




Serotonergic neuron ADF modulates avoidance behaviors by inhibiting sensory neurons in *C. elegans*

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Abstract

Serotonin plays an essential role in both the invertebrate and vertebrate nervous systems. ADF, an amphid neuron with dual ciliated sensory endings, is considered to be the only serotonergic sensory neuron in the hermaphroditic *Caenorhabditis elegans*. This neuron is known to be involved in a range of behaviors including pharyngeal pumping, dauer formation, sensory transduction, and memory. However, whether ADF neuron is directly activated by environmental cues and how it processes these information remains unknown. In this study, we found that ADF neuron responds reliably to noxious stimuli such as repulsive odors, copper, sodium dodecyl sulfonate (SDS), and mechanical perturbation. This response is mediated by cell-autonomous and non-cell autonomous mechanisms. Furthermore, we show that ADF can modulate avoidance behaviors by inhibiting ASH, an amphid neuron with single ciliated ending. This work greatly furthers our understanding of 5-HT's contributions to sensory information perception, processing, and the resulting behavioral responses.

Keywords Serotonergic neuron · Avoidance behavior · Sensory information perception · *Caenorhabditis elegans*

Introduction

In *Caenorhabditis elegans*, serotonin (5-HT) modulates a variety of behaviors including locomotion, egg laying, defecation, pharyngeal pumping, dauer formation, sensory transduction, and memory formation [8, 9, 12, 19, 20, 29]. 5-HT is also an essential component in mammalian nervous system function and has been implicated in several human diseases such as Parkinson's disease, schizophrenia, and depression [9]. In *C. elegans*, 5-HT is generated by eight types of serotonergic neurons including ADF, HSN, and RIH, of which ADF is the only serotonergic neuron presumed to play a sensory role in the hermaphroditic worms. ADF modulates neuronal activity postsynaptically [12, 15, 20,

33] and has been shown to respond to cAMP, copper, and sodium [4, 12]. However, it remains unknown whether this neuron can be directly activated by sensory cues or if it depends on the upstream activation of other sensory neurons.

Worms detect a variety of environmental cues mostly through the amphid, a polymodal sensory organ located in the tip of their nose that contains 12 pairs of sensory neurons [2, 18, 26]. Numerous studies have shown that the amphid sensory neurons ASH, AWC, AWB, and AWA are the major contributors to chemosensation [3, 5, 25]. ASH is the main primary sensor for chemical repellents and nose touch, and thereby mediates avoidance behaviors [13, 18]; 5-HT has been shown to modulate this ASH activity [12]. However, the mechanism through which ADF regulates ASH activity and the resulting behaviors remains largely unexplored.

Here, by combining in vivo calcium (Ca^{2+}) imaging, genetic manipulation, and behavioral assays, we established that ADF responds to a variety of noxious stimuli including Cu^{2+} , detergent, repulsive odors, and mechanical stimulation. We found that ADF can respond to noxious stimuli in a both cell-autonomous and non-cell autonomous fashion. Silencing ADF neuron significantly prolongs the duration of aversive stimulation-induced backward movement in *C. elegans*. However, activation of ADF delays the initiation of avoidance behavior in response to repulsive stimulation.

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Ca²⁺ imaging further revealed that ADF activation depresses aversive stimuli-induced ASH Ca²⁺ transients.

