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名古屋市立大学 博士学位論文

Electric shock causes a fleeing-like persistent behavioral response in the nematode *Caenorhabditis elegans*

A thesis submitted in fulfilment of the requirements

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Abstract

Behavioral persistency reflects internal brain states, which are the foundations of multiple brain functions. However, experimental paradigms enabling genetic analyses of behavioral persistency and its associated brain functions have been limited. Here, I report novel persistent behavioral responses caused by electric stimuli in the nematode *Caenorhabditis elegans*. When the animals on bacterial food are stimulated by alternating current, their movement speed suddenly increases 2- to 3-fold, persisting for more than 1 minute even after a 5-second stimulation. Genetic analyses reveal that voltage-gated channels in the neurons are required for the response, possibly as the sensors, and neuropeptide signaling regulates the duration of the persistent response. Additional behavioral analyses implicate that the animal's response to electric shock is scalable and has a negative valence. These properties, along with persistence, have been recently regarded as essential features of emotion, suggesting that *C. elegans* response to electric shock may reflect a form of emotion, akin to fear.

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Chapter 1: Introduction

Animal behaviors, such as feeding, mating, aggression, and sleeping, are strongly related to internal states in the brain, namely motivation, arousal, drive, and emotion (Anderson, 2016; Berridge, 2004; Kennedy et al., 2014). Because animals can produce different behavioral responses to the same stimulus depending on their brain state, these states are considered to be the foundation from which a variety of behavioral responses emerge (Chen & Hong, 2018; Maimon, 2011). The brain states persist for a certain period of time and transit to a different state based on internal and/or external triggers, which can be observed as transitions among different persistent behavioral states. The neural mechanisms of brain/behavioral states are starting to be revealed: For example, the behavioral states of mating and aggressiveness have been shown to be controlled by relatively small circuits in mice and flies (Anderson, 2016; Hoopfer et al., 2015; Lee & Dan, 2012). However, the mechanisms of persistent brain/behavioral states have been revealed in only limited studies, and, moreover, the molecular basis that generates persistent states is still unclear.

The nematode *Caenorhabditis elegans* has been widely used in neurobiological research because of the feasibility of molecular, physiological, and behavioral analyses of neural functions (Bargmann, 2006; de Bono & Maricq, 2005; Sasakura & Mori, 2013). Recently, persistent behavioral states have also been studied in these animals, especially roaming/dwelling and sleep/arousal. Roaming/dwelling are states of locomotion on bacterial food that involve either moving over long distances at a constant speed or moving back and forth over short distances (Ben Arous et al., 2009; Fujiwara et al., 2002). Sleep in *C. elegans* is a phenomenon observed just before molt, and meets the definition of sleep in higher animals such as humans, rodents, fishes and flies (Raizen et al., 2008). Both the neural

circuits and genes that control these phenomena are being revealed (Flavell et al., 2020). However, much remains unknown about *C. elegans'* behavioral states.

In this study, I report that *C. elegans* exhibits a novel type of persistent behavioral response to electric stimulus. The animals respond to alternating current (AC) stimulus by immediately increasing their speed, and the speed increase persists for minutes even when an electric stimulus as short as 5 seconds is provided: This result suggests that the response is caused not by direct stimulation of the motor system for rapid movement but by persistent activity of a specific set of neurons to generate the behavioral response. Further behavioral analyses suggest that the speed increase to AC stimulus is scalable and has negative valence. Because persistent behavioral response is one of the most prominent characteristics of emotions of animals (Abbott, 2020; Anderson & Adolphs, 2014; Nettle & Bateson, 2012; Paul & Mendl, 2018; Perry & Baciadonna, 2017), and persistency, scalability and valence are 3 of the 4 key features of animal emotions proposed by Anderson and Adolphs (2014), the speed increase caused by the electric shock may reflect a form of emotion. A series of candidate genetic analyses reveal that the response is not mediated by any single well-known chemo- or mechanosensory mechanisms. Instead, it requires voltage-gated calcium and potassium channel genes, which are required for electro-sensation in cartilaginous fishes (Bellono et al., 2017, 2018), suggesting an evolutionarily conserved mechanism for electro-sensation. Furthermore, neuropeptide signaling is found to regulate the duration of persistence. These results indicate that the response of *C. elegans* to electric shock can be a suitable paradigm to reveal genetic and physiological mechanisms of electro-sensation as well as persistent brain/behavioral states.

Chapter 2: Materials and Methods

2.1 *C. elegans* strains

C. elegans strains were maintained with standard procedures (Brenner, 1974). In brief, for regular cultivation, animals were grown on standard 6-cm nematode growth medium (NGM) agar plates spread with *Escherichia coli* strain OP50 and incubated at 19.0-19.5°C. Strains used were the wild-type strain Bristol N2, mutant strains PR678 *tax-4(p678)*, CX4652 *osm-9(ky10);ocr-2(ak47)*, CB1033 *che-2(e1033)*, TU253 *mec-4(u253)*, ZB2551 *mec-10(tm1552)*, TQ296 *trp-4(sy695)*, MT1212 *egl-19(n582)*, DA995 *egl-19(ad995)*, JD21 *cca-1(ad1650)*, CB55 *unc-2(e55)*, VC854 *unc-2(gk366)*, NM1968 *slo-1(js379)*, BZ142 *slo-1(eg142)*, KDK11 *cat-2(tm2261)*, MT7988 *bas-1(ad446)*, GR1321 *tph-1(mg280),* RB993 *tdc-1(ok914),* VC671 *egl-3(ok979)*, MT1219 *egl-3(n589*) and ZM5438. ZM5438 is the strain in which *Pmyo-3-egl-19* N-terminal cDNA was coinjected with a fosmid WRM0629dG07 in *egl-19(n582)* to produce the recombined *egl-19* minigene (Gao & Zhen, 2011).

2.2 *C. elegans* cultivation for electric shock behavioral assay

Before the behavioral assay, animals were cultivated as described previously (Kimura et al., 2010). In brief, 4 adult wild-type animals were placed onto NGM agar plates with OP50 and kept at 19.5°C for 7.5 hours before being removed. After removal, these plates were incubated at 19.0–19.5°C for 3 days until the assay day. On the assay day, about 100 synchronized young adult animals were grown on each plate. As some mutant animals had slower growth or laid fewer eggs than wild-type animals did, the incubation temperature and number of these mutant animals were adjusted and increased accordingly in order to obtain a developmental stage (i.e. young adult) and worm number comparable to the wild-type animals. All behavioral assays were carried out with young adult hermaphrodites.

2.3 Experimental instruments for electric shock behavioral assay

The following electric instruments (Figure 1) were utilized for the electric shock behavioral assay. A 50-MHz Arbitrary Waveform Generator (FGX-295, Texio Technology Corporation) was used to generate different types of electric waveforms over a wide range of frequencies. This waveform generator has an output limit of 10 V. Thus, an AC Power Supply (PCR500MA, Kikusui Electronics Corp.) was used to amplify the voltage supply. A Digital Storage Oscilloscope (DCS-1054B, Texio Technology Corporation) was also used in parallel to measure the voltage and observe the electric waveforms produced as well as a Digital Multimeter (PC720M, Sanwa Electric Instrument Co., Ltd.) to measure current. A USB camera (DMK72AUC02, The Imaging Source Co., Ltd.) with a lens (LM16JC5M2, Kowa) was used to record trajectories produced by the animals.

