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Glutamate-input fluctuation evokes a sporadic Ca$^{2+}$ response in AIY

The membrane-potential depolarization precedes Ca$^{2+}$ responses in AIY

Ca$^{2+}$ increases in AIY when the glutamate input decreases, and vice versa

Mutation of glutamate signaling abolished Ca$^{2+}$ response in AIY

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The Input-Output Relationship of AIY Interneurons in *Caenorhabditis elegans* in Noisy Environment

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**SUMMARY**

Determining how neurotransmitter input causes various neuronal activities is crucial to understanding neuronal information processing. In *Caenorhabditis elegans*, AIY interneurons receive several sources of sensory information as glutamate inputs and regulate behavior by integrating these inputs. However, the relationship between glutamate input and the Ca²⁺ response in AIY under environmental noise, in other words, without explicit stimulation, remains unknown. Here, we show that glutamate-input fluctuations evoke a sporadic Ca²⁺ response in AIY without stimulation. To ensure that Ca²⁺ response can be considered AIY output, we show that the membrane-potential depolarization precedes Ca²⁺ responses in AIY. We used an odor as model stimulation to modulate the sensory inputs. Simultaneous imaging of glutamate input and Ca²⁺ response, together with glutamate transmission mutants, showed that glutamate-input fluctuations evoke sporadic Ca²⁺ responses. We identified the input-output relationships under environmental noise *in vivo*, and our results address the relationship between sensory-input fluctuations and behavioral variability.

**INTRODUCTION**

Information processing from sensory inputs to regulate behavior in noisy environments is a fundamental topic in neuroscience. To improve our understanding of such information processing, the relationship between the noise level of the stimulation and animal perception has been studied in depth (Faisal et al., 2008; Nienborg and Cumming, 2009; Renart and Machens, 2014). However, at the neuronal level these relationships remain largely unclear. Although the input-output relationship in neurons has also been thoroughly investigated (Augustine et al., 2003; Cichon and Gan, 2015; Destexhe, 2011; Frick et al., 2004; Losonczy et al., 2008; Ogawa and Oka, 2015; Stuart and Spruston, 2015), it remains unknown how neurotransmitter inputs affect postsynaptic neuronal activity *in vivo* in noisy environments, because simultaneous measurement of input and output *in vivo* is technically difficult due to the complexity of the networks involved (Ogawa and Oka, 2015; Presern et al., 2015; Stuart and Spruston, 2015).

The nematode *Caenorhabditis elegans* is a suitable model for investigating this question. It has a simple neuronal circuit (White et al., 1986), as well as optical transparency, making it well suited for visualization with various imaging techniques (Kerr and Schafer, 2006). The AIY interneurons of *C. elegans* are particularly suitable for this purpose; they receive various sensory inputs, including gustatory, olfactory, and thermal information (Chalasani et al., 2007; Clark et al., 2006; Satoh et al., 2014), which regulate the animal’s behavior following integration (Kocabas et al., 2012; Li et al., 2014; Satoh et al., 2014). Furthermore, AIY interneurons show a sporadic Ca²⁺ response regardless of the presence of explicit stimulation (Chalasani et al., 2007; Clark et al., 2006), whereas sensory neurons show a deterministic Ca²⁺ response to environmental stimulation (Chalasani et al., 2007; Clark et al., 2006; Kato et al., 2014; Tsukada et al., 2016). Both glutamate receptor mutations and the ablation of sensory neurons have been shown to abolish the Ca²⁺ response to sensory stimulation in AIY (Chalasani et al., 2007; Clark et al., 2006). This suggests that glutamate input to AIY can evoke the sporadic Ca²⁺ response as a result of sensory input integration under natural noise.

We used several simultaneous fluorescence imaging techniques to identify the input-output relationship in AIY in a noisy environment *in vivo*. To ensure that Ca²⁺ spikes could be considered AIY output, we first showed that depolarization of the membrane potential precedes Ca²⁺ spikes, by simultaneously imaging both in AIY. We used an odor, isoamyl alcohol (IAA) as model stimulation to modulate the sensory inputs, and simultaneous imaging of glutamate input and the Ca²⁺ response showed that glutamate input decreases when the Ca²⁺ spikes occur, with or without odor stimulation, and vice versa. We also investigated these relationships in both the glutamate receptor and glutamate-defective mutants and showed that...
fluctuations in glutamate input evoke the sporadic Ca²⁺ response in AIY. As far as we know, this is the first report identifying the input-output relationship for fluctuations under natural environmental noise in vivo. Glutamate inputs represent sensory inputs, and AIY neurons have been reported to regulate behavior; our results thus suggest that fluctuation in sensory input induces behavioral variability.

