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In vivo calcium imaging of OFF-responding ASK chemosensory neurons in *C. elegans*

Tokumitsu Wakabayashi,[1] Yukihiro Kimura,[1] Yusuke Ohba,[1] Ryota Adachi,[1] Yoh-ichi Satoh,[2] and Ryuzo Shingai[1*]

[1], Laboratory of Bioscience, Faculty of Engineering, Iwate University, 4-3-5 Ueda, Morioka, Iwate 020-8551, Japan.

[2], Department of Cell Biology and Functional Morphology, Iwate Medical University, 19-1 Uchimaru, Morioka, Iwate 020-8505, Japan.

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*Corresponding author. Laboratory of Bioscience, Faculty of Engineering, Iwate University, 4-3-5 Ueda, Morioka, Iwate 020-8551, Japan. Tel/Fax: +81-19-621-6479.

E-mail: shingai@iwate-u.ac.jp (R. Shingai)

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Abstract

BACKGROUND How neurons and neuronal circuits transform sensory input into behavior is not well understood. Because of its well-described, simple nervous system, *Caenorhabditis elegans* is an ideal model organism to study this issue. Transformation of sensory signals into neural activity is a crucial first step in the sensory-motor transformation pathway in an animal's nervous system. We examined the properties of chemosensory ASK neurons of *C. elegans* during sensory stimulation.

METHOD A genetically encoded calcium sensor protein, G-CaMP, was expressed in ASK neurons of *C. elegans*, and the intracellular calcium dynamics of the neurons were observed.

RESULTS After application of the attractants L-lysine or food-related stimuli, the level of calcium in ASK neurons decreased. In contrast, responses increased upon stimulus removal. Opposite responses were observed after application and removal of a repellent.

CONCLUSION The observed changes in response to external stimuli suggest that the activity of ASK neurons may impact stimulus-evoked worm behavior. The stimulus-ON/activity-OFF properties of ASK neurons are similar to those of vertebrate retinal photoreceptors.

GENERAL SIGNIFICANCE Analysis of sensory-motor transformation pathways based on the activity and structure of neuronal circuits is an important goal in neurobiology and is practical in *C. elegans*. Our study provides insights into the mechanism of such transformation in the animal.

Keywords: *Caenorhabditis elegans*; G-CaMP; calcium; imaging; sensory neuron; OFF-response

1. Introduction

Neural circuits transform sensory input into behavior. During this process, sensory receptor cells convert stimuli in the environment into neuronal activity. Either via ionotropic or metabotropic pathways, sensory receptor cells depolarize (e.g. olfactory neurons, taste cells, nociceptors etc.) or hyperpolarize (e.g. photoreceptors) according to the sensory stimuli. The nematode *Caenorhabditis elegans* has at least 12 chemosensory and/or thermosensory neurons in their head region [1-4]. Understanding the physiological properties of these sensory receptor cells is an important step toward the understanding of the sensory-motor transformation pathway in this simple animal.

The *C. elegans* genome has no voltage-gated sodium channel [5, 6], yet *C. elegans* neurons have rapidly activating voltage-gated calcium channel. Thus, calcium transients in worm neurons are thought to correlate with neuronal activities [7, 8]. The genetically encoded calcium probe proteins, cameleon and G-CaMP have been used for *in vivo* calcium imaging of worm sensory neurons. In chemosensory neurons called ASH and ASEL, intracellular calcium transiently increased upon presentation of sensory stimuli (stimulus-ON / neural activity-ON) [9-11]. Thermosensory AFD neurons respond to both upstep and downstep of ambient temperature by increasing and decreasing intracellular calcium, respectively [12, 13]. More recently, olfactory AWC neurons and chemosensory ASER neurons show a transient increase of intracellular calcium upon removal of the sensory stimuli (stimulus-OFF / activity-ON) [11, 14, 15]. These neurons also show a stimulus-evoked decrease of calcium (stimulus-ON / activity-OFF), that resembled vertebrate photoreceptor cells [11, 15]. Analysis of a small subset of the *C. elegans* sensory neurons revealed a variety of physiological responses to sensory stimuli. Therefore, it is possible that other sensory neurons in the worm have different physiological properties yet undescribed.

Chemosensory ASK neurons of *C. elegans* are required for the chemotaxis toward L-lysine [1] and food-dependent stimulation of egg-laying [16], suggesting a role for these neurons in food detection.

Well-fed young adult *C. elegans* that have recently been removed from food sources exhibit frequent backward movements for 10 to 15min. The area explored within this period is restricted because of short forward movements and random reorientation (area-restricted search, pivoting, or local search behavior) [17-20]. Cell ablation studies demonstrated that loss of ASK neurons caused a decrease of backward movements during this period, suggesting that ASK neurons regulate a backward movement [19, 20]. In this study, we observed intracellular calcium transients in ASK neurons in response to L-lysine or some other stimulus. ASK neurons showed OFF-responding properties similar to those reported for AWC and ASER neurons. The ASK neurons also showed opposite response to repulsive sensory cue.

2. Materials and Method

2.1 Strain and culture.

Wild-type animals were *Caenorhabditis elegans var.* Bristol, strain N2. Worms were grown under standard conditions at 20˚C [21].