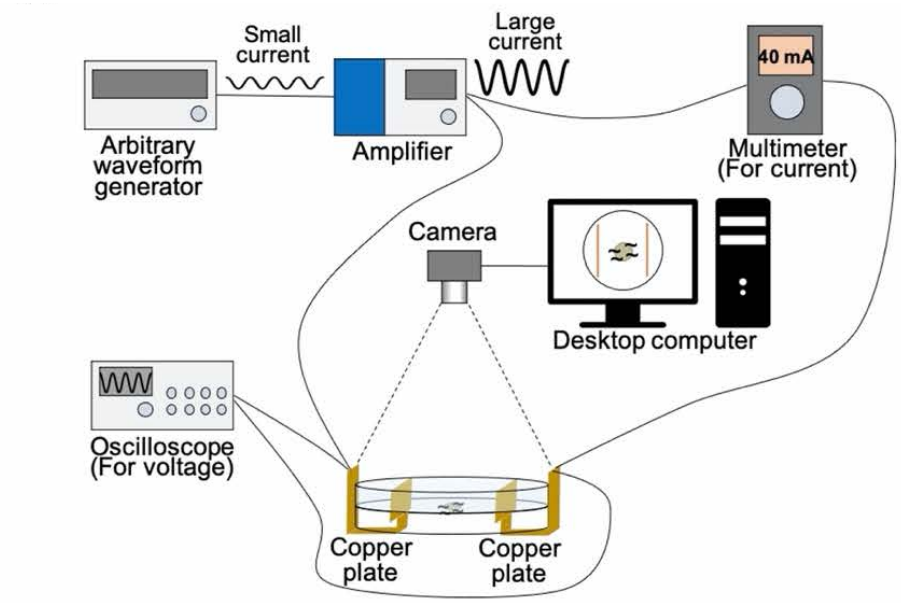

Figure 1. Experimental setup of electric shock experiment. This setup consists of an arbitrary waveform generator, amplifier, multimeter, oscilloscope, camera, and desktop computer.

2.4 Electric shock behavioral assay with small OP50 bacterial food patch

Most of the behavioral assays were conducted on 9-cm NGM agar plates seeded with a small food patch unless otherwise indicated. For the food patch, the bacteria OP50 was grown in 100 mL of LB culture overnight at 37°C, spun down and resuspended in 10 volumes of NGM buffer, and 5 μL of the suspension was applied at the center of the plate to create a food patch 3 × 10 mm in size on the assay day. This process was used to minimize the thickness of the food patch as it prevents clear images of worms in the patch. Four animals per plate were placed in the food patch 1-3 hours before the assay to accustom the animals to the environment and to reduce their movement speed to the basal level. The assay plates were then inverted and placed onto a custom-made copper plate bridge, whose distance is 6 cm (Figure 1). The images were acquired 2 frames per second, and electric shock was delivered with the conditions described in each figure. The assay was repeated 3–5 days per condition in general. Move-tr/2D software (Library Inc., Japan) was used to calculate the *x*-*y* coordinates of the animal centroids in each image frame, which were then analyzed using Excel (Microsoft) or R (The R Project) to calculate the animal's speed. The moving median for ±1 frame was calculated to remove noise for each animal and then ensemble averaged for each condition. Baseline speed was calculated from the average speed over 30 seconds before the stimulation, and Δspeed was calculated by subtracting the baseline value from each animal's speed during or after the stimulus.

2.5 Electric shock behavioral assay with full or strip-like OP50 bacterial food lawn

For the assays conducted with full food lawn, the region of the assay plates between the copper plates were fully seeded (about 5.5 × 5.5 cm^2) or seeded in a 3 stripe-shape (about 5.5 × 1 cm^2; Figure 2) with OP50 and kept on the bench overnight until the assay began. A total of 60 μL or 20 × 3 μL of the OP50 suspension (see above) were used for the full and 3 stripe-

shape food plates, respectively. Animals grown in regular cultivation plates were washed in 2 droplets of NGM buffer and then transferred to the center of the assay plate and left for 5 minutes. The rest of the procedures were the same as for assays conducted with small food patch.

To detect outward and inward movement on the food stripes (Figure 2), the food positions were first indicated on each image series by the experimenter and moments when the animal's centroid crossed a boundary was automatically detected by a custom-made program.

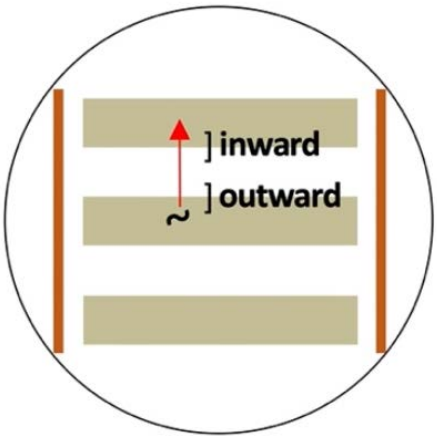

Figure 2. Illustration showing worms' movement across multiple food strips. When worms leave food strip and enter no food area, this movement is defined as "outward movement". When worms enter food strip from no food area, this movement is defined as "inward movement".

2.6 Suppression of EGL-19 activity

Because *egl-19* is expressed in many neurons as well as muscles, a series of experiments are performed to clarify whether *egl-19* functions in neurons or muscles. For pan-neuronal RNAi by the expression of double-stranded RNA (Esposito et al. 2007), the sense or antisense "Fragment 3" of *egl-19* was fused with the *rab-3* promoter (Stefanakis et al., 2015) and *unc-54* 3'UTR using the PCR fusion method (Hobert, 2002). The sense and antisense PCR fusion products (10 ng/µL each), IR101 (*rps-0p*::HygR::mCherry, 2 ng/µL), and sonicated OP50

genome (78 ng/μL) were coinjected (Mello et al., 1991) into wild-type animals to obtain the transgenic strains KDK55167 and KDK55221. IR101 was used for the selection of transgenic animals with hygromycin B (see below) (Radman et al., 2013). Injection of higher concentrations of the PCR fusion products of *egl-19* Fragment 3 did not generate transgenic animals for unknown reasons. Control transgenic lines (KDK55038 and KDK55054) without the *egl-19* plasmids were also obtained from the injection. For the behavioral analysis, 20 μL of 40-mg/μL hygromycin B (FUJIFILM Wako Chemicals Corp.) was added to an OP50-seeded NGM plate 1 day before egg-laying to obtain only animals with the transgene. The electric shock behavioral assays were conducted as described earlier.

2.7 Data analysis and statistics

All the statistical analyses were performed in R (The R Project). Generally, data of 20–50 animals in total from 9 plates from 3 days of experiments for each condition were pooled and analyzed together. This sample number was chosen based on a large scale behavioral analysis of *C. elegans* (Yemini et al., 2013). Data are presented as means ± SD unless otherwise specified. Experimental conditions, such as the electric stimulation or different strains were randomized on a daily basis.

Chapter 3: Results

3.1 *C. elegans*' speed is increased by AC stimulation

Initially, I started this project by studying *C. elegans* behavioral responses to AC stimuli. The animals are known to respond to direct current (DC), migrating along the electric field from the positive end to the negative end (Sukul & Croll, 1978), and a few classes of chemosensory neurons (ASH, ASJ and AWC) were found to be required for their ability to align themselves according to the DC field (Chrisman et al., 2016; Gabel et al., 2007). However, the animal's migratory response to AC stimulus has not been reported yet. In the original setup for this study (Figure 1), several adult wild-type animals were placed onto 9-cm agar plates seeded with a small bacterial food patch and subjected to AC stimulation. The complete trajectories produced by the animals were video-recorded, and their speed was calculated.