Results

ADF responds to a variety of noxious stimuli

Previous reports that Cu²⁺ elevates Ca²⁺ levels in the ADF neuron led us to wonder whether ADF can be activated by other environmental cues [12]. Toward assessing intracellular Ca²⁺ fluctuations, we expressed a genetically encoded Ca²⁺ indicator GCaMP5.0 exclusively in ADF under the control of the *srh-142* promoter [10, 29, 30]. First, we confirmed that Cu²⁺ induced a Ca²⁺ response in ADF. Consistent with previous studies [12], Ca²⁺ reliably increased in ADF when the worm's nose tip was exposed to 50 mM Cu²⁺ (Fig. 1a). This occurred with a latency of about 17 s (Fig. 1j), and took about 60 s to reach peak Ca²⁺ levels (Fig. 1k). Considering that Cu²⁺ is a repulsive factor for worms, we next checked whether ADF responds to other repulsive odors. *C. elegans* avoid high concentrations of odors that are attractive at low concentrations [28]. We recorded Ca²⁺ elevations in ADF upon exposure to repulsive concentrations of isoamyl alcohol (IAA, 1/100 in bath solution), benzaldehyde (BZ, 1/300), and 2,4,5-trimethylthiazol (TZ, 1/300) (Fig. 1b–d, i). Notably, an initial decay, namely the first phase, was observed when 1/100 IAA was applied (Fig. 1b, i). Interestingly, the latency (Fig. 1j) and the peak time (Fig. 1k) in response to these odors were much shorter than in response to Cu²⁺. The odorless detergent SDS (0.1%), a known repellent, also induced robust intracellular Ca²⁺ elevation in ADF (Fig. 1e, i) with a latency and peak time similar to that induced by Cu²⁺ (Fig. 1j–k). We then examined whether ADF is activated by mechanical stimulation, a response that helps worms find food sources and avoid adverse environments [11, 17]. Interestingly, Ca²⁺ levels in ADF dramatically increased with a short delay and fast peak time, after we delivered mechanical stimulation with a displacement of 10 μm to the dendritic endings of ADF on the nose tip (Fig. 1f, i). We further investigated whether ADF is susceptible to attractive cues as well. Surprisingly, ADF showed no detectable Ca²⁺ elevation in response to attractive concentrations of odors including 1/10000 IAA and 1/100 diacetyl [28] (Fig. 1g–i). These data suggest that ADF's role in chemosensation is the detection and avoidance of repelling, rather than attracting, cues.

ADF responds to noxious stimulation in both a cell-autonomous and non-cell autonomous fashion

It was hereto unknown whether ADF can be directly activated by sensory cues, or if its activity is dependent on upstream sensory neurons. The loss-of-function mutants *unc-13(e51)* and *unc-31(e928)* are traditionally used to exclude the contribution of other

neurons because *unc-13* is essential for synaptic vesicle (SV) exocytosis and *unc-31* is necessary for neuropeptide release from dense-core vesicles (DCVs) [1, 27]. The Cu²⁺-induced Ca²⁺ elevation in ADF was remarkably reduced in the *unc-13* mutants but not in the *unc-31* mutants, suggesting that ADF responds to Cu²⁺ non-cell autonomously (Fig. 2a, b). Interestingly, the 1/100 IAA-induced Ca²⁺ elevation (the second phase) was not impaired in either mutant, indicating that ADF may respond to IAA cell-autonomously (Fig. 2c, d). Of note, the IAA-induced Ca²⁺ decrease (the first phase) was abolished in the *unc-13* mutant worms, likely due to the neurotransmitter released from other neurons suppressing the activation of ADF. Taken together, these data suggest that ADF is capable of responding to noxious stimuli both cell-autonomously and non-cell autonomously.

Noxious stimulation-induced avoidance behaviors are modulated by ADF

After establishing that ADF neurons use dual mechanisms to respond to noxious stimulation, we next sought to characterize the physiological function of ADF in sensory processing. Worms initiate reversal behavior upon noxious stimulation; thus, we hypothesized that ADF may be involved in this behavior. Interestingly, the duration of reversals induced by both Cu²⁺ and IAA was largely extended when the light chain of tetanus toxin (TeTx), a protease of synaptobrevin that blocks transmitter release [21], was exclusively expressed in ADF (Fig. 3a, b). *tph-1* encodes tryptophan hydroxylase and is required for the biosynthesis of 5-HT [9]. The duration of reversals induced by both Cu²⁺ and IAA was also remarkably extended in the *tph-1* mutants, implicating 5-HT in the determination of reversal duration (Fig. 3a, b). These data suggest that ADF neurons prolong the duration of reversal when worms encounter noxious stimulation. However, there was no detectable difference in delay, i.e., the latency period prior to initiating reversal behavior, upon exposure to by either Cu²⁺ or IAA between the wild-type and *ADF::TeTx* transgenic animals (Fig. 3c, d).

Next, we investigated noxious stimulation-induced avoidance behaviors when ADF was pre-activated. We transgenically expressed the rat capsaicin receptor TRPV1, which can be reliably activated by exogenous capsaicin [23], in ADF. Unexpectedly, the duration of reversals induced by either Cu²⁺ or IAA was not affected when ADF neurons were preactivated for 10 min with 200 μM capsaicin in the *ADF::TRPV1* worms (Fig. 3a, b), but the reversal latency was remarkably extended; this shows that the activation of ADF neurons suppresses noxious stimulation-induced avoidance behavior (Fig. 3c, d). Consistent with previous reports, a loss-of-function mutation *cat-2*, which encodes tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines such as dopamine, caused prolonged reversal latency [9]. Together, these behavioral data indicate that both Cu²⁺- and IAA-induced avoidance behaviors are modulated by ADF.