2.2 Molecular biology.

Details of plasmid constructions are available upon request. Promoters, coding sequences, 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). *srd-23p* and *srg-2p* promoters were used for ASK-selective expression of G-CaMP and DsRed, respectively [22, 23]. G-CaMP cDNA was kindly provided by Dr. Junichi Nakai [24].

2.3 Germline transformation.

A mixture of *srd-23p::G-CaMP* (45 ng / µl), *srg-2p::DsRed* (45 ng / µl) and transgenic marker *F28A12.1p::GFP* (10 ng / µl, expressed in a part of intestine; T.W. unpublished) plasmids was introduced into wild-type gonads by microinjection. Two independent transgenic lines for *Ex [srd-23p::G-CaMP, srg-2::DsRed, F28A12.1p::GFP]* were obtained, and the consistent expression of G-CaMP and DsRed in ASK neuron pair was confirmed. One of the resulting transgenic lines was used for in vivo imaging.

2.4 Buffer and worm preparations.

In the imaging experiments, we used a polydimethylsiloxane (PDMS) microfluidic device known as an olfactory chip [25]. Single, well-fed transgenic worm expressing G-CaMP and DsRed in ASK neurons was injected into the worm inlet of the chip and restrained. Stimuli were dissolved into the imaging buffer (5 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM D-glucose, 25 mM sucrose, 20 mM KH_2PO_4-KOH pH6.0) [9] and were delivered via regulation of gatings of buffer inlet channels [25]. With this chamber system, dissolved stimuli could be delivered or removed from the tip of worm's nose within a second. For the bacterial-conditioned media experiments, the medium was prepared by suspending *E. coli* strain OP50 in imaging buffer. This suspension was incubated at room temperature for 12 hrs, centrifuged, and the supernatant was recovered. In SDS (sodium dodecyl sulfate, 0.1 %) application experiments, SDS buffer (20 mM D-glucose, 25 mM sucrose, 10 mM Tris-HCl pH 7.5) was used instead of imaging buffer.

119*2.5 Calcium imaging*

120

121 Optical recording and analysis were performed with LSM510 meta / ConfoCor2 system (ZEISS) 122under a 40X / 0.75 objective. Images were taken every 2s for 5-8min. The region of interest (ROI) 123was selected manually in the image stack, and fluorescence intensities of G-CaMP and DsRed were 124analyzed with LSM-FCS software. To minimize the effect of motion artifact, the simultaneously- 125obtained fluorescence intensities of G-CaMP and DsRed in each image were calculated into a ratio. 126The average of the 30s prestimulus baseline ratio was set as R_0. The percent change in ratio relative 127to R_0 were calculated and plotted for all image stacks $[(R_t-R_0)/ R_0$, where R_t means a ratio at the time 128t].

129

130*2.6 Behavioral analysis.*

131

132 Chemotaxis behavior in wild-type and transgenic worms toward 2 M L-lysine (pH 6.0 adjusted with 133 acetic acid) were examined as described [26], except that worms were placed at the 134 center of the assay plate, 1.5 cm apart from the point source of the chemoattractant.

135

136**3. Results**

137

138*3.1 Intracellular calcium in ASK neurons was decreased in response to L-lysine addition.*

139

140 Cell ablation of the ASK chemosensory neurons led to less frequent spontaneous backward 141movements, which suggests that the activation of ASK neurons stimulate backward movements [19, 14220]. In wild-type worms, frequent backward movements occur immediately after removal from a 143bacterial food, suggesting that the stimulation of backward movements is taken place upon removal 144of sensory stimuli. Therefore, we hypothesized that ASK neurons are activated in the absence of the

145sensory stimuli, and are suppressed in the presence of sensory stimuli. To examine whether it is the
146case, we constructed a transgenic strain expressing G-CaMP and DsRed in ASK neurons (*Ex [srd-*
147*23p::G-CaMP, srg-2p::DsRed]*, Fig. 1A). G-CaMP is a genetically encoded calcium sensor protein
148which elevates its fluorescence intensity upon binding calcium [24]. Bilateral ASK neurons were
149involved in chemotaxis behavior toward L-lysine [1]. Since the transgenic worm showed normal
150chemotaxis toward L-lysine, expression of these fluorescent proteins in ASK neurons did not disrupt
151the function of the neuron (Fig. 1B).

152 We diluted L-lysine in saline buffer and used it as a chemosensory stimulus. After a 10 min
153prestimulus application of saline buffer without the amino acid, the stimulus was delivered to the
154transgenic worm. Upon exposure to 30 mM L-lysine, we observed reliable decreases in fluorescence
155intensity of G-CaMP and the ratio of G-CaMP to DsRed fluorescence (Fig. 2A, C, E, Supplementary
156Fig. S1A), which was not observed in the buffer exchange control experiments (Fig. 2B, D, E,
157Supplementary Fig. S1C), demonstrating the OFF-response of the ASK neuron (stimulus-ON,
158activity-OFF). The fluorescence ratio was decreased for 10 s and persisted at a low level during the
159stimulation (Fig. 2A, C and E). Previous studies of OFF-responding chemosensory neurons in *C.*
160*elegans* have shown that sustained stimulation causes a sustained calcium decrease [11, 15]. The
161persistent, non-recovering (or non-adapting) property observed in the ASK neurons might be similar
162to these OFF-responding sensory neurons. These results also suggest that the intracellular calcium
163level in the absence of sensory stimulus is above the lowest level that ASK neurons can achieve,
164similar to that shown in AFD, AWC and ASER neurons [11, 13, 15].