I first studied the response to AC stimulation covering a range between 15 and 105 V at 60 Hz (the commercial power frequency in Japan), and found that the animals increased their average speed during electric stimulation by varying amounts (Figure 3). I then conducted a series of systematic analyses with different voltages and frequencies at 30–75 V and 0.25–256 Hz, and noticed that an interesting characteristic of this behavioral phenotype is most apparent when using 4-Hz stimuli: When animals were stimulated with 30 V, their average speed of movement suddenly increased more than 2-fold, and this persisted during and after the electric admission. This behavior was named as the “ON response” (Figure 4A and C). During this running behavior, the animals engage in rapid body bends as well as rapid head movements. In the ON response, I did not detect a statistical bias in direction (Figure 5) Unexpectedly, when a stronger electric stimulus of 75 V was applied, it caused a significant

increase in average speed not during but immediately after the stimulus, which was named the “OFF response” (Figure 4B).

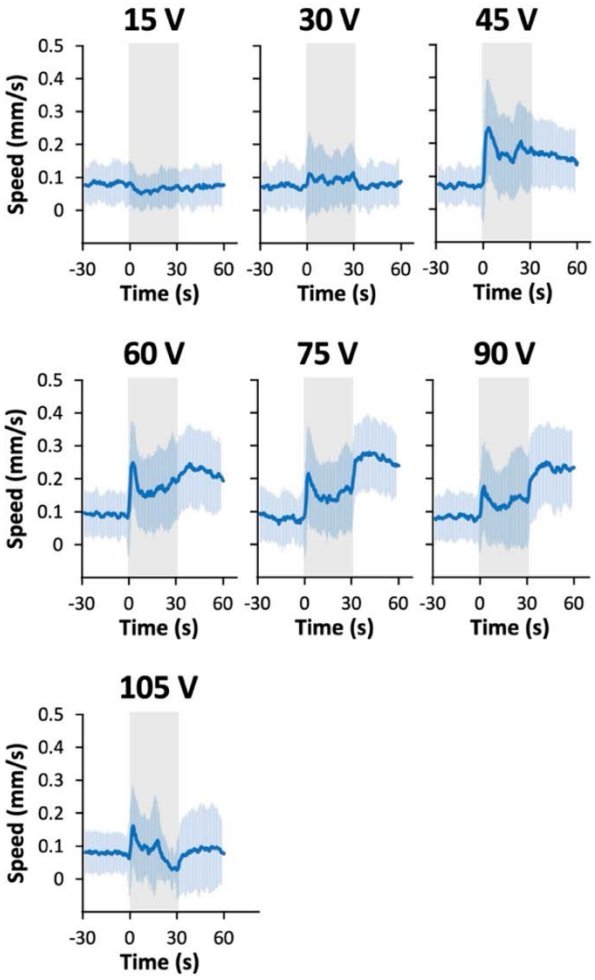

Figure 3. Speed-time graphs with different voltage stimulation at 60 Hz. Gray indicates the duration of electric stimulation (0–30 seconds). The thick line and the shaded region indicate the average ± SD. Sample numbers were 57–58 per condition.

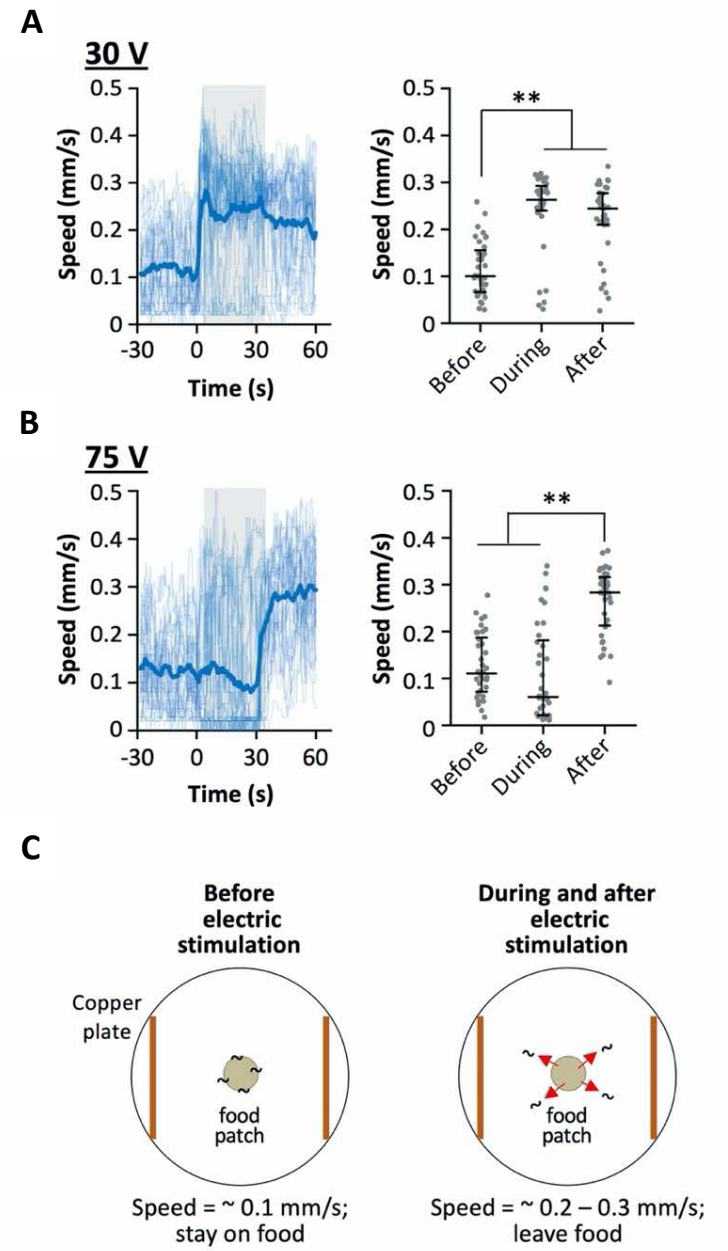

Figure 4. Animals' speed is increased by AC stimulation. **A,** (Left) Speed-time graph with 30-V stimulation at 4 Hz. Thin and thick lines are for individual and average values, respectively. Gray indicates the duration of electric stimulation (0–30 s). (Right) Scatter plot showing average speed of individual animals before, during and after electric stimulation. Each period is 30 seconds. n = 35. **B,** Speed-time graph (left) and scatter plot (right) with 75-V stimulation at 4 Hz. n = 36. **C,** Cartoons of worm's response to the electric shock. (Left) Before electric stimulation, the animals stay on food patch and maintain their speed at around 0.1 mm/second. (Right) During electric stimulation delivery, the animals increase speed to around 0.2–0.3 mm/second and leave the food patch, which persists even after the stimulus is terminated. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. $^{**}P < 0.001$.