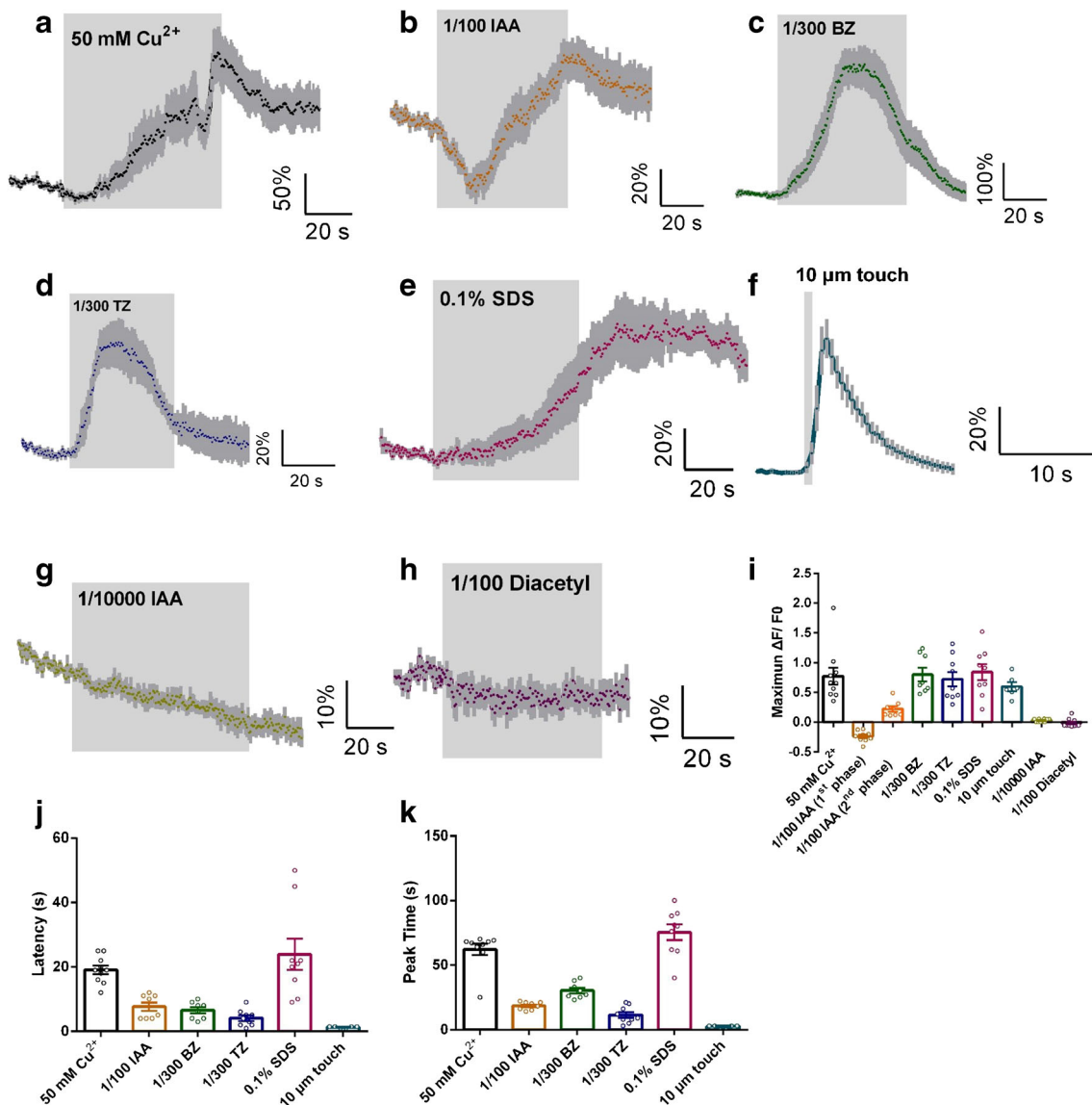


Fig. 1 ADF neuron specifically responds to noxious stimuli. **a–h** Shown are calcium transients in ADF evoked by noxious stimuli including 50 mM Cu²⁺ (**a**), 1/100 isomyl alcohol (IAA) (**b**), 1/300 benzaldehyde (BZ) (**c**), 1/300 2,4,5-trimethylthiazol (TZ) (**d**), 0.1% SDS (**e**), and mechanical stimulation with a displacement of 10 μm delivered to the dendritic endings of ADF on the nose tip (**f**). Notably,