RESULTS

Membrane-Potential Spike Precedes Ca²⁺ Response in AIY

To quantify the input-output relationship in a single neuron in vivo, we investigated AIY interneurons in C. elegans. AIY neurons receive environmental information from sensory neurons via glutamate as a neurotransmitter and show a large sporadic Ca²⁺ response (hereafter, a “spike”) as a result of sensory integration (Chalasani et al., 2007; Clark et al., 2006). In C. elegans, Ca²⁺ is thought to be the main charge carrier in membrane-potential changes, due to the lack of voltage-gated Na⁺ channels in its neurons (Faumont et al., 2011, 2006; Gao and Zhen, 2011; Goodman et al., 1998; Mellem et al., 2008; Shidara et al., 2013). Recently, the relationship between membrane potential and Ca²⁺ channels has been thoroughly investigated, and it has been shown that Ca²⁺ channels are necessary for membrane-potential spikes (Liu et al., 2018; Shindou et al., 2019). Ca²⁺ responses in AIY regulate the animal’s behavior (Li et al., 2014), so the results of information processing should be expressed as membrane-potential changes. However, the relationship between Ca²⁺ and membrane potential in AIY remains unclear (Liu et al., 2018, 2009; Shidara et al., 2013), because it has not been measured simultaneously in AIY. We therefore measured both simultaneously, using a genetically encoded voltage indicator, ArcLight (Jin et al., 2012), and a genetically encoded Ca²⁺ indicator, R-GECO (Zhao et al., 2011) (Figure 1A). As previously suggested (Faumont et al., 2012), changes in membrane potential appeared as a spike-like response under ArcLight fluorescence, similar to a Ca²⁺ spike (Figure 1B). Importantly, these membrane-potential spikes were coincident with Ca²⁺ spikes. Membrane-potential changes were detected at the neurite and even at the soma, as reported in our earlier study (Shidara et al., 2013).

To investigate the relationship between membrane-potential spikes and Ca²⁺ spikes, we collected and summarized the data (Figure 1C). Membrane-potential spikes preceded Ca²⁺ spikes, and their amplitudes were not modulated by odor presentation (Figure 1D). However, the amplitudes of Ca²⁺ spikes and membrane-potential spikes were not correlated (Figure 1E). Based on these results, membrane-potential changes precede Ca²⁺ changes. In subsequent analyses, therefore, we consider Ca²⁺ spikes to be AIY output.

Glutamate Input and Ca²⁺ Response to Odor Stimulation in AIY Interneurons

In AIY interneurons, glutamate input reflects environmental information from sensory neurons, and sporadic Ca²⁺ spikes occur as a result of sensory integration (Chalasani et al., 2007; Clark et al., 2006). These glutamate inputs emanate from gustatory, olfactory, and thermal sensory neurons (Chalasani et al., 2007; Clark et al., 2006; Satoh et al., 2014; White et al., 1986); therefore, in the absence of explicit changes of stimulation, glutamate inputs to AIY presumably reflect environmental noise, such as fluctuations in environmental factors such as temperature, local salt concentration, and olfactory molecules. AIY neurons receive inhibitory glutamate inputs via a glutamate-gated chloride channel, GLC-3 (Clark et al., 2006; Horoszok et al., 2001; Serrano-Saiz et al., 2013). AIY neurons exhibit Ca²⁺ spikes in response to odor stimulation because sensory neurons reduce their Ca²⁺ response and neurotransmitter release upon addition of odor (Chalasani et al., 2007; Ventimiglia and Bargmann, 2017) (Figure S1). Mutation of glc-3 or glutamate transmission abolishes Ca²⁺ spikes in response to odor in AIY neurons (Chalasani et al., 2007). Therefore, sporadic Ca²⁺ spikes in AIY could occur in response to variable glutamatergic inputs representing environmental noise. To demonstrate this directly, we simultaneously measured glutamatergic inputs and Ca²⁺ responses in AIY interneurons, using a genetically encoded glutamate indicator, iGluSnFR (Marvin et al., 2013), and a genetically encoded Ca²⁺ indicator, R-GECO (Zhao et al., 2011), respectively (Figure 2A).

As a previous study hypothesized that odor (IAA) stimulation could modify glutamate input to AIY (Chalasani et al., 2007), we selected IAA as model sensory stimulation. We measured glutamate input and the Ca²⁺ response with and without odor stimulation (Figure 2B). The glutamate input to AIY decreased following odor application and transiently increased following odor removal, in a deterministic manner (Figure 2C). These responses to odor correspond to the responses of sensory neurons (Chalasani et al., 2007; Shidara et al., 2017). Although there were deterministic changes in glutamate input, large Ca²⁺ spikes were observed even without odor, and often at the onset of odor stimulation, as reported previously (Chalasani et al., 2007). Odor application typically evoked Ca²⁺ spikes immediately in wild-type animals (see N2...
Figure 1. Simultaneous Imaging of Membrane Potential and Ca\textsuperscript{2+} in A1Y Interneurons

(A) Confocal image of the voltage indicator (ArcLight: green, left) and Ca\textsuperscript{2+} indicator (R-GECO magenta, middle). The merged image is on the right. Scale bar, 10 \( \mu \text{m} \). A, anterior; L, left; P, posterior; R, right.

(B) Representative results of Ca\textsuperscript{2+} (red) and membrane potentials (black) at the soma (left) and neurite (right).

(C) Averaged Ca\textsuperscript{2+} spike (red) and membrane-potential spike (black) without (left) and with (right) odor. The shadows around the solid lines indicate the standard error of the mean (SEM) (no odor, \( N = 20, n = 16 \); odor, \( N = 10, n = 15 \)).