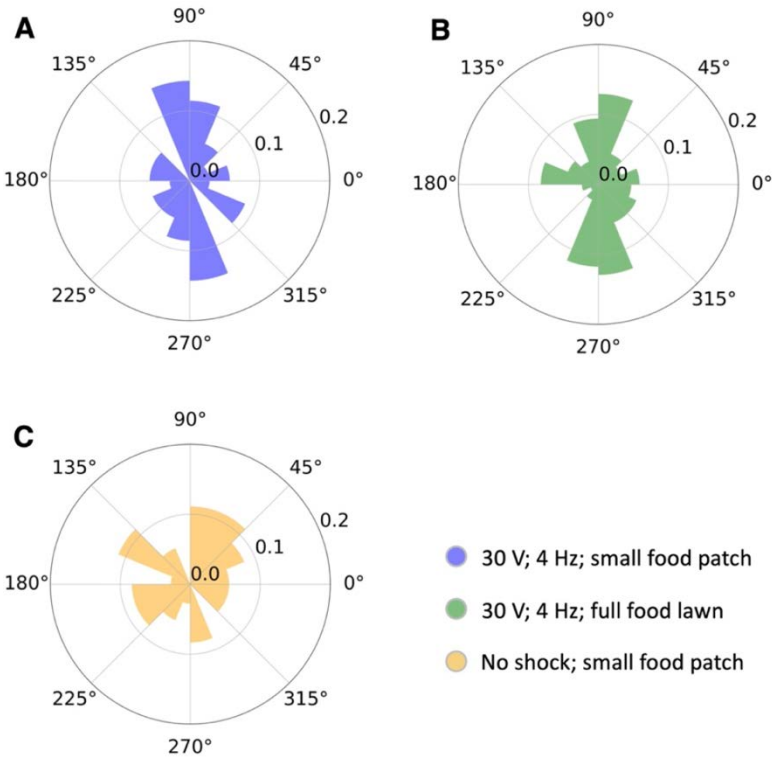

Figure 5. Movement directions of animals during the response. The angles of movement vectors from the beginning to the first 2 minutes of the stimulation were plotted. **A-C,** Rose plot for animals which were assayed on plate with small food patch (**A**, n = 35; Group 1) or full food lawn (**B**, n = 85; Group 2) with 30 V at 4 Hz, or small food patch without electric stimulation (**C**, n = 36; Group 3). Bin number for each chart is set at 16 bins. Statistical analysis performed is Watson U2 test, and *P* values for Groups 1 vs 2, 1 vs 3, and 2 vs 3 were all >0.1.

A fraction of the animals responded during the stimulus in the OFF response condition, while in the majority of the animals, the speed was suppressed during the stimulus and then increased immediately after its removal (Figure 6); this behavioral difference may stem from variation in the threshold required to elicit the response. With other frequencies, ON and OFF responses were also observed but were less clear compared to those with 4 Hz (Figure 7). The range of voltage per length (30–75 V/6 cm = 5–12.5 V/cm) is similar to the range previously shown to elicit responses to DC (3–12 V/cm) (Gabel et al., 2007), suggesting that these electric stimuli are physiologically meaningful for the animals.

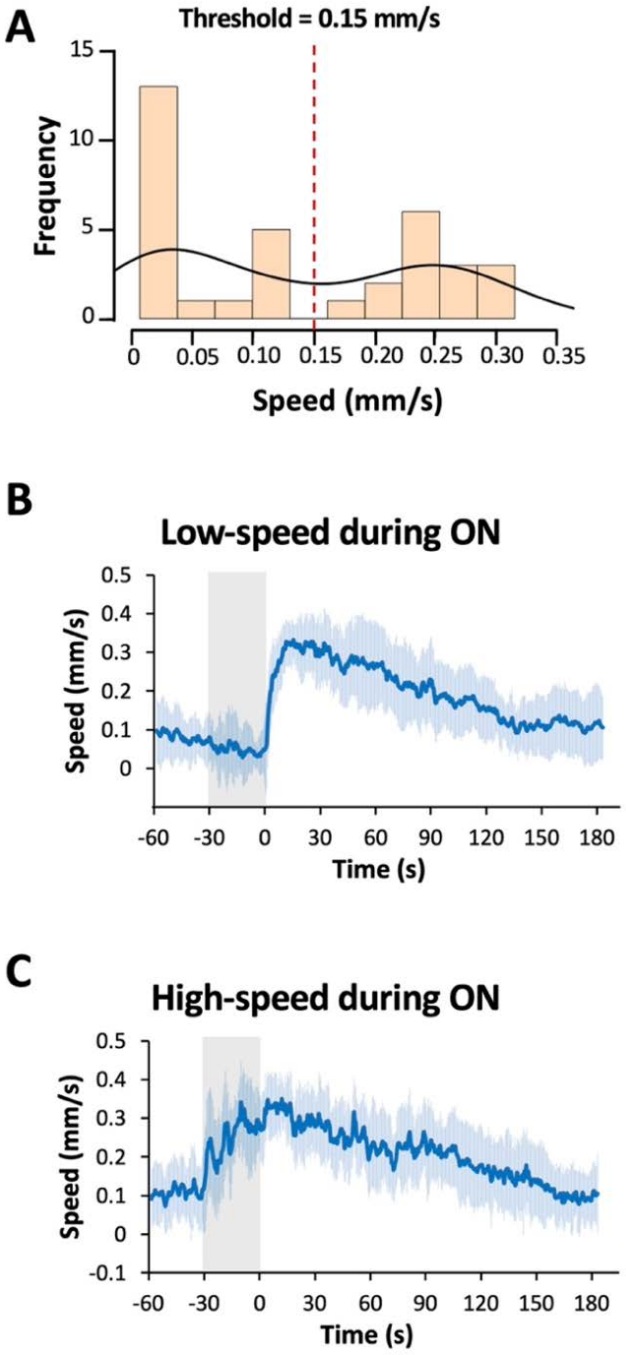

Figure 6. Low and high speed groups during 75 V stimulation. **A**, Histogram and its density (black line) indicates speed of each animal during the electric shock. From the histogram, I set the threshold as 0.15 mm/second to separate the low- (**B**) and high-speed (**C**) groups. Sample numbers were 20 and 15 for lower and higher speed groups, respectively.