ADF showed no detectable Ca²⁺ response in response to attractive concentrations of odors including 1/10000 IAA (**g**) and 1/100 diacetyl (**h**). Shading around traces indicates error bars (SEM). Gray regions indicate duration of stimulation. **i–k** Quantitative statistics of maximum ΔF/F₀ (**i**), latency (**j**), and peak time (**k**) for calcium responses

ADF attenuates noxious stimulation-induced Ca²⁺ elevation in ASH

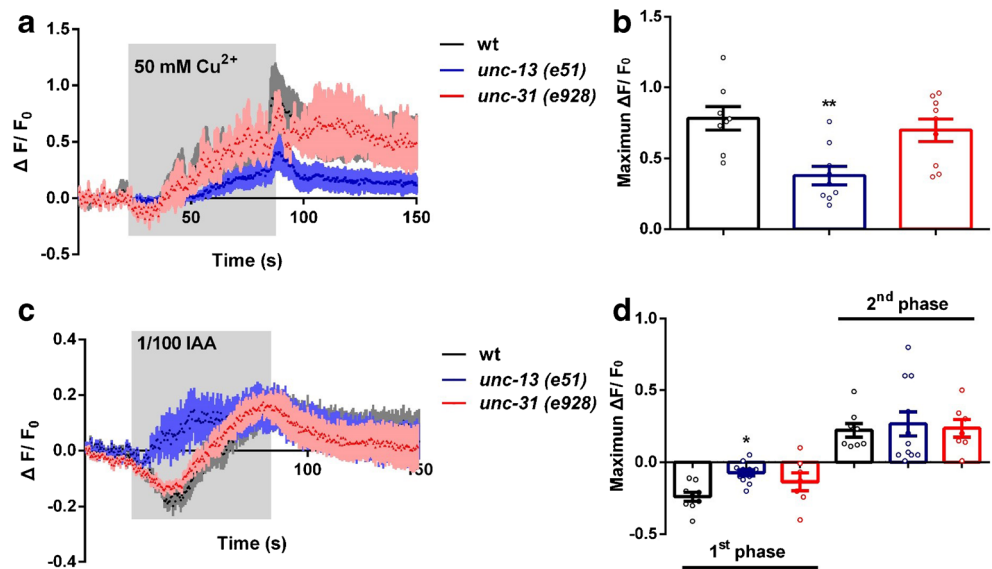
Next, we aimed to determine how ADF modulates noxious stimulation-induced avoidance behaviors. Since ASH is the main sensory neuron responsible for detecting a wide range of noxious stimuli [13, 28], we hypothesized that ADF affects avoidance behaviors by inhibiting ASH. Consistent with this hypothesis, pre-activation of ADF with 100 μM capsaicin in *ADF::TRPV1* worms dramatically diminished both Cu²⁺- and IAA-induced Ca²⁺ elevations in ASH (Fig. 4a–d). These data demonstrate that the activation of ADF inhibits the noxious

stimulation-induced Ca²⁺ responses in ASH and thereby results in prolonged delay of avoidance behaviors.

Discussion

Here, we report that the serotonergic ADF neuron responds to noxious stimulation in both cell-autonomous and non-cell autonomous fashions. Furthermore, the activation of ADF modulates noxious stimulation-induced avoidance behaviors through inhibiting ASH neuron.