165 Two types of OFF-responding sensory neurons in *C. elegans*, chemosensory AWC and ASER
166neurons, exhibit a remarkable increase in the calcium transient upon removal of their specific
167stimuli, and decrease upon addition of the same stimuli [11, 15]. We next examined the effect of L-
168lysine removal on ASK neuron activity (Fig. 3A, C, Supplementary Fig. S1B). After 10 min pre-

169exposure to 30 mM L-lysine, the stimulus was removed from the imaging chamber. The intracellular
170calcium level in ASK neurons was transiently increased. The highest ratio occurred about 10 s after
171stimulus removal, and decreased for 90 s thereafter (Fig. 3A, C). In many cases, gradual increases of
172fluorescence in oscillating intensities were observed after the first immediate response (Fig 3A).
173Precise oscillation dynamics were not observable with the low image acquisition rate used in our
174experiments. However, these variable fluorescence intensities and ratio values were a consistently
175observed in ASK neurons after stimulus removal. The onset of oscillation was apparently random,
176and begun between one and four (or more) minutes after stimulus removal (Fig. 3A, Supplementary
177Fig. S1B, E). Consistent with this notion, the fluorescence ratios in the ASK neurons immediately
178before stimulus addition (Fig. 2C, Supplementary Fig S1A, first 60s of the traces) were more
179variable than they were in the sustained presence of a stimulus. The fluorescence intensity of the
180ASK neurons were unaffected by the buffer exchange control experiments (Fig. 3B, C,
181Supplementary Fig. S1D). Therefore, chemosensory ASK neurons in *C. elegans* show OFF-
182responses to stimuli, 1) with a sustained decrease in fluorescence ratio upon L-lysine addition, and 2)
183a transient increase followed by a gradual oscillating increase in fluorescence ratio upon stimulus
184removal. The response of ASK neurons was similar to AWC and ASER neurons in their ON/OFF
185properties, but slightly different in their pattern of intensity, especially after prolonged stimulus
186removal.
187
188*3.2 ASK neurons were activated by the second stimulus removal after brief stimulus delivery and*
189*inactivated by the second stimulus addition after brief stimulus removal.*
190
191 ASK neurons were activated by stimulus removal and inactivated by stimulus addition. To further
192observe these opposing forces, we re-applied the stimulus 15 s after the initial removal. The

193 activation response of ASK neurons was suppressed by the second stimulus addition (Fig. 4A,
194 Supplementary Fig. S2B). Furthermore, the inactivation response was suppressed by the second
195 stimulus removal (Fig. 4B, Supplementary Fig. S2A). After the second stimulus removal following a
196 brief stimulus addition, the fluorescence ratio was increased above the pre-stimulus level. ASK
197 neuron responses to brief stimulus additions were dose-dependent, and increased in the 10 to 100
198 mM range (Fig. 4C, Supplementary Fig. S2C).

199

200 *3.3 Responses of ASK neurons to bacterial-conditioned medium.*

201

202 The ASK neurons of *C. elegans* are necessary for the detection of L-lysine, a chemosensory cue
203 related to the environmental food source [1], and for the food-dependent stimulation of egg-laying
204 [16]. Therefore, we examined the responses of ASK neurons to food-derived stimulus. Consistent
205 with their role in food detection, ASK neurons showed responses to bacterial-conditioned medium
206 that were similar to responses following L-lysine addition and removal (Fig. 5 A, B, Supplementary
207 Fig. S3A, B). ASK neurons might respond similarly to the attractive sensory cues. .

208

209 *3.4 Responses of ASK neurons to SDS, a repulsive sensory stimulus.*

210

211 Previous work has shown that ASK neurons are also involved in avoidance behavior, prompted by
212 repulsive sensory cues, such as SDS [27]. It became of particular interest to see how the neurons
213 respond to the chemosensory cues causing opposite behavioral outputs (*i. e.* avoidance instead of
214 attraction). In contrast to L-lysine responses, ASK neurons were activated by SDS addition and
215 inactivated by SDS removal (Fig. 6). The responses of ASK neurons to SDS were slower than those
216 to L-lysine, with a 4-6 s delay (see also Supplementary Fig. S4A, B). One possible explanation for

this result is that ASK neurons are not primary sensory neurons for SDS, so they are not respond quickly. Possibly, ASK response was evoked indirectly by the other neurons which have neuronal connections with ASK. Alternatively, ASK neurons may have sensory receptor molecule for SDS having slow activation kinetics. Overall, this result suggests that ASK neurons contribute to avoidance behavior prompted by SDS, either directly or indirectly, by responding oppositely upon addition and removal of the repellent compare to the case for chemoattractants.