(D) Membrane potential (left) and Ca\textsuperscript{2+} spike (right) intensities, with (filled boxes) and without (hollow boxes) odor. Box plots include the median (center line), quartiles (boxes), and range (whiskers). The statistical metrics are as follows: membrane potential, \( p = 0.38 \); Ca\textsuperscript{2+}, \( p = 0.85 \), paired t tests (no odor, \( N = 20, n = 16 \); odor, \( N = 10, n = 15 \)).
in Figure 6C, suggesting that glutamate decreases, induced by odor application, evoke Ca²⁺ spikes. Moreover, odor presence increased the frequency of Ca²⁺ spikes (see N2 in Figure 6D). This suggests that reducing glutamate input via odor application causes Ca²⁺ spikes.

Glutamatergic Input Decreases when the Ca²⁺ Response Increases in AIY under Natural Noise, and Vice Versa

When quantifying the average response of glutamatergic input and Ca²⁺ in AIY to odor, we found that decreases in glutamatergic input appeared to be related to Ca²⁺ spikes (Figure 3A). To investigate this...
Figure 3. Glutamate Input When the Ca\(^{2+}\) Spike Occurs

(A) Representative responses of Ca\(^{2+}\) and glutamate input (left). Magnifications of the dotted boxes showing the responses without (middle) and with (right) odor stimulation. The blue and red lines are the glutamate input and Ca\(^{2+}\) response, respectively. The shaded region indicates IAA stimulation.

(B) Definition of the response categories.

(C) The averaged glutamate responses coincident with Ca\(^{2+}\) spikes. Time 0 indicates the onset of Ca\(^{2+}\) spikes. All glutamate responses were normalized to zero at time 0. The purple, blue, and green lines indicate the responses under the no odor, early odor, and later odor conditions, respectively (defined in B). The error bars indicate the SEM (N = 15; no odor, n = 15; early odor, n = 11; later odor, n = 21).

(D) Glutamate inputs before the Ca\(^{2+}\) spike and at spike onset. "Pre" indicates the average intensity from \(-3\) to \(-2\) s in (C), and "onset" indicates the average intensity from \(-0.5\) to \(+0.5\) s. The statistical metrics are as follows: no odor, p = 0.010; early odor, p = 0.0024; later odor, p = 0.0037; paired t test (N = 15; no odor, n = 15; early odor, n = 11; later odor, n = 21).

* \(p < 0.05\), ** \(p < 0.01\).

(E) Correlation between glutamate decreases and Ca\(^{2+}\) increases. The purple dots, blue diamonds, and green triangles are correlations under the no odor, early odor, and later odor conditions, respectively. The statistical metrics are as
relationship further, we classified conditions into three categories: no odor, early odor, and later odor (Figure 3B). Glutamatergic inputs were collected and aligned with the timing of Ca²⁺ spikes (Figure 3C). Decreasing levels of glutamate did not show a strong correlation with Ca²⁺ spike amplitude (Figure 3D) in any of the three conditions. These results imply that decreases in glutamatergic inputs are necessary to trigger Ca²⁺ spikes. We also compared the extent of glutamate decreases in the presence or absence of Ca²⁺ spikes (Figure 3F) and found that glutamate input decreased significantly when Ca²⁺ spikes occurred, with or without odor, which suggests that decreases in glutamate evoke Ca²⁺ spikes.

To support the idea that decreases in glutamate induce a Ca²⁺ response, we investigated whether Ca²⁺ increases when glutamate decreases. We collected and aligned Ca²⁺ traces with the timing of decreases in glutamatergic inputs (Figure 4A) and found that Ca²⁺ reliably increases when glutamatergic input decreases, with or without odor (Figure 4B). Moreover, the degree of increase was significantly larger than in the randomly resampled data (Figures 4C and 4D). These findings show that Ca²⁺ increases when glutamate decreases and support the idea that decreases in glutamate induce a Ca²⁺ response.

The GLC-3 Receptor Primarily Mediates a Sporadic Ca²⁺ Response in AIY under Odor Stimulation

We demonstrated that glutamate decreases when Ca²⁺ increases, and vice versa. Previous research suggests that the glutamate-gated chloride channel, GLC-3, is crucial for the sensory-input-evoked Ca²⁺ response in AIY (Aoki et al., 2017; Chalasani et al., 2007; Horoszok et al., 2001; Ohnishi et al., 2011). Therefore, we investigated the relationship between Ca²⁺ and glutamate input in a glc-3 mutant. We detected the same type of changes in glutamate input following odor stimulation in glc-3 mutants as in wild-type (N2) animals (Figures 5A and 5B), and found that GLC-3 mutation had a limited effect on the Ca²⁺ response evoked by odor application (see glc-3 in Figure 6C). However, the Ca²⁺ spike frequency decreased significantly compared with N2, both with and without odor stimulation (see glc-3 in Figure 6D). Next, glutamatergic inputs were collected and aligned with the timing of Ca²⁺ spikes, as was done for the N2 animals (Figure 5C). Glutamate decreases were abolished under odor stimulation, but not in the absence of odor stimulation (Figure 5D). The extent of the decrease when accompanied by a Ca²⁺ spike was not significantly different from that without a Ca²⁺ spike under the odor stimulation, but was significantly larger in the absence of odor stimulation (Figure 5E). AIY neurons have a metabotropic glutamate receptor, MGL-1 (Jeong and Paik, 2017; Kang and Avery, 2009), which could play a role in initiating Ca²⁺ spikes. These results suggest that fluctuations in glutamate input via the GLC-3 receptor are important for the sporadic Ca²⁺ spikes.