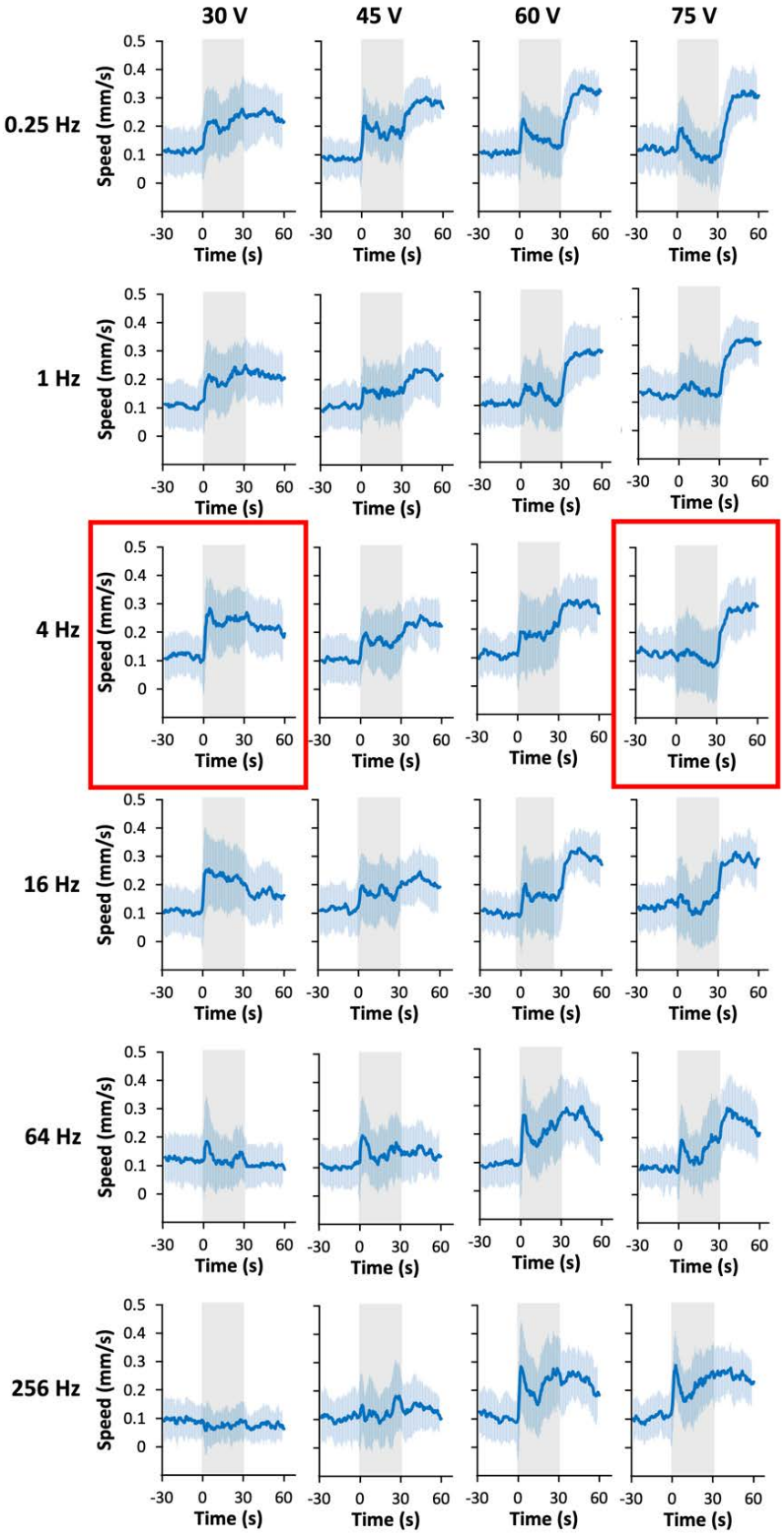

Figure 7. Speed-time graphs with different voltage stimulation at different frequencies. Gray indicates the duration of electric stimulation (0–30 seconds). The thick line and the shaded region indicate the average ± SD. Thirty and 75 V at 4 Hz (red rectangles) were chosen for further analyses. Sample numbers were 33–37 per condition.

3.2 Speed increase lasts for several minutes

Next, I examined how long the increased speed persists during and after the stimulus. When 30 V was applied for 0.5–2 minutes, significant speed increases were maintained during the stimulus, lasted for ~1 minute after the stimulus, then went back to the baseline level (Figure 8A). Interestingly, when 30 V was applied for only 5 seconds, the speed increase still lasted for 1.5 minutes. When 4-minute stimulus was applied, the increase was maintained during the stimulus but went back to the baseline level 30 seconds after the stimulus. During 10-minute stimulation, the significant speed increase was observed only for 5.5 minutes. Thus, I concluded that the ON response caused by 30-V stimulation persists ~5 minutes at most.

This result suggested that the speed increase may decline after several minutes because of fatigue in motor systems. However, animals stimulated intermittently 5 times for 30 seconds per stimulation maintained a speed increase for a much longer time than those under the continuous stimulus (Figure 8B versus "10 minutes" in A). This result supports the idea that the decrease in speed during the long ON stimulation period is not caused by fatigue in the motor system, but possibly by sensory adaptation, which is widely known to adjust the animal's sensory response to new environments (Wark et al., 2007).

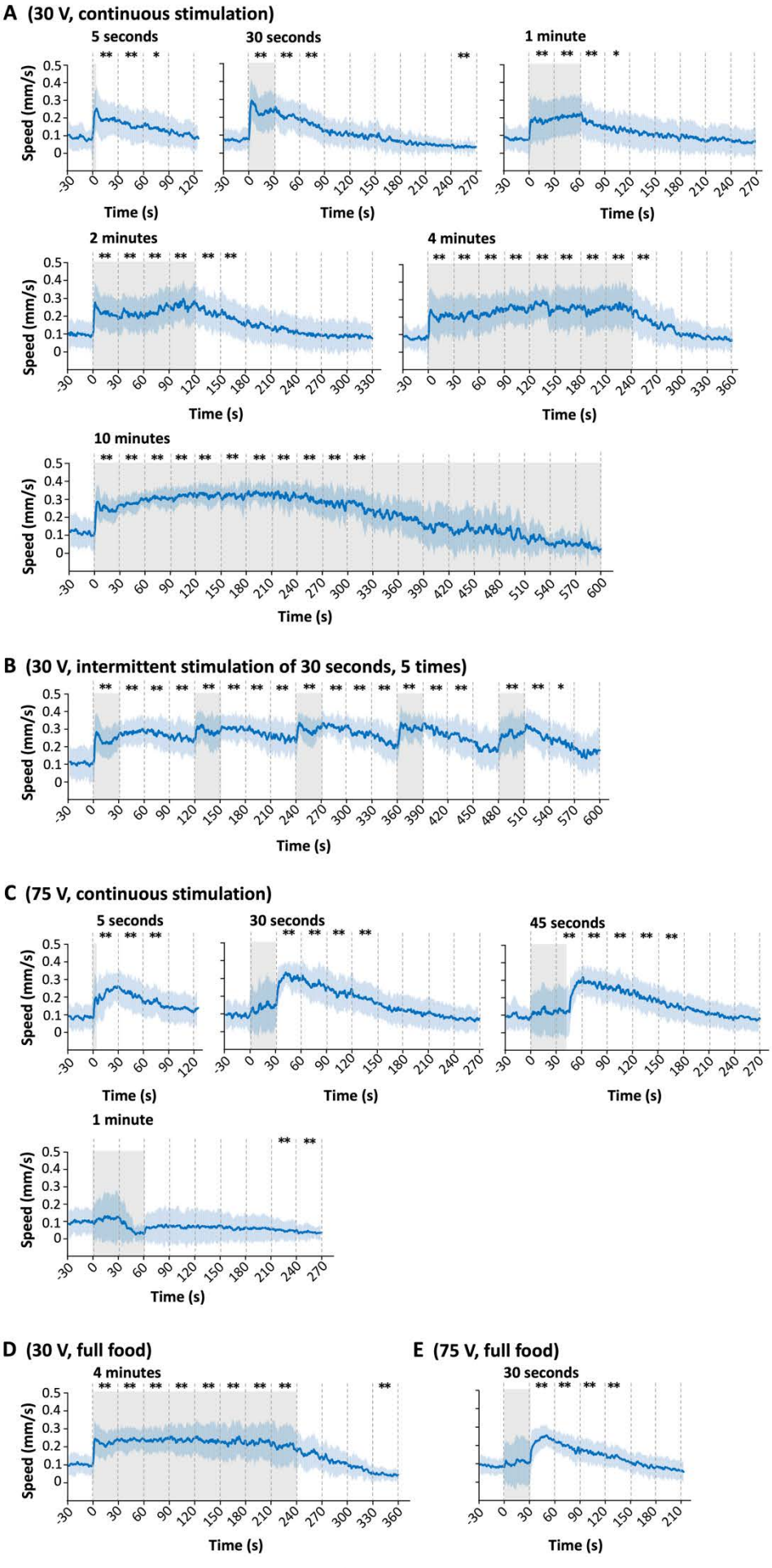
A (30 V, continuous stimulation)
5 seconds
30 seconds
1 minute
2 minutes
4 minutes
10 minutes
B (30 V, intermittent stimulation of 30 seconds, 5 times)
C (75 V, continuous stimulation)
5 seconds
30 seconds
45 seconds
1 minute
D (30 V, full food)
4 minutes
E (75 V, full food)
30 seconds
Speed (mm/s)
Time (s)