4. Discussion

Transformation of the chemical (*e.g.* odor, taste) and physical (*e.g.* temperature, light *etc*.) sensory information into neural activity is a crucial first step of the sensory-motor transformation pathway in the animal's nervous system. In this study, we analyzed the intracellular calcium dynamics in *C. elegans* chemosensory ASK neurons with a genetically encoded calcium probe protein, and found that these neurons are more active in the absence of chemosensory stimuli than they are in the presence of such stimuli. The OFF-response (*i.e.* stimulus-ON / activity-OFF and stimulus-OFF / activity-ON) of ASK neurons resembled responses in vertebrate photoreceptor cells, which hyperpolarize upon illumination. Vertebrate photoreceptor cells control their activity by regulating cytoplasmic cGMP levels as well as the target molecules of cGMP [28]. Worm ASK neurons express genes required for these processes, including guanylate cyclase (*odr-1*, *daf-11*) [29, 30], heterotrimeric G-proteins (*gpa-2*, *gpa-3*, *gpa-14*, *gpa-15*) [31], cyclic nucleotide-gated channel (*tax-2*, *tax-4*) [32, 33], and cGMP-dependent protein kinase (*egl-4*) [34]. The presence of this analogous repertoire of molecules in *C. elegans* suggests that the OFF-response of ASK neurons may be mediated by cGMP.

Our imaging study revealed several remarkable features of the ASK neuron response; a long-lasting inhibitory response upon stimulus, and a transient activation followed by gradual increase of

241 the activity upon removal of the stimulus. Previous works have shown that backward movements are
242 enhanced immediately after worms are removed from food and these backward movements are
243 greatly reduced by ASK ablation [17-20]. How can these calcium dynamics in ASK neurons relate to
244 regulation of the backward movements in this period? The present study suggests that ASK neurons
245 stay in a state of low activity during the presence of food stimuli, yet upon removal from food, these
246 neurons show an immediate increase of activity. Therefore, activation in ASK neurons may be
247 responsible for the facilitation of frequent backward movements during the local search. In the
248 sustained absence of food for about 10 to15 min, ASK neuron activity may increase gradually with
249 the fluctuation, and during this gradual activation period, backward movements are kept at a high
250 frequency. After prolonged starvation, when ASK activity is saturated, ASK neurons may acquire an
251 alternative high activity state. During this state, backward movements are no longer enhanced
252 (traveling or long range dispersal) [19, 20], possibly because there are no activity dynamics or the
253 adaptation of downstream pathway to sustained ASK activity. Thus, ASK neurons may have multiple
254 basal states reminiscent of dark-adapted or light-adapted states of retinal photoreceptor cells [28].
255 *C. elegans* is capable of detecting millimolar or submillimolar difference in L-lysine concentration
256 in a gradient [26], and can reorient themselves toward an attractant within several seconds. The
257 observed stimulus-ON/activity-OFF and stimulus-OFF/activity-ON responses in ASK neurons allow
258 worms to detect both increases and decreases in chemoattractant levels. As ASK neurons have a
259 function promoting backward movements [19, 20], suppression of these neurons in the presence of
260 chemoattractant is logically linked to behavioral strategies of the worm to reach the point source of
261 chemoattractant. Similarly, activation (suppression) of the neurons upon addition (removal) of
262 repellent is also logically linked to avoidance behavior. Thus, ASK neurons may serve as
263 bidirectional sensory detectors that transform bidirectional changes in environmental sensory cues
264 into backward movements.

265

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395

396**Figure legends**

397

398**Figure 1.** A transgenic strain expressing G-CaMP and DsRed in ASK neurons. (A) Expression of G-
399CaMP and DsRed in ASK neurons (arrow head) of *Ex [srd-2p::G-CaMP; srg-2p::DsRed]* transgenic
400worm. G-CaMP was also expressed in AWB but DsRed was not (arrow). Asterisk indicates the
401expression of transgenic marker gene at the most anterior position of the intestine. (B) Chemotaxis
402behavior of wild-type (W.T.) and the transgenic worm (*Ex*) toward 2M L-lysine. A layout of the assay
403plate is indicated at left. 2M L-lysine was spotted at the center of region A. Chemotaxis index at
40490min was calculated by $(N_A-N_B)/N_{total,}$ where N_A, N_B and N_{total} indicate the number of worms in
405region A, B and the total number of worms on the assay plate. Worms harboring extrachromosomal
406array were selected under fluorescence microscope and assayed by placing at the center of the assay
407plate (six assays, about 30 worms/assay). The difference between the wild-type and the transgenic
408worm was examined by Students *t*-test. N.S. not significant.

409

410**Figure 2.** Responses of ASK neurons upon stimulus addition. In this and subsequent figures,
411stimulus conditions are indicated by horizontal bars at the top or bottom of each panel; light bar
412indicates saline only, and a dark bar indicates the addition of 30 mM L-lysine in saline. (A)(B)
413Changes in G-CaMP and DsRed fluorescence are shown alongside the ratio of these two
414fluorescence signals. Scale bars represent fluorescence magnitude in the vertical axis (% change),
415and time in the horizontal axis (s). (A) Representative fluorescence responses in ASK neurons after
416addition of L-lysine. The time interval of only saline at the tip of worm's nose is followed by the
417addition of 30 mM L-lysine. (B) Representative fluorescence responses in ASK neurons during
418buffer exchange control experiments. The same procedures were followed as in (A), but saline was
419continued in the second interval. (C) Three example traces of fluorescence ratio change in ASK