Glutamate Input Evokes a Sporadic Ca²⁺ Response in AIY

We thus showed that the GLC-3 receptor plays a crucial role in the sporadic Ca²⁺ spikes. We used a glutamate-defective mutant, eat-4, to directly reveal the effect of glutamate input on AIY. EAT-4 is a vesicular glutamate transporter, and eat-4 mutants are defective in glutamate transmission (Chalasani et al., 2007; Lee et al., 1999). In contrast to the N2 animals and glc-3 mutants, the glutamate response following odor application and removal was abolished (Figures 6A and 6B). Moreover, the Ca²⁺ response to odor stimulation was also abolished (Figure 6C), and in fact the Ca²⁺ spike rarely occurred at all, with or without odor stimulation (Figure 6D). We collected and aligned glutamate input with the timing of Ca²⁺ spike initiation (Figure 6E), but no decreases were observed (Figure 6F), and the extent of decrease in the presence of a Ca²⁺ spike did not differ from that without a Ca²⁺ spike (Figure 6G). These results show that glutamate-input fluctuations evoke sporadic Ca²⁺ spikes in AIY neurons.
In this article, we present two sets of simultaneous imaging investigations into the effects of environmental fluctuations on the interaction between Ca$^{2+}$ and membrane potentials and Ca$^{2+}$ and neurotransmitter (glutamate) inputs in AIY interneurons. By combining this approach with the use of mutant strains, we succeeded in determining the input-output relationship. We showed that membrane-potential spikes precede Ca$^{2+}$ spikes in AIY, a previously unknown relationship. Moreover, decreases 

![Figure 4. Ca$^{2+}$ Response to Decreases in Glutamate Input](image)

Figure 4. Ca$^{2+}$ Response to Decreases in Glutamate Input
(A) Ca$^{2+}$ response to decreases in glutamate input. Time 0 indicates the time when the glutamate decreases. All Ca$^{2+}$ responses were normalized to zero at time 0. The red and yellow lines indicate the response under the no odor and later odor conditions, respectively. The shadows indicate the SEM (for the Ca$^{2+}$ response under later odor condition, the shadows are too small to see; N = 15; no odor, n = 43; later odor, n = 61).
(B) Ca$^{2+}$ intensities before (“pre”; averaged from −3 to −2 s in A) and after (“onset”; averaged from −0.5 to +0.5 s in A) glutamate decrease. The statistical metrics are as follows: no odor, p = 0.014; later odor, p = 0.014; paired t test (N = 15; no odor, n = 43; later odor, n = 61). *p < 0.05.
(C) Ca$^{2+}$ responses to decreases in glutamate input relative to randomly selected data with (right) and without odor (left). The gray lines indicate the averaged randomly selected data ± 3 SD (see Methods).
(D) Ca$^{2+}$ increases with glutamate decreases (colored boxes) and by random selection (gray boxes). Box plots indicate the median (center line), quartiles (boxes), and range (whiskers). The statistical metrics are as follows: no odor, p = 0.045; later odor, p = 0.0015. Wilcoxon rank-sum test (N = 15; no odor, decrease, n = 43; no odor, random, n = 100; later odor, decrease, n = 61; later odor, random, n = 100). *p < 0.05, **p < 0.01.

**DISCUSSION**
In this article, we present two sets of simultaneous imaging investigations into the effects of environmental fluctuations on the interaction between Ca$^{2+}$ and membrane potentials and Ca$^{2+}$ and neurotransmitter (glutamate) inputs in AIY interneurons. By combining this approach with the use of mutant strains, we succeeded in determining the input-output relationship. We showed that membrane-potential spikes precede Ca$^{2+}$ spikes in AIY, a previously unknown relationship. Moreover, decreases

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ing l u t a m a t ei nr e s p o n s et oe n v i r o n m ental fluctuations evoke sporadic Ca\(^{2+}\) spikes. We thus succeeded, for the first time, in identifying the relationship between input and output fluctuations in response to environmental fluctuations in vivo.