Figure 8. Speed increase persisted for minutes even after the stimulation. **A,** Speed-time graphs of ON response with 30-V stimulation of different time periods, ranging from 5 seconds to 10 minutes. **B,** Speed-time graph for intermittent electric stimulation of 30 seconds, 5 times with 90-second intervals. **C,** Speed-time graphs of OFF response with 75-V stimulation of different time periods, ranging from 5 seconds to 1 minute. **D** and **E,** Speed-time graphs for electric stimulation of 30 V for 4 minutes (**D**) or 75 V for 30 seconds (**E**) with animals placed on full food lawn. Shaded regions around the lines represent standard deviation. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction for the differences from the average speed before the stimulation. *$P < 0.01$, **$P < 0.001$. Sample numbers were 32–46 per condition.

I then tested the persistence of speed increase in the OFF response with 75 V. Five- and 30-second stimuli caused similar or longer persistent responses after the stimulus than 30 V did (Figure 8C). Remarkably, 45-second stimulus caused >2 minutes persistent response, which is the longest among the responses to 30- and 75-V stimuli after the stimulus. When animals were stimulated for 1 minute, no ON or OFF responses were observed. The fact that the larger stimulus (75 V) caused longer persistent responses than the smaller one (30 V) suggests that the response to electric shock is "scalable" (i.e. different strength of stimulus causes different strength of behavioral response), one of the critical "emotion primitives" together with persistence (Anderson & Adolphs, 2014).

I next tested the effect of food presence on the speed increase. *C. elegans* move slowly on the bacterial food lawn and faster out of the lawn (Sawin et al., 2000). As I used a small food lawn to localize the animal's initial positions to the center of the plate (Figure 1 and 4C), it was possible that the electric stimulus caused the animals to move away from the food lawn, which then caused increased speed due to the absence of food. If this is the case, the animal's speed would be considerably lower with the electric stimulus when the plates were fully covered with a bacterial lawn. To test this hypothesis, I compared the time course of speed changes on plates with a small patch of food lawn and with a full food lawn. As shown in Figure 8D and E (compare Figure 8A "4 minutes" and C " 30 seconds", respectively), there

was no substantial difference in the time course of speed change between the small food and the full food plates in ON as well as OFF responses, demonstrating that the speed increase is not caused by the food absence but by the electric stimulation itself.

To further confirm that result, I analyzed the animals' speed on a 3-stripe food pattern (Figure 2). I did not observe a significant difference in speed when the animals moved into or out of the food area (Figure 9). This result suggests that the electric stimulus may have negative valence that is more influential to the animal's behavior than the food signal, even though food is critical for their survival. It further suggests that animals prioritize moving away from a harmful condition, such as the electric shock, to protect themselves.

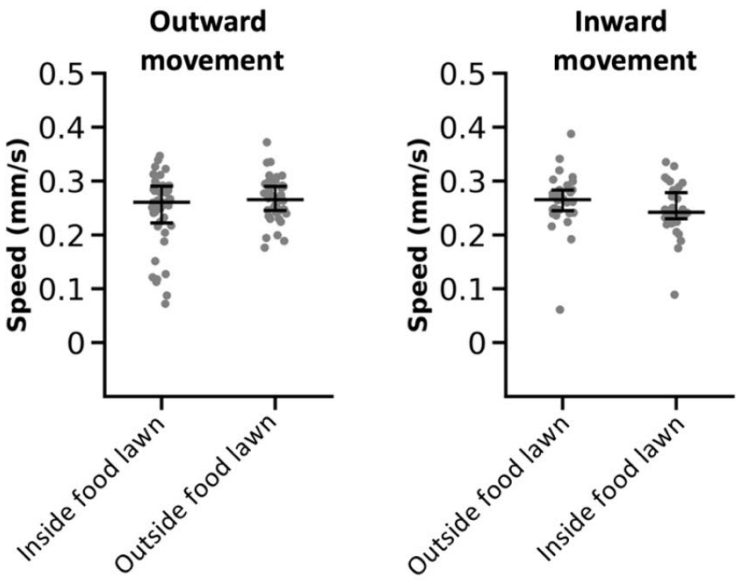

Figure 9. Scatter plot showing average speed of individual animals with outward (left; n = 44) or inward (right; n = 32) movement during 30-V stimulation for 4 minutes. The average speed was calculated 10 seconds before and after the food exit/entry. Statistical analysis was performed by Wilcoxon signed-rank test, and no significant difference was observed.

3.3 Voltage-gated ion channel genes required for the AC response

The molecules required for responses to electric signals have only been revealed in cartilaginous fishes: Bellono et al. (2017, 2018) reported that electrosensory cells in little skate and chain catshark use L-type voltage-gated calcium channels (VGCCs) and voltage-gated big-conductance potassium (BK) channels. To identify gene(s) required for the response to electric shock in *C. elegans*, I analyzed a series of mutant strains of candidate genes. Specifically, I tested mutants of genes involved in the animals' chemo- and mechanosensation, and the homologs of genes involved in electroreception in the cartilaginous fishes.

C. elegans' chemo-sensation is largely mediated by the 12 pairs of amphid sensory neurons in the head, classified into the ones using TAX-2 and TAX-4 cyclic nucleotide-gated channel (CNGC) subunits or the others using OSM-9 and OCR-2 transient receptor potential (TRP) channel subunits for depolarization (Coburn & Bargmann, 1996; Colbert et al., 1997; Komatsu et al., 1996; Tobin et al., 2002). In addition to loss-of-function mutants for the above-mentioned genes, I tested mutants for *che-2*, a gene required for the proper formation and function of the sensory cilia (Fujiwara et al., 1999). For mechanosensation, I analyzed loss- or reduction-of-function alleles of *mec-4*, *mec-10*, and *trp-4*. *mec-4* and *mec-10* genes encode DEG/ENaC proteins and are responsible for the response of touch receptor neurons (Goodman and Sengupta, 2019), while *trp-4* encodes TRPN (NOMPC) for harsh touch response (Kang et al., 2010). These mutant strains exhibited wild-type- like responses (Figure 10 and 11, panels A and C); some mutants (*osm-9;ocr-2*, *che-2*, *mec-4*, *mec-10*, and *trp-4*) exhibited statistical differences in either ON or OFF response, suggesting the partial involvement of these genes, although the defects in speed increase (i.e. Δspeed) were not as severe as the ones of VGCC mutants (see next page). The noninvolvement of *tax-4* also

