the sl2-GFP or unc-54 3’ UTR and sequences and recombined with the PDONR-P2R-P3 vector. All primers for cloning these promoters and genes are listed in Table S2.

Behavioral assays

All behavioral experiments were performed using young adult animals maintained at 20°C.

Cu2+ trap tests

6 cm nematode growth medium (NGM) plates were used. CuSO4 was dissolved in M13 buffer consisting of (in mM) Tris 30, NaCl 100, and KCl 10 (pH 7.2, adjusted by 1 M HCl or 1 M NaOH). 10 μL of CuSO4 solution (10 mM) was applied to form an annular ring with an inner diameter of 10 mm and width of 2 mm on the center of the standard NGM plate (Culotti and Russell, 1978; Ghosh et al., 2016). Ten adult worms were transferred onto the center of the area encircled by the Cu2+ ring after being cleaned for 2 minutes in an NGM plate without food bacteria. Worm locomotion on the test plate was imaged at 1 frame (for most tests) or 10 (for assays of avoidance delay) frames per second for 25 minutes to 50 minutes until all worms left the ring under a Zeiss Discovery V8 stereo microscope (Carl Zeiss Microlmaging GmbH, Göttingen, Germany), using an Andor iXon™+ DV885K EMCCD camera (Andor Technology plc., Springvale Business
Park, Belfast, UK). The time of the animal exiting the Cu^{2+} trap (exit time) was manually calculated by the replay of the locomotion video.

**Wet drop test**

6 cm nematode growth medium (NGM) plates were used. The assay was performed as previously described (Hilliard et al., 2004; Krzyzanowski et al., 2016). About 20 young adults were transferred onto NGM plates without food, OP50 bacteria lawn. Three minutes later, the individual animal was stimulated by a droplet (approximately a few hundreds of nano-liters) of 1 mM or 10 mM CuSO_{4} solution (dissolved in M13) applied via a glass micropipette onto the tail of a forward-moving worm. Avoidance response to Cu^{2+} was scored as the percentage of animals that displayed reversal or U turn for more than one-half of body length within 4 seconds in all tested animals.

**Confocal fluorescence imaging**

Confocal fluorescence imaging was performed using an Andor (Andor Technology plc., Springvale Business Park, Belfast, UK) Revolution XD laser confocal microscope system based on a spinning-disk confocal scanning head CSU-X1 (Yokogawa Electric Corporation, Musashino-shi, Tokyo, Japan), under the control of the Andor IQ 1.91 software. The confocal system was constructed on an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan). The fluorescent images were imaged by a 60× objective lens (numerical aperture = 1.45, Olympus) and captured by an Andor iXonEM+ DU-897D EMCCD camera. The images were displayed and analyzed using Image J 1.43b software (Wayne Rasband, National Institutes of Health, USA).

**Calcium imaging**

Cytosolic calcium transients in the soma of tested neurons were measured by detecting changes in the fluorescence intensities of genetically encoded Ca^{2+} indicators G-CaMP3 or G-CaMP6s. The calcium indicators were 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 (ML5102, InBio Life Science Instrument Co. Ltd., Wuhan, China) and filtered with a Semrock FF01-520/35-25 emission filter (IDEX Health & Science, LLC, Oak Harbor, WA, USA). Fluorescence images were captured with an Andor iXonEM+ DU885K EMCCD camera with a 100-ms exposure time and 256 × 256 pixels at 10 frames per second under an Olympus IX-70 inverted microscope (Olympus, Tokyo, Japan) equipped with a 40× objective lens (numerical aperture (NA) = 1.3). A homemade PDMS microfluidic device was used to trap worms and deliver solutions (Guo et al., 2015; Liu et al., 2019; Wang et al., 2016; Wen et al., 2020).

We employed Day-1 adult worms for all calcium imaging and used them once only. Because C. elegans worms are sensitive to the blue light used for Ca^{2+} imaging, we exposed the tested animal to fluorescent excitation light for 2 min before recording to decrease the impact of light on Ca^{2+} fluorescence for all Ca^{2+} fluorescence imaging tests. The average fluorescence intensity of the region of interest (ROI) of the tested neuron soma was captured and analyzed using Image-ProPlus 6.0 (Media Cybernetics Inc., Rockville, MD, USA). A nearby region with an area similar to that of the tested soma was used to measure background signals. The percent change of average background-subtracted fluorescence intensity ΔF = (F - F₀)/F₀ was plotted as a function of time for all curves. The averaged fluorescence intensity within the initial 5 s (ASK and ASH) or 30 s (AIA) before stimulation was taken as the basal signal F₀.

**Quantification and Statistical Analysis**

Exit-time data are displayed as box plots, with each dot representing the data from each individual tested animal or each test. The Ca^{2+} signal data are expressed as heatmaps, box plots, or as the means ± SEM indicated by solid traces ± gray shading. Data were statistically analyzed using software packages in GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). When the comparison was limited to 2 groups, an unpaired t-test was used to analyze differences and calculate p values. When more than two groups of data were compared, data were analyzed by ordinary one-way or two-way ANOVA (analysis of variance), with recommended post hoc tests in the GraphPad Prism 8 software package. Dunnett’s multiple comparison correction was applied when multiple samples were compared to a single sample, i.e., wild-type N2 or other controls. Tukey’s multiple comparison correction was used when multiple samples were compared. The p value is indicated as follows: ns = not significant, *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001, and in different colors for varied comparisons.