420neurons during 30 mM L-lysine addition. Stimulation procedure is the same as in (A). (D) Three
421example traces of fluorescence ratio change in ASK neurons during buffer exchange control
422experiments. Stimulation procedure is the same as in (B). (E) Average fluorescence ratio changes
423observed under two conditions. Bottom trace, before and after addition of 30 mM L-lysine. Top trace,
424the equivalent time period in only saline, the buffer exchange control experiments (12 recordings in
4256 worms for each condition). Gray shadings indicate S.E.M. in this and subsequent figures.
426
427**Figure 3.** Responses of ASK neurons upon stimulus removal. (A) Four representative traces of ASK
428neuron responses to the removal of 30 mM L-lysine. (B) Three representative traces of ASK neuron
429responses during buffer exchange control experiments. (C) Average fluorescence ratio change under
430the two experimental conditions. Top trace, after removal of 30 mM L-lysine (14 recordings from 8
431worms). Bottom trace, buffer exchange control experiments (9 recordings from 7 worms).
432
433**Figure 4.** Responses of ASK neurons upon brief stimulus addition or removal. (A) Average
434fluorescence ratio change upon brief (15 s) removal of 30 mM L-lysine (7 recordings from 4 worms).
435(B) Average fluorescence ratio change upon brief (15 s) addition of 30 mM L-lysine (10 recordings
436from 4 worms). (C) Dose-dependence of ASK neuron responses upon brief (15 s) addition of 10 -
437100 mM L-lysine. Lines and shadings are as in (A), (15-17 recordings from 8-10 worms). The L-
438lysine concentrations (mM) are indicated above the stimulus bars.
439
440**Figure. 5** Responses of ASK neurons upon addition or removal of bacterial-conditioned medium. In
441these experiments, bacterial-conditioned medium (dark bar) was followed by saline (light bar), or
442vice versa. (A) Average fluorescence ratio change upon removal of bacterial-conditioned medium (9
443recordings from 4 worms). (B) Average fluorescence ratio change upon addition of bacterial-

444conditioned medium (9 recordings from 6 worms).

445

446**Figure 6.** Responses of ASK neurons upon addition and removal of 0.1 % SDS. After a period of

447exposure to buffer (light bar), 0.1 % SDS was delivered (dark bar). (A) Average fluorescence ratio

448change upon addition of 0.1% SDS, followed by removal (9 recordings from 4 worms). (B) Average

449fluorescence ratio change during buffer exchange control experiments (9 recordings from 6 worms).

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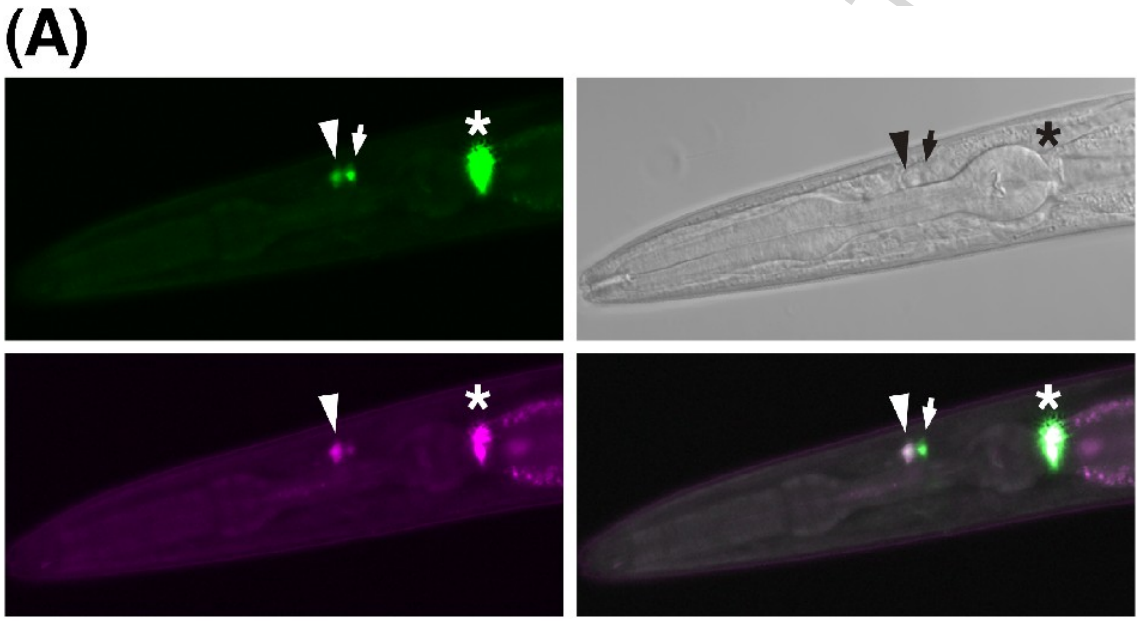