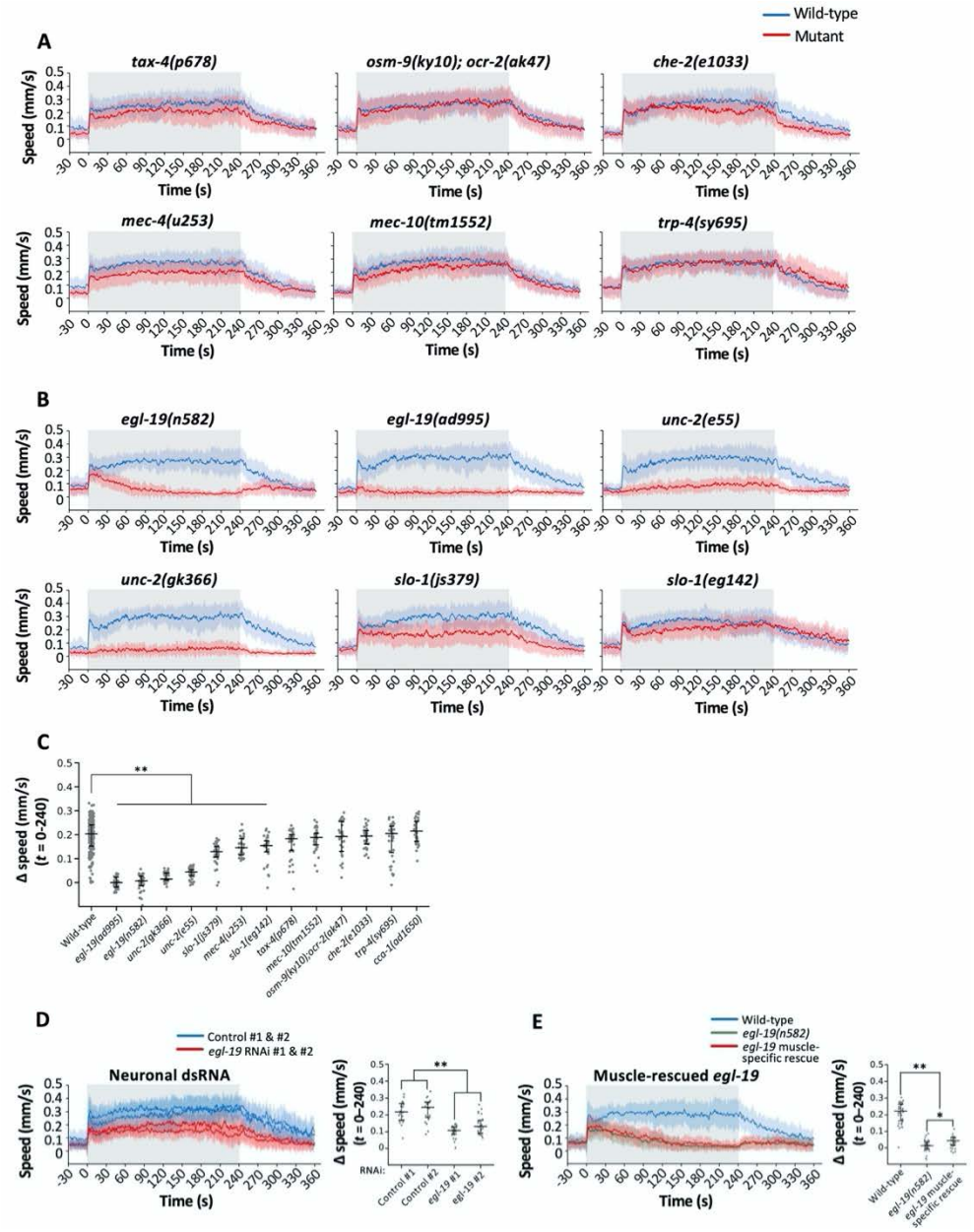

Figure 10. Genetic analysis of ON response. **A** and **B,** Speed-time graphs of ON response with 30-V stimulation of 4 minutes on mutants of sensory signaling (**A**) and of voltage-gated channels (**B**). **C**, Scatter plot showing Δspeed of individual animals during 4 minutes of the stimulation (i.e. t = 0–240 seconds). In a series of daily experiments, wild-type animals and 3 to 5 mutant strains were analyzed in parallel. All the wild-type data are combined, and the mutant strains are arranged in ascending order of median values in **C**. **D**, Speed-time graphs (left) and scatter plot showing Δspeed (right) of ON response with 30-V stimulation of 4 minutes of 2 independent transgenic strains with (red) or without (blue) dsRNA of *egl-19* expressed under pan-neuronal promoter. **E**, Speed-time graphs (left) and scatter plot showing Δspeed (right) of ON response with 30-V stimulation of 4 minutes of wild-type (blue) or *egl-19(n582)* (green), or *egl-19(n582)* animals expressing the *egl-19* minigene only in muscles (red). Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. *P < 0.01, **P < 0.001. Sample numbers were 20–36 per mutant strain.

indicates that the temperature increase caused by the electric stimulus is not responsible for the speed increase.

I then tested *egl-19*, the ortholog of the L-type VGCC alpha subunit (Lee et al., 1997), which functions in the sensory organ for environmental electric signals for cartilaginous fishes (Bellono et al., 2017, 2018). I found that 2 reduction-of-function alleles of *egl-19* mutants exhibited strong defects in ON and OFF responses (Figure 10 and 11, panels B and C).

Because *egl-19* is expressed in many neurons as well as muscles, a series of experiments were performed to clarify whether *egl-19* functions in neurons or in muscles. My laboratory member and I conducted RNAi using the expression of double-stranded *egl-19* RNA under a pan-neuronal promoter, which causes neuron-specific RNAi (Esposito et al., 2007). As shown in Figure 10D, this caused significant suppression of the ON response. Furthermore, expressing an *egl-19* minigene only in the muscles of *egl-19* mutants (Gao & Zhen, 2011) did not rescue the phenotype (Figure 10E). All of these results are consistent with the idea that *egl-19* functions in neurons but not in muscles. One allele of *egl-19* mutants exhibited movement speed comparable to wild-type animals before stimulation (Figure 12), suggesting that the defect in the speed increase is not caused by a problem in the basal locomotory system.

The involvement of *egl-19* in the response to electric shock further motivated I to test two other types of VGCCs, namely, N-type (UNC-2) and T-type (CCA-1) VGCCs (Schafer & Kenyon, 1995; Steger et al., 2005), although only L-type VGCC had been found to be involved in electrical responses in the cartilaginous fishes. Unexpectedly,