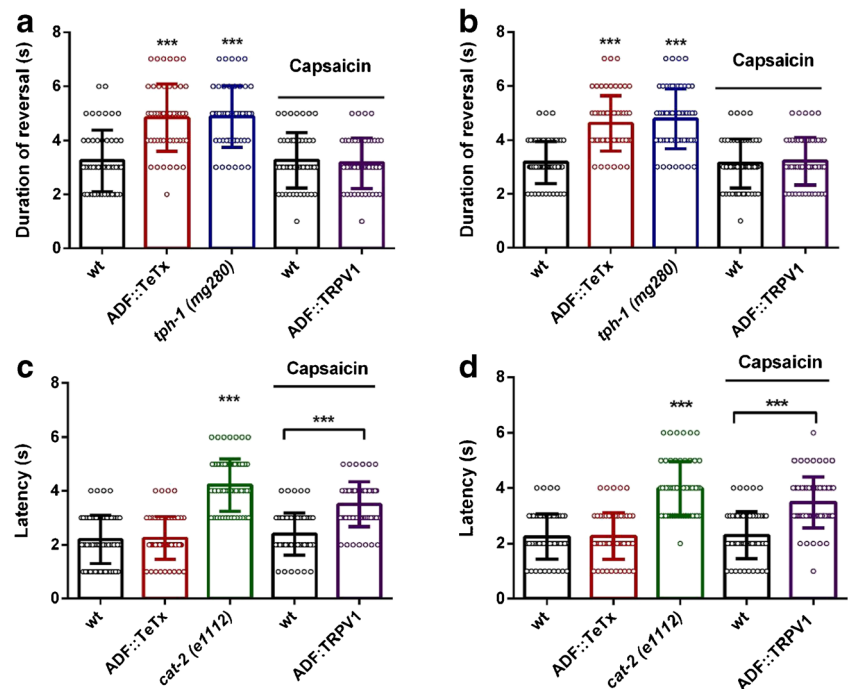
Fig. 2 ADF responds to noxious stimulation in both a cell-autonomous and non-cell autonomous fashion. **a, b** Fifty millimolar Cu^{2+} -induced calcium responses (**a**) and maximum $\Delta F/F_0$ (**b**) in ADF neuron in the wild-type worms ($n = 11$), *unc-13* ($n = 13$) mutants, and *unc-31* ($n = 7$) mutants. **c, d** 1/100 IAA-induced calcium responses (**c**) and maximum $\Delta F/F_0$ (**d**) in ADF in wild type ($n = 8$), *unc-13* ($n = 11$), and *unc-31* ($n = 9$) mutant worms. Shading around traces indicates error bars (SEM). * $p < 0.05$; ** $p < 0.01$



Avoiding adverse environmental conditions through the detection of noxious cues is essential for survival. ADF has been shown to promote hyperoxia avoidance [7, 22], modulate the chemosensory avoidance response to octanol [8], and couple environmental food signals with 5-HT neurotransmission [4]. In addition, the ciliated dendritic endings of ADF neuron are exposed to the external environment [26], which is highly suggestive and traditionally characteristic of a sensory chemoreceptor neuron. However, it

remains unknown whether this neuron directly detects chemicals or other sensory cues. In this study, we examined intracellular Ca^{2+} fluctuations within ADF upon exposure to a variety of chemical and mechanical stimuli. Our data empirically establish, for the first time, that ADF is only activated by noxious chemical stimuli and not by attractive odors, and highlight their involvement in avoidance responses in *C. elegans*. Notably, our data show that ADF responds to noxious stimulation both cell-

Fig. 3 ADF modulates noxious stimulation-induced avoidance behaviors. **a, b** The duration of reversals induced by both 50 mM Cu^{2+} (**a**) and 1/100 IAA (**b**) was largely extended when the light chain of tetanus toxin (TeTx), a protease of synaptobrevin that blocks transmitter release, was exclusively expressed in ADF. However, activation of ADF with transgenic TRPV1 by 200 μM capsaicin had no effect on duration of reversals. $n \geq 50$ for each genotype. **c, d** The latency of reversals induced by 50 mM Cu^{2+} (**c**) and 1/100 IAA (**d**) was remarkably extended when ADF was pre-activated with capsaicin in the *ADF::TRPV1* worms. However, TeTx expressed in ADF caused no effect. $n \geq 50$ for each genotype. *** $p < 0.001$