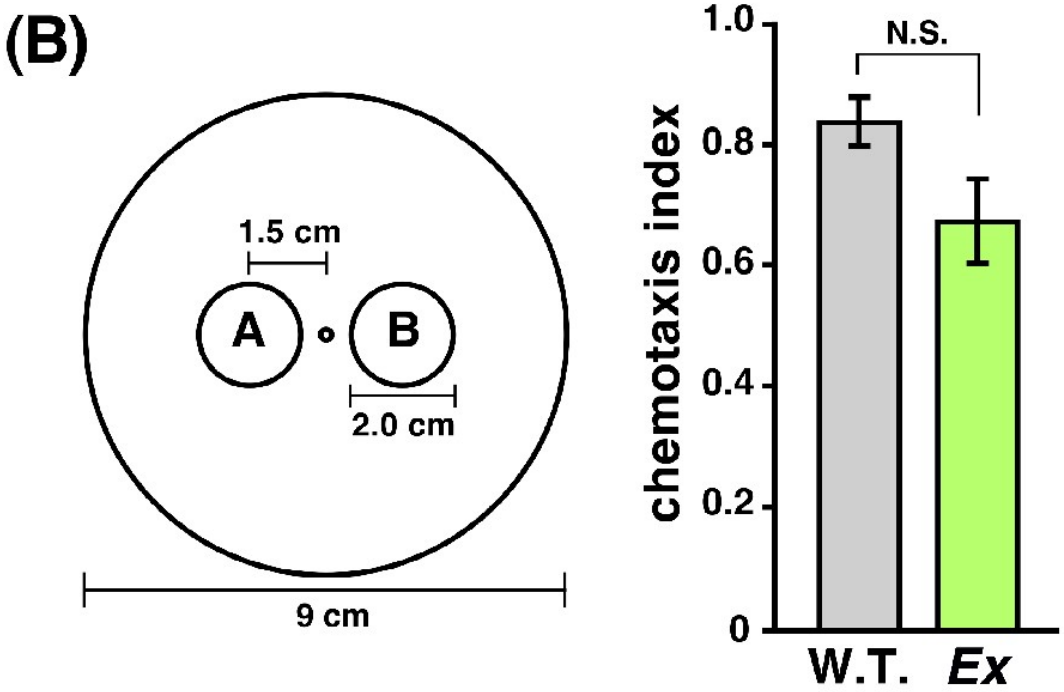

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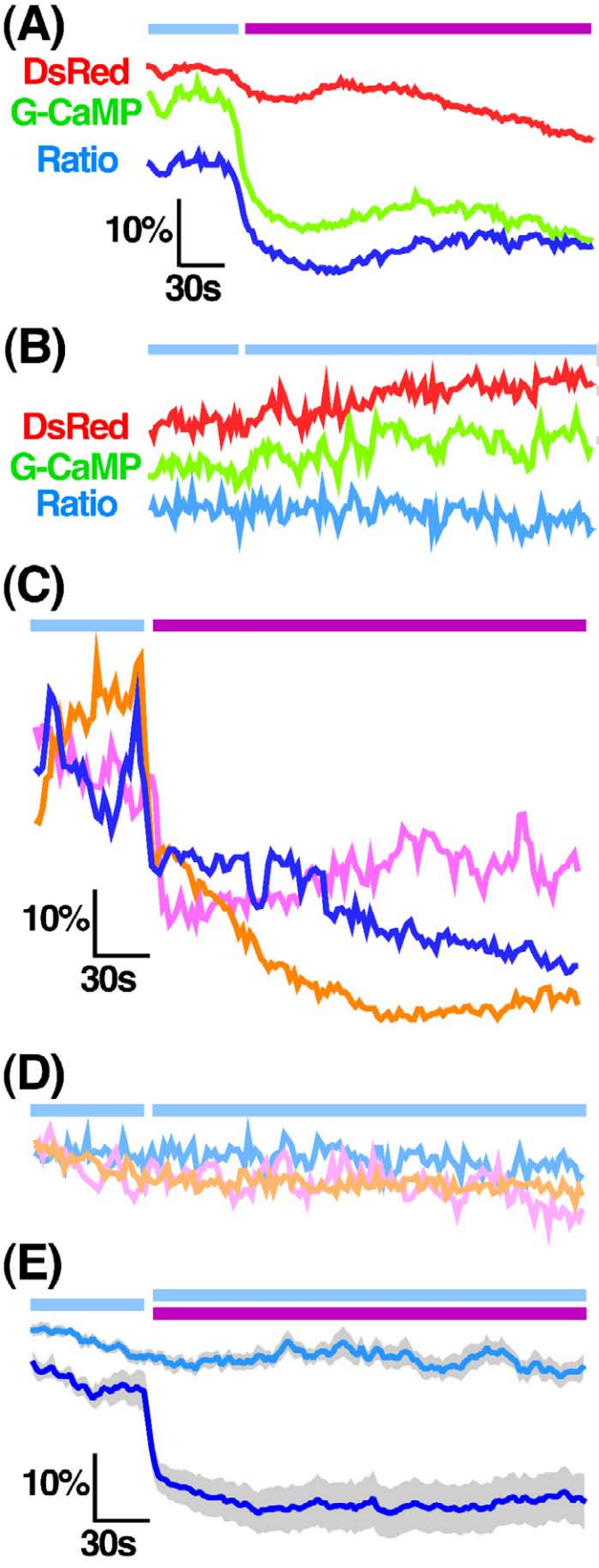