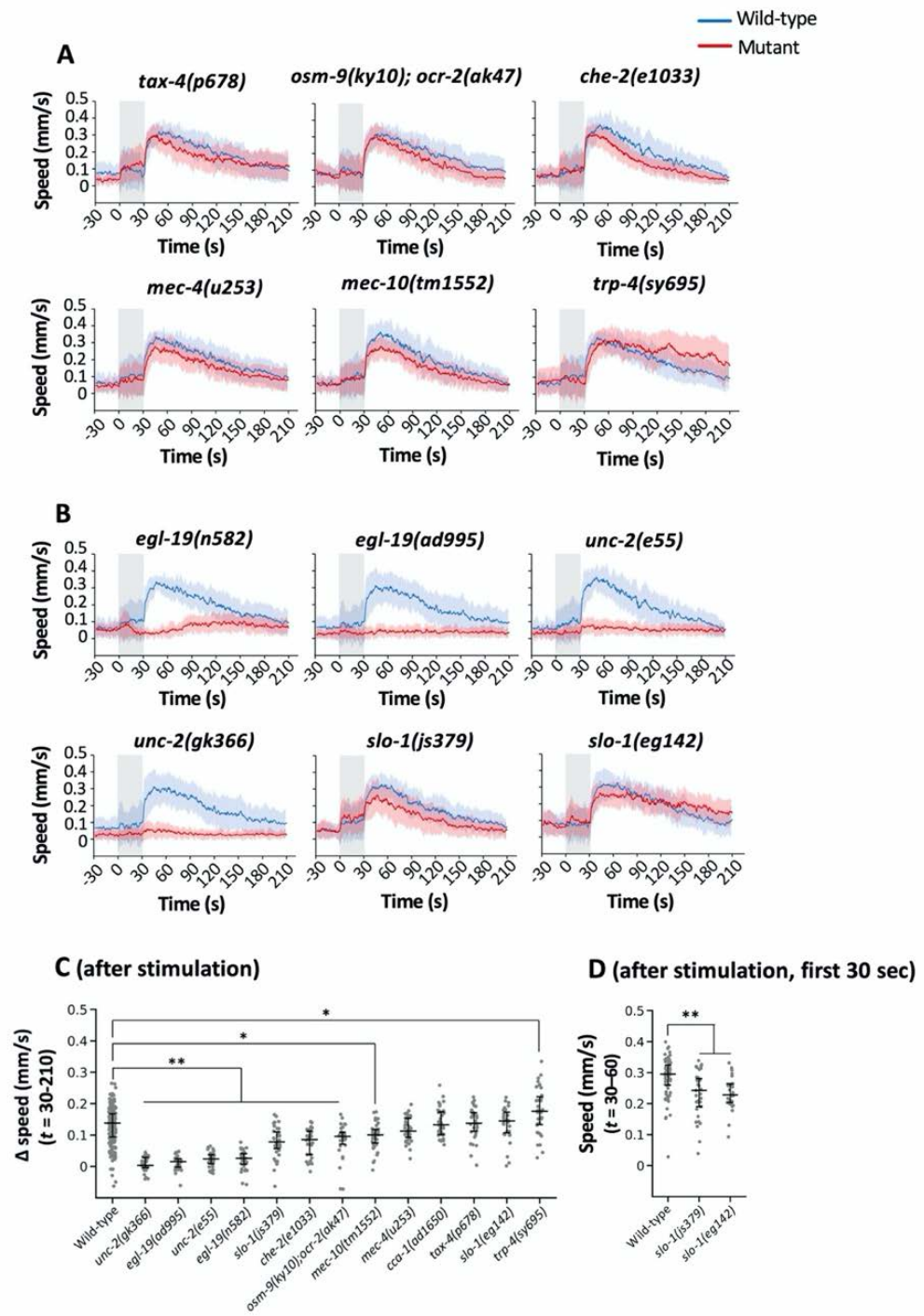

Figure 11. Genetic analysis of OFF response. **A** and **B,** Speed-time graph of OFF response with 75-V stimulation of 30 seconds on mutants of sensory signaling (**A**) and of voltage-dependent channels (**B**). **C**, Scatter plot showing Δspeed of individual animals during 3 minutes after the stimulation (i.e. t = 30–210 seconds). In a set of daily experiments, wild-type and 3 to 5 mutant strains were analyzed in parallel. All the wild-type data are combined, and the mutant strains are arranged in ascending order of median values in **C**. **D**, Scatter plot showing the average speed of individual wild-type and *slo-1* mutant animals during 30 seconds after the stimulation (i.e. t = 30–60 seconds). Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. *P < 0.01, **P < 0.001. Sample numbers were 30–36 per mutant strain.

mutants for 2 alleles of *unc-2* were defective in both ON and OFF responses, while *cca-1* mutants behaved similar to the wild-type controls (Figure 10 and 11, panels B and C, and Figure 13).

I then investigated the involvement of the BK channel, a voltage-gated potassium channel, also known to be involved in electro-sensation in cartilaginous fish (Bellono et al., 2017, 2018). Interestingly, 2 alleles of *slo-1*, the sole ortholog of BK channels in *C. elegans* (Davies et al., 2003; Wang et al., 2001), also exhibited statistical differences in the ON as well as the OFF response, at least in some aspects (Figure 10B and C for ON response, and 11B–D for OFF response). The possible involvement of BK channels in addition to the L-type VGCC in *C. elegans'* electrical response suggest that the molecular mechanisms of electro-sensation may be evolutionally conserved, although a novel component (N-type VGCC) is also involved.

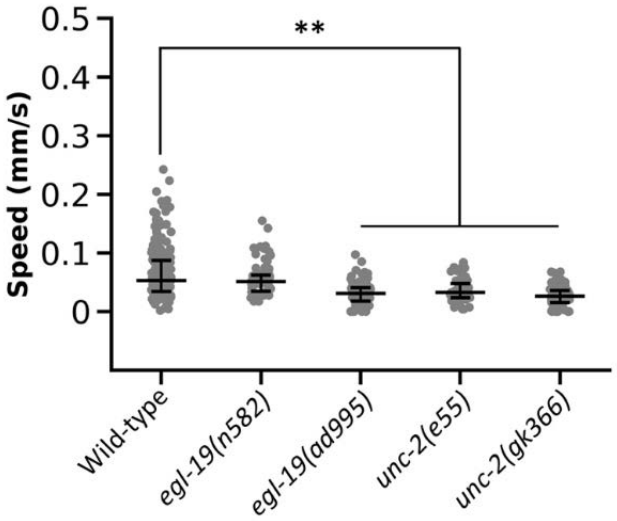

Figure 12. Basal speeds of wild-type and VGCC mutants before the 30-V and 75-V stimulations. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. $^{**}P < 0.001$.

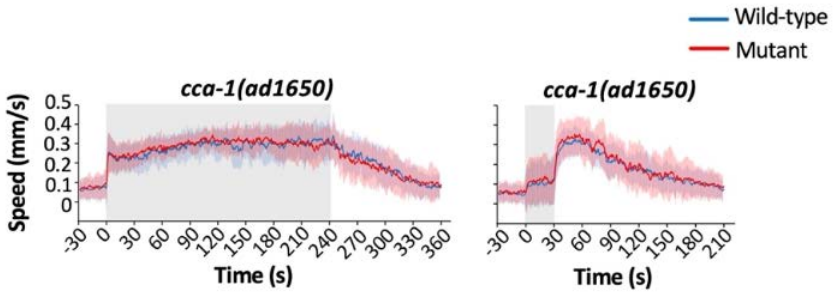

Figure 13. Speed-time graphs of ON (30 V, left) and OFF (75 V, right) responses of *cca-1*.

3.4 Neuropeptide signaling down-regulates the duration of persistent response

Lastly, I attempted to identify genes required for behavioral persistency, and considered the genes involved in the biosynthesis of neuromodulators as candidates. I tested *cat-2* (dopamine), *tph-1* (serotonin), *bas-1* (dopamine and serotonin) and *tdc-1* (tyramine and octopamine) mutant animals (Alkema et al., 2005; Lints & Emmons, 1999; Loer & Kenyon, 1993; Sze et al., 2000), and most of these mutants exhibited wild-type-like responses, indicating that these neuromodulators are not involved (Figure 14A, B, G, and H); although *tph-1* mutants exhibited a statistical difference in OFF response, its contribution does not appear substantial. Because dopamine and serotonin signaling are known to be required for the feeding status-dependent modulation of migratory speed, these results are also consistent with the fact that feeding status is not the causal reason for the speed increase (Figure 8D and E, and Figure 9).