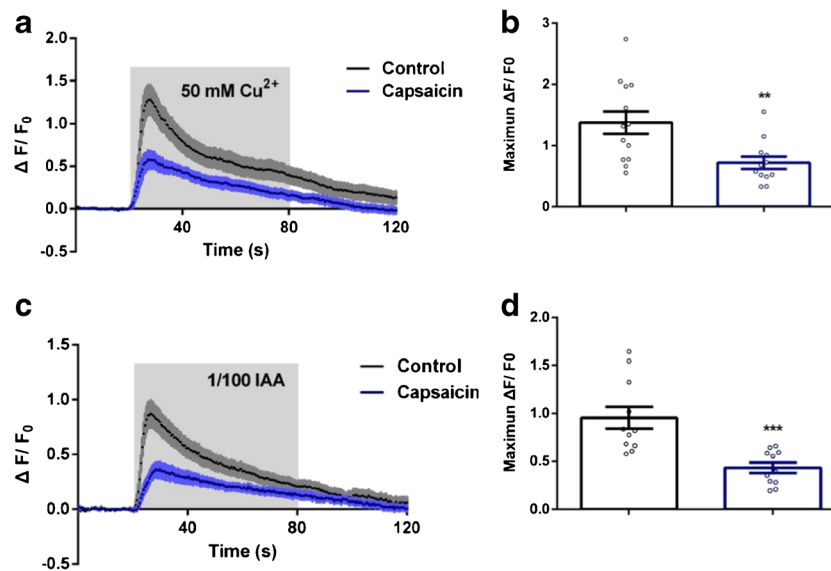


Fig. 4 ADF attenuates noxious stimulation-induced Ca^{2+} elevations in ASH. **a, b** Pre-activation of ADF with 100 μM capsaicin in *ADF::TRPV1* worms dramatically diminished 50 mM Cu^{2+} -induced Ca^{2+} elevations in ASH neuron. **a** Calcium responses. **b** Maximum $\Delta F/F_0$. $n = 13$ and $n = 12$ for wild-type and *ADF::TRPV1* transgenic animals, respectively. Shading around traces indicates error bars (SEM). **c, d** Pre-activation of

ADF with 100 μM capsaicin in *ADF::TRPV1* worms dramatically diminished 1/100 IAA-induced Ca^{2+} elevations in ASH neuron. **c** Calcium responses. **d** Maximum $\Delta F/F_0$. Shading around traces indicates error bars (SEM). $n = 11$ for each group. ** $p < 0.01$; *** $p < 0.001$

autonomously and non-cell autonomously, suggesting that ADF has dual roles as both a sensory receptor and a modulator of the avoidance response to environmental factors. Additionally, our results are the first to provide evidence that ADF also responds to nose touch, suggesting ADF's polymodal role in *C. elegans*' sensory perception and processing. Previous studies have detailed that ASH neurons' ability to respond to both chemical and mechanical stimulation [13] and a recent study showed that mouse olfactory sensory neurons (OSNs) can also detect multiple sensory modalities including mechano-sensation in addition to olfaction [16]. Our study contributes to a better understanding of the molecular and cellular mechanisms underlying sensory perception in organisms from *C. elegans* to mammals. It should be noted that the response of ADF to 1/100 IAA was partially suppressed by other neurons, though ADF may also detect this concentration of IAA cell-autonomously. This mechanistic duality likely equips *C. elegans* with more acute, adaptable, and robust responses to environmental challenges, thereby increasing their resilience and likely contributing to their biological success. The interplay between the neuronal subtypes is highly nuanced and requires additional examination. Further studies will further elucidate these complex interactions, both within cell types and environmental fluctuations, and reveal their physiological relevance in detecting aversive odorants. Furthermore, it will be interesting to explore the

conservation of these mechanisms in higher organisms, including humans.

5-HT is extensively involved in regulating *C. elegans*' behaviors [8, 9, 12, 29]. However, 5-HT's role in modulating behavior is largely established through analyzing of mutants for the genes encoding the serotonin biosynthetic enzymes such as *bas-1* and *tph-1*, or through behavioral studies of wild-type animals treated with drugs altering 5-HT signaling [9]. Here, we showed that directly blocking 5-HT release from ADF by TeTx promoted noxious stimulation-induced backward movement, which may be mediated by a prolonged Ca^{2+} response in ASH [12], whereas activation of ADF inhibited this reversal behavior, which is correlated with diminished ASH Ca^{2+} elevation responding to noxious stimuli (Fig. 4). It is likely that the Ca^{2+} elevation in ADF induced by aversive environmental stimulation triggers 5-HT release, which in turn inhibits the Ca^{2+} responses in ASH and, thereby, facilitates sensory adaptation.