Figure 5. Ca\(^{2+}\) Responses and Glutamate Inputs in glc-3 Mutants
(A) Averaged glutamate inputs (blue) and Ca\(^{2+}\) responses (red) to odor. The shaded region indicates IAA stimulation, and the shadows around the solid lines indicate the SEM (N = 12).
(B) Glutamate intensities before (pre-addition) and after (post-addition) odor stimulation onset (left), and average intensities before (pre-removal) and after (post-removal) odor stimulation removal (right). The statistical metrics are as follows: left, p = 0.0030; right, p = 0.0018; paired t test (N = 12). **p < 0.01.
(C) Averaged glutamate responses coincident with Ca\(^{2+}\) spikes. Time 0 indicates the onset of Ca\(^{2+}\) spikes. All glutamate responses were normalized to zero at time 0. The purple, blue, and green lines indicate the responses under the no odor, early odor, and later odor conditions, respectively. Shadows indicate the SEM (N = 12, no odor, n = 15; early odor, n = 6; later odor, n = 20).
(D) Glutamate input before the Ca\(^{2+}\) spike and at spike onset. “Pre” indicates the average intensity from \(-3\) to \(-2\) s in (C), and “onset” indicates the average intensity from \(-0.5\) to +0.5 s. The statistical metrics are as follows: no odor, p = 0.0012; early odor, p = 0.65; later odor, p = 0.89; paired t test (N = 12, no odor, n = 15; early odor, n = 6; later odor, n = 20). **p < 0.01.
(E) Averaged glutamate decreases with (filled boxes) and without (hollow boxes) Ca\(^{2+}\) spikes. Box plots indicate the median (center line), quartiles (boxes), and range (whiskers). The statistical metrics are as follows: no odor, p = 0.0027; later odor, p = 0.58; Welch’s t test (N = 12, no odor, spike, n = 15; no odor, no spike, n = 114; later odor, spike, n = 20; later odor, no spike, n = 82). **p < 0.01.

in glutamate in response to environmental fluctuations evoke sporadic Ca\(^{2+}\) spikes. We thus succeeded, for the first time, in identifying the relationship between input and output fluctuations in response to environmental fluctuations in vivo.
Figure 6. Ca²⁺ Responses and Glutamate Inputs in eat-4 Mutants

(A) Averaged glutamate input (blue) and Ca²⁺ response (red) to odor. The shaded region indicates IAA stimulation and the shadows around the solid lines indicate the SEM (N = 12).

(B) Average glutamate intensity before (pre-addition) and after (post-addition) odor stimulation onset (left), and average intensity before (pre-removal) and after (post-removal) odor stimulation removal (right). Error bars indicate the SEM. The statistical metrics are as follows: left, p = 0.41; right, p = 0.055; paired t test (N = 12).

(C) Ca²⁺ spike probability following odor addition. Comparison between N2 animals and glc-3 mutants: p = 0.13; comparison between N2 animals and eat-4 mutants: p = 0.014. Fisher’s exact test with Holm correction (N2, N = 15; glc-3, N = 12; eat-4, N = 12); *p < 0.05.

(D) Ca²⁺ spike frequency in N2 (filled), glc-3 (striped), and eat-4 mutants (hollow) with and without odor. Box plots indicate the median (center line), quartiles (boxes), and range (whiskers). The statistical metrics are as follows: N2 data comparison...
Glutamate inputs to AII represent sensory neuronal outputs (Chalasani et al., 2007; Serrano-Saiz et al., 2013; White et al., 1986), and Ca\(^{2+}\) responses in AII correlate with velocity and turn behavior (Li et al., 2014). Therefore, our results suggest that fluctuations in sensory input induce behavioral variability. It is difficult to address the relationship between sensory input and behaviors under environmental noise, because of the low signal-to-noise ratio (Faumont et al., 2012). Our results thus provide novel insights into the relationship between sensory-input fluctuation and behavioral variability.

In previous research, noise has been applied artificially (Nienborg and Cumming, 2009; Renart and Machens, 2014) and is thus not reflective of real environmental fluctuation. Controlling noise is an appropriate strategy for understanding perception, but it is not suitable for evaluating spontaneous variability. Here, we showed that Ca\(^{2+}\) spikes are caused by glutamate-input fluctuations. AII neurons play an important role in sensory integration (Chalasani et al., 2007; Clark et al., 2006; Yoshida et al., 2012) and regulate behavior, and Ca\(^{2+}\) responses are correlated with reversal behavior and locomotion speed (Kocabas et al., 2012; Li et al., 2014). Therefore, changing behaviors under natural conditions are presumably caused by fluctuations in sensory input under environmental noise. Our approach of evaluating the input-output relationship in AII under environmental fluctuations aids understanding of resting-state dynamics in complex neural circuits.

We identified the relationship between neurotransmitter inputs and the Ca\(^{2+}\) response. In previous research (Branco and Häusser, 2010; Callaway and Katz, 1993; Kramer et al., 2013), caged compounds and the stimulation of presynaptic neurons were used to investigate these relationships. However, such stimulation is often intense and artificial, and neurotransmitter inputs are not directly measurable. To address these shortcomings, we first demonstrated the relationship between neurotransmitter inputs and Ca\(^{2+}\) responses under naturally occurring physiological conditions, and then quantified the input-output relationships using simultaneous imaging of neurotransmitter inputs and neuronal activity.