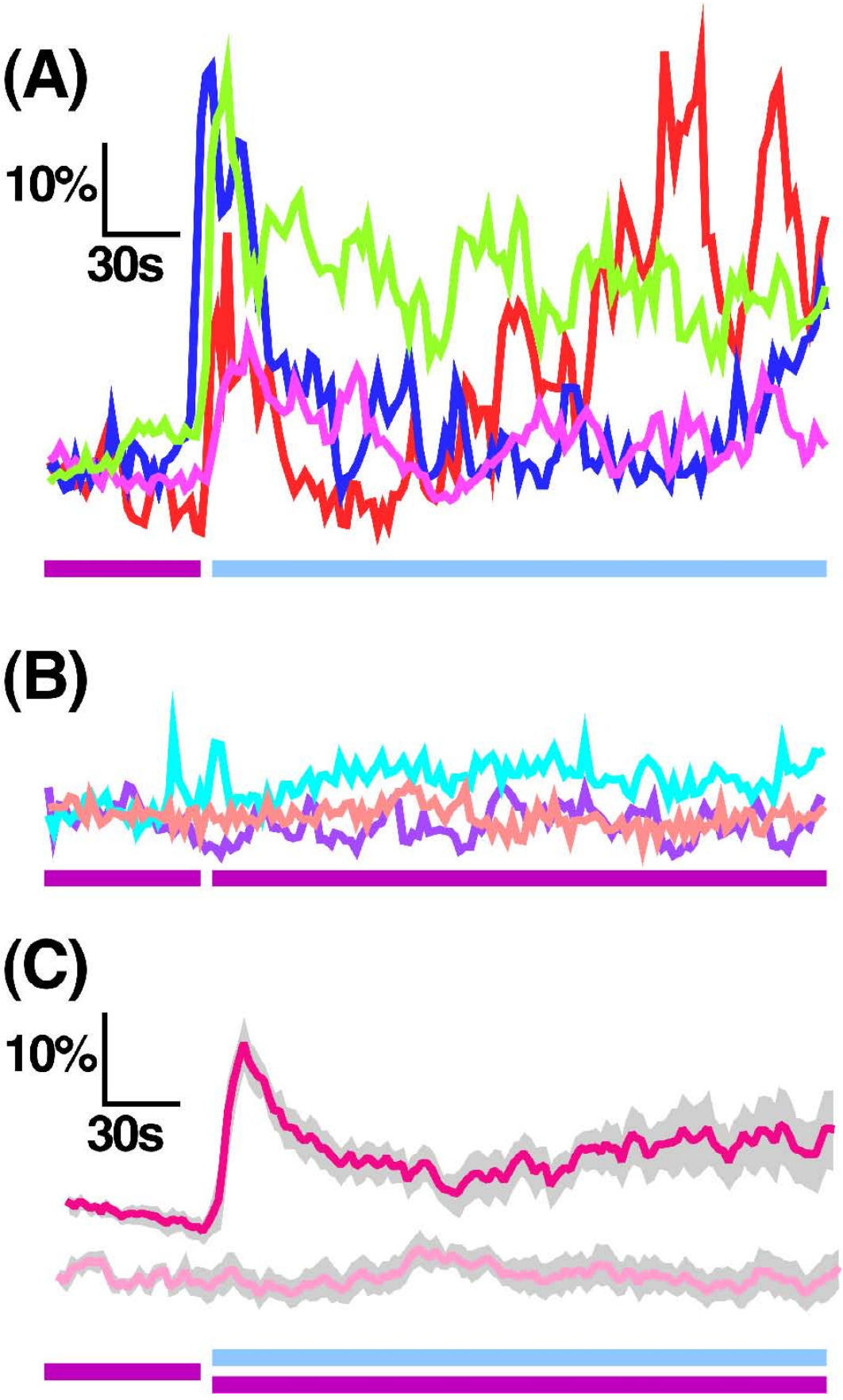

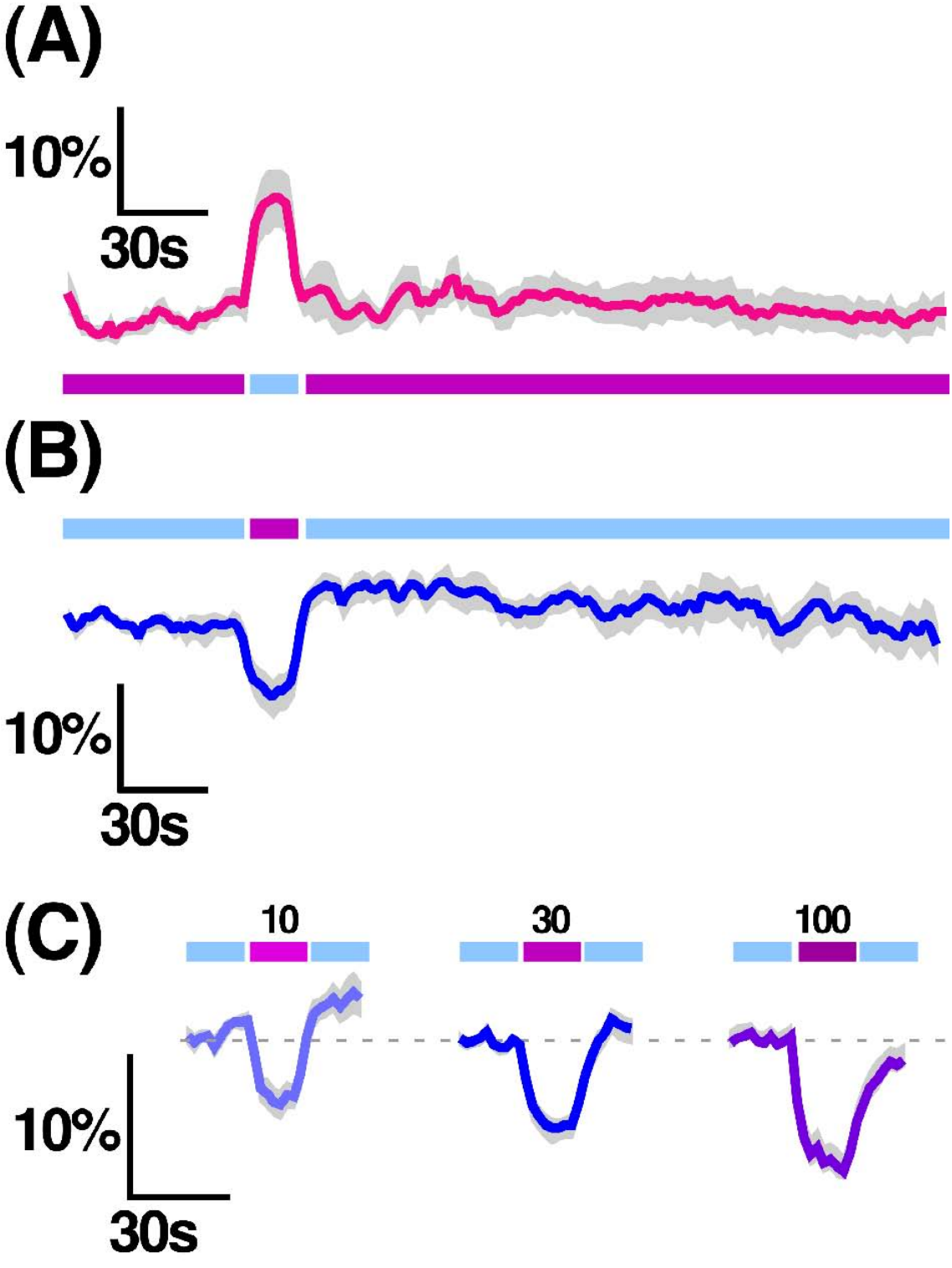

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