In summary, our study provides evidence of a serotonergic neuron responding to aversive environmental cues. Furthermore, we directly correlated the cellular activity of this serotonergic neuron with a defined behavioral sensory output in an in vivo setting. This work lays the foundation for further understanding the many roles of serotonergic neurons in sensory information perception, processing, and integration.

Methods

Strains and media

C. elegans strains were maintained under standard conditions [6]. Strains used in this study were listed below.

ST2325	<i>kanEx709[Psrh-142::GCaMP5.0 + Psrh-142::mcherry + Plin-44::gfp]</i>
ST2340	<i>unc-13(e51);kanEx709[Psrh-142::GCaMP5.0 + Psrh-142::mcherry + Plin-44::gfp]</i>
ST2341	<i>unc-31(e928);kanEx709[Psrh-142::GCaMP5.0 + Psrh-142::mcherry + Plin-44::gfp]</i>
ST2353	<i>kanEx733[Psrh-142::TeTx::sl2::tagRFP + Plin-44::gfp]</i>
ST2476	<i>kanEx790[Psrh-142::TRPV1::sl2::tagRFP + Psra-6::GCaMP5.0 + Plin-44::gfp]</i>
ST1837	<i>tph-1(mg280)</i>
ST930	<i>cat-2(e1112)</i>

To prepare capsaicin-containing NGM plates, capsaicin (Sigma-Aldrich histamine-dihydrochloride) was added to sterilized NGM to a final concentration of 200 μ M right before pouring plates.

Calcium imaging

For in vivo calcium imaging, individual day 2 (D2) adult hermaphroditic worms were glued with a medical grade cyanoacrylate-based glue (Glutire Topical Tissue Adhesive, Abbott Laboratories) to cover glasses in extracellular saline (145 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM D-glucose, and 10 mM HEPES, 325–335 mOsm, PH adjusted to 7.3 with NaOH) as previously described [29, 31, 32]. Fluorescent images were acquired using an Olympus microscope (IX71) under a $\times 40$ objective lens coupled with an Andor DL-604M EMCCD camera. Data were collected using the Micro-manager software (developed in Ron Vale's laboratory at UCSF based on ImageJ). Nose-touch stimulus was delivered using a tip diameter ~ 5 μ m borosilicate glass capillary driven by a piezoelectric actuator (PI) triggered by SD9 stimulator [30]. GCaMP5.0 was excited by a ThorLabs blue light (460–480 nm) LED lamp, and the fluorescent signals were collected at the rate of 2 Hz. The average GCaMP5 signal from the first 3 s before stimulus was taken as F_0 , and $\Delta F/F_0$ was calculated for each data point. The data was analyzed using the ImageJ.

Behavioral assays

Behavioral assays were performed with day 1 (D1) adult hermaphroditic worms at room temperature and undertaken blind to the genotypes. Assay plates were freshly seeded. Wild-type

and transgenic worms were allowed to crawl around on capsaicin-contained NGM plates for 10 min for effective activation of ADF and then transferred to assay plates. For copper stimulation, a dry drop test protocol was adopted [14]. Briefly, copper drop was delivered about 0.1 mm anterior to the animal during forward locomotion. The time from initiating head reversal to ceased tail backward movement was defined as duration. The initiation time was calculated as the time from worm encountered copper solution to initiate head reversal. IAA stimulation was assessed by the “smell-on-a-stick” assay as previously described [24]. We used a micropipette, of which the tip was dipped in pure IAA, to deliver IAA in front of the animal during forward locomotion. At least 30 animals per strain were tested in each trail, and each experiment was repeated for at least three times on independent days.

Statistical analysis

Data analysis was performed using GraphPad Prism 5. All data were presented in mean \pm SEM. Unpaired two-tailed *t* test was used for data comparison. If the data do not meet the normal distribution, the Wilcoxon test was used. $p < 0.05$ was considered as statistically significant.

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Author contribution Jiajie Shao and Lijun Kang designed the experiments. Jiajie Shao, Xiaoyan Zhang, Hankui Cheng, Xiaomin Yue, and Wenjuan Zou conducted the experiments. Jiajie Shao, Wenjuan Zou, and Lijun Kang analyzed and interpreted the results. Jiajie Shao and Lijun Kang wrote the manuscript, and modification was provided by all the authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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