Limitations of the Study

Here, we identified the relationship between glutamate input and the Ca\(^{2+}\) response. In the eat-4 mutants, however, the Ca\(^{2+}\) responses were rare but were not completely abolished. We acknowledge that other neurotransmitters, such as neuropeptides, also affect the Ca\(^{2+}\) response in AII (Aoki et al., 2017; Kuhara et al., 2011; Rabinowitch et al., 2016). Although our results show that decreases in glutamate evoke Ca\(^{2+}\) responses in AII under natural noise, cooperation between several neurotransmitters could modulate the responsiveness of AII to glutamate inputs, which would modulate sensory information and the behavioral response.

In this study, we revealed the relationships with and without odor condition. However, it is difficult to generalize for the other sensory stimulation, and modulation of the other environmental modalities could have yielded different results. In addition to the different neurotransmitters involved, AII neurons receive glutamate from several types of sensory neurons (Clark et al., 2006; Kuhara et al., 2011; Kunitomo et al., 2013; Serrano-Saiz et al., 2013; Wang et al., 2017; White et al., 1986). For example, high concentration of
salt affects odor sensory neurons (Leinwand and Chalasani, 2013). So, the other sensory stimulation could change the input-output relationship in AY.

We showed that mutation of glc-3 and eat-4 reduced the frequency of Ca²⁺ spikes. Because GLC-3 is a glutamate-gated chloride channel (Horoszok et al., 2001), ablation of the glutamate signal should depolarize the membrane potential of AY neurons. However, shapes of Ca²⁺ spikes were identical (Figure S2). This indicates that AY neurons retain the function of Ca²⁺ spike generation even without glutamate inputs. This suggests that the AY neurons should have maintained their equilibrium potential even in the mutant animals because depolarization is necessary for generating Ca²⁺ spikes (Liu et al., 2018; Shindou et al., 2019). In the mutants, glutamate inputs could not cause the depolarization of membrane potential beyond the threshold in response to environmental fluctuations by glutamate inputs, so the Ca²⁺ spike frequency decreased. However, the mutations could change the physiological property in AY; we could not dismiss these effects in the mutants.

We also noted that C. elegans does not have voltage-gated Na⁺ channels in its neurons (Faumont et al., 2011, 2006; Gao and Zhen, 2011; Goodman et al., 1998; Mellem et al., 2008; Shidara et al., 2013). This fact indicates that the mechanism of spike generation should be different from that of the other animals. Therefore, it is difficult to directly extrapolate our conclusions beyond C. elegans.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.07.028.

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AUTHOR CONTRIBUTIONS
K.A. designed the research. K.A. performed all experiments and analysis. K.A. wrote the first draft of the manuscript. K.A., K.O., and K. H. edited the manuscript. K.A., K.O., and K. H. contributed to data interpretation. K.O. and K. H. supervised the research.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

The Input-Output Relationship of AIY Interneurons in *Caenorhabditis elegans* in Noisy Environment

Keita Ashida, Kohji Hotta, and Kotaro Oka
Supplemental Information

Transparent methods

C. elegans strains

Worms were cultured at 20°C on nematode growth medium agar plates with *Escherichia coli* OP50 bacteria under standard conditions (Brenner, 1974). Hermaphrodites were used for all experiments. The following transgenic strains were created via microinjection into the Bristol strain N2 (wild type), RB594 *glc-3* (ok321) V, and MT6308 *eat-4* (ky5) III. The transgenic strains used for this research were as follows: okaEx5\[pttx-3::iGluSnFR, 50 ng/µL + pttx-3::R-GECO1, 50 ng/µL], okaEx6\[pttx-3::ArcLight, 50 ng/µL + pttx-3::R-GECO1, 50 ng/µL], okaEx10\[pttx-3::GCaMP6, 50 ng/µL + pttx-3::dimer2, 50 ng/µL], okaEx15; *glc-3* (ok321) X \[pttx-3::iGluSnFR, 50 ng/µL + pttx-3::R-GECO1, 40 ng/µL + punc-122::dsRed, 15 ng/µL], and okaEx16; *eat-4* (ky5) X \[pttx-3::iGluSnFR, 50 ng/µL + pttx-3::R-GECO1, 40 ng/µL + punc-122::dsRed 15 ng/µL].

Plasmids

For ArcLight expression, codon-optimized ArcLight was synthesized using *C. elegans* Codon Adapter (Redemann et al., 2011) (Integrated DNA Technologies Genes). This codon-optimized ArcLight was cloned into a Gateway Destination vector (Thermo Fisher Scientific). Gateway Destination vectors for expressing proteins and Gateway Entry vectors (Thermo Fisher Scientific) for cell-specific promoters were obtained from the Comprehensive Brain Science Network. All plasmids used to express proteins in the animals were generated from these vectors using Gateway Cloning Technology (Thermo Fisher Scientific).