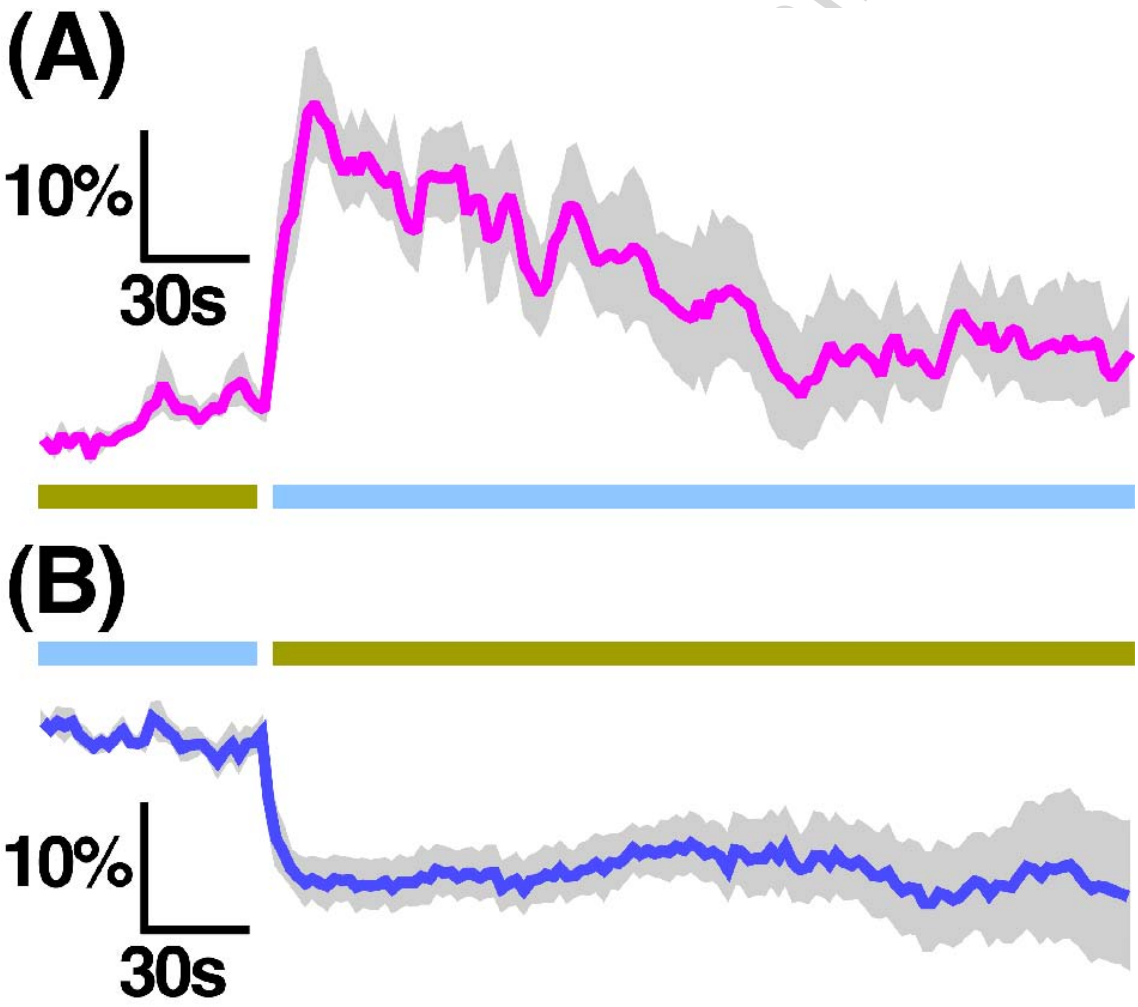

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