Confocal imaging

Confocal images of detailed neuronal structures (Figures 1A and 2A) were acquired using a confocal laser scanning microscopy system (FluoView FV1000, Olympus) mounted on an inverted microscope (IX81, Olympus) using a 40× objective oil-immersion lens (UPLFLN 40XO, Olympus) for animals expressing ArcLight and R-GECO or iGluSnFR and R-GECO in their AIY neurons. Animals were immobilized with 20 mM sodium azide and mounted in a 1% low-melting-point agarose gel (UltraPure, Invitrogen). The imaging conditions were excitation at 488 nm and detection at 500–545 nm for ArcLight and iGluSnFR, and excitation at 559 nm and detection at 570–670 nm for R-GECO.
**Ca$^{2+}$, glutamate and voltage imaging**

We used a genetically encoded Ca$^{2+}$ indicator, R-GECO1 (Zhao et al., 2011), a genetically encoded Ca$^{2+}$ indicator, G-CaMP6 (Ohkura et al., 2012) with dimer 2 (Campbell et al., 2002) (RFP as a reference), a genetically encoded glutamate indicator, iGluSnFR (Marvin et al., 2013), and a genetically encoded voltage indicator, ArcLight (Jin et al., 2012) with olfactory chips (Chronis et al., 2007). The olfactory chips are microfluidic devices that can control stimulation temporally via valve switching. The animals were stimulated with S-basal buffer and isoamyl alcohol (IAA) diluted with S-basal buffer ($9.2 \times 10^{-4}$ mol/L). For Ca$^{2+}$ and glutamate imaging, the animals were stimulated with 60 s S-basal buffer, 60 s diluted IAA, and again 60 s S-basal buffer (as shown in Figure 3B). To simultaneously image Ca$^{2+}$ and membrane potential under odor stimulation, images were acquired over 70 s immediately after switching from S-basal to diluted IAA. For the no-odor condition, images were acquired without changing the buffer. To reduce the effects of photobleaching, we used images taken after 60 s for analysis. Images were acquired every 100 ms for Ca$^{2+}$ and glutamate imaging, and every 20 ms for Ca$^{2+}$ and membrane potential imaging; the exposure time was equal to the interval duration (100 or 20 ms) in each case.

We used an inverted microscope (IX71, Olympus) with a LED light source (SOLA, Lumencor), and a 3CCD camera (C7800-20, Hamamatsu Photonics). Images were acquired using an AQUACOSMOS (Hamamatsu Photonics) with a 20× objective lens (UCPLFLN 20X, Olympus) and a 1.6× zoom lens. A BrightLine GFP/DSRED-A (Semrock) cube was used. We noted that ArcLight becomes dim when the membrane potential depolarizes, while R-GECO becomes bright when Ca$^{2+}$ increases. The membrane potential spikes in Figures 1B and 1C are thus not artifacts of fluorescent leakage by R-GECO. In addition, photobleaching causes an increase of the ArcLight traces in Figure 1B because we showed $-\Delta F/F_0$ for the ArcLight traces. To reduce noise caused by movement of the animals, a cholinergic agonist, levamisole (2 mM), was used in the S-basal buffer. In preliminary experiments, we demonstrated that levamisole did not affect the AIY spike frequency (Figure S1).

**Image analysis**

We analyzed the imaging data using the same semi-automated custom software written in MATLAB (MathWorks) that we used in a previous study (Shidara et al., 2017). Regions
of interest were determined based on fluorescence intensities and the morphology of the neurons (Figures 1A and 2A). We measured the Ca²⁺ response and glutamate input at the AIY neurite, because these interneurons show a clear Ca²⁺ response at the neurite but not at the soma (Chalasani et al., 2007; Clark et al., 2006). All fluorescence intensity data were normalized by the average intensities for the first 2 s, and are presented as ΔF/F.

Data analysis and statistical tests
Statistical tests were performed in Excel 2011 (paired t-test and Welch’s t-test, using the “TTEST” function), Python 3 (version: 3.7.0; tests for Pearson correlation coefficients were conducted using the “stats.pearsonr” function in the SciPy library (version 1.1.0)), and R (version: 3.4.3; Wilcoxon signed rank tests were conducted using the “wilcoxon_test” function with the “exact” option, and Wilcoxon rank sum tests were conducted using the “wilcox_test” function with the “exact” option in the coin package).

The average intensities of the Ca²⁺ and membrane potential spikes in Figure 1C were generated by detecting Ca²⁺ spikes as described below. The peak values of membrane potential in Figures 1D and 1E were calculated by subtracting the average values for the 1 s period from −1 s to −2 s in Figure 1C from the maximal values for the 1 s period from −1 s to 0 s. The Ca²⁺ increases were calculated by subtracting the average intensities for the 1 s period from −1 s to 0 s from the maximal intensities for the 1 s period from 0.5 s to 1.5 s.

The averaged intensities over the 20 s before and after odor addition are shown in Figure 2C (left), and the average intensities over the 20 s before and after odor removal are shown in Figure 2C (right).

We classified the odor stimulation conditions into two groups, early odor and later odor, for Figures 3–6. The early odor condition comprised the data from the first 10 s after the onset of odor stimulation, and the later odor condition comprised the data from 10–60 s after odor addition (see Figure 3B). Odor addition reduced glutamate-input levels immediately (Figure 2B), so we considered two conditions to be transient and stable, respectively.

We classified the data based on whether Ca²⁺ spikes were present or absent (with or without) for Figures 3, 5, and 6. The Ca²⁺ spikes in Figure 3C were extracted as described below, and data that did not include Ca²⁺ spikes were considered non-Ca²⁺
spiking data. All glutamate responses were normalized to zero at time 0. The intensities before the Ca\(^{2+}\) spike (“pre” in Figure 3D) are the average intensities for the 1 s period from \(-3\) s to \(-2\) s in Figure 3C, and the average intensities during the spike (“onset” in Figure 3D) are the average intensities for the 1 s period from \(-0.5\) s to \(+0.5\) s in Figure 3C. The glutamate decreases in Figure 3E and 3F were calculated by subtracting the “onset” intensities in Figure 3D from the “pre” intensities. The Ca\(^{2+}\) increases in Figure 3E were calculated by subtracting the average intensities for the 1 s period from \(-1\) s to 0 s (as in Figure 3C) from the average intensities for the 1 s period from 2 s to 3 s.

The Ca\(^{2+}\) responses to glutamate decreases, shown in Figure 4A were collected as described below. All Ca\(^{2+}\) responses were normalized to zero at time 0. The intensities before and during the response in Figure 4B were calculated as for Figure 3D. The randomly selected data in Figures 4C and 4D are described below. The Ca\(^{2+}\) increases shown in Figure 4D were calculated by subtracting the average intensities before the response (“pre” in Figure 4B) from those during the response (“onset” in Figure 4B).

The average intensities of the glutamate input shown in Figure 5B were calculated as for Figure 2C; Figures 5C–5E were generated in the same manner as Figures 3C, 3D, and 3F.

The average intensities of glutamate input shown in Figure 6B were calculated as for Figure 2C. The probability of a Ca\(^{2+}\) spike in response to odor stimulation shown in Figure 6C was calculated by counting the Ca\(^{2+}\) spikes during the early-odor period. The frequency of the Ca\(^{2+}\) spikes in Figure 6D was calculated by counting the Ca\(^{2+}\) spikes detected as described below. Figures 6E–6G were generated in the same manner as Figures 3C, 3D, and 3F.

**Extracting Ca\(^{2+}\) spikes and glutamate decreases**

To extract the Ca\(^{2+}\) spikes, we selected a data point at near the beginning of the Ca\(^{2+}\) data, and calculated the difference between the average intensities for the 5 s period before and after before that point. If the difference was larger than the average intensity over the 5 s period before the point plus three standard deviations, the point was determined to be the onset of a Ca\(^{2+}\) spike, after visual verification. To extract the glutamate-decrease point shown in Figure 4, we selected a data point at near the beginning of the glutamate data and calculated the difference between the averaged intensities for the 5 s period before and after that point. If the difference was larger than the threshold (0.025; determined
from the values in Figure 3F), the point was automatically determined to be the onset of a glutamate decrease. For Ca\(^{2+}\) and glutamate decreases, we collected the data for 5 s periods before and after the points that had been detected as described above. There was no overlap between the collected data. Finally, we defined all data that did not include a Ca\(^{2+}\) spike as the data without a Ca\(^{2+}\) spike.

In Figure S1, we counted the Ca\(^{2+}\) spikes manually, because the signal-to-noise ratio differed between the with- and without-levamisole conditions. We counted the spikes from 70 s to 120 s for Figure S1B, and from 60 s to 70 s for Figure S1C.

**Random resampling**

Randomly resampled data are used in Figures 4C and 4D. One hundred time points were selected randomly using the “randint” function (in Python numpy.random in NumPy library (version 1.15.2)), and we extracted 10 s of data after each of these points. We initialized the seed of the random number generator using the current system time.
Figure S1. Levamisole does not significantly affect Ca$^{2+}$ spikes in AIY. Related to Figure 1.

(A) The Ca$^{2+}$ response in AIY to odor with (red) and without (blue) levamisole. The shaded region is odor stimulation.

(B) Spike frequency without (from 0 s to 60 s) and with (from 70 s to 120 s) odor stimulation, with (red) and without (blue) levamisole. Box plots indicate the median (center line), quartiles (boxes), and range (whiskers). The statistical metrics are as follows: no odor, $p = 0.75$, later odor, $p = 0.80$. Wilcoxon rank sum test (without levamisole, $N = 10$; with levamisole, $N = 10$).

(C) Ca$^{2+}$ spike probability immediately after odor stimulation with and without levamisole. The statistical metrics are as follows; $p = 1.0$, Fisher's exact test (without levamisole, $N = 10$; with levamisole, $N = 10$).
Figure S2. Averaged Ca\textsuperscript{2+} responses. Related to Figures 3, 5 and 6.
Time 0 indicates the onset of Ca\textsuperscript{2+} spikes. All Ca\textsuperscript{2+} responses were normalized to zero at time 0. The purple, blue and green lines indicate the responses under the no-odor, early-odor, and later-odor conditions, respectively. Shadows indicate the SEM (N2: N = 15; no odor, n = 15; early odor, n = 11; later odor, n = 20; glc-3: N = 12, no odor, n = 15; early odor, n = 6; later odor, n = 20; eat-4: N = 12, no odor: n = 9; early odor, n = 3; later odor, n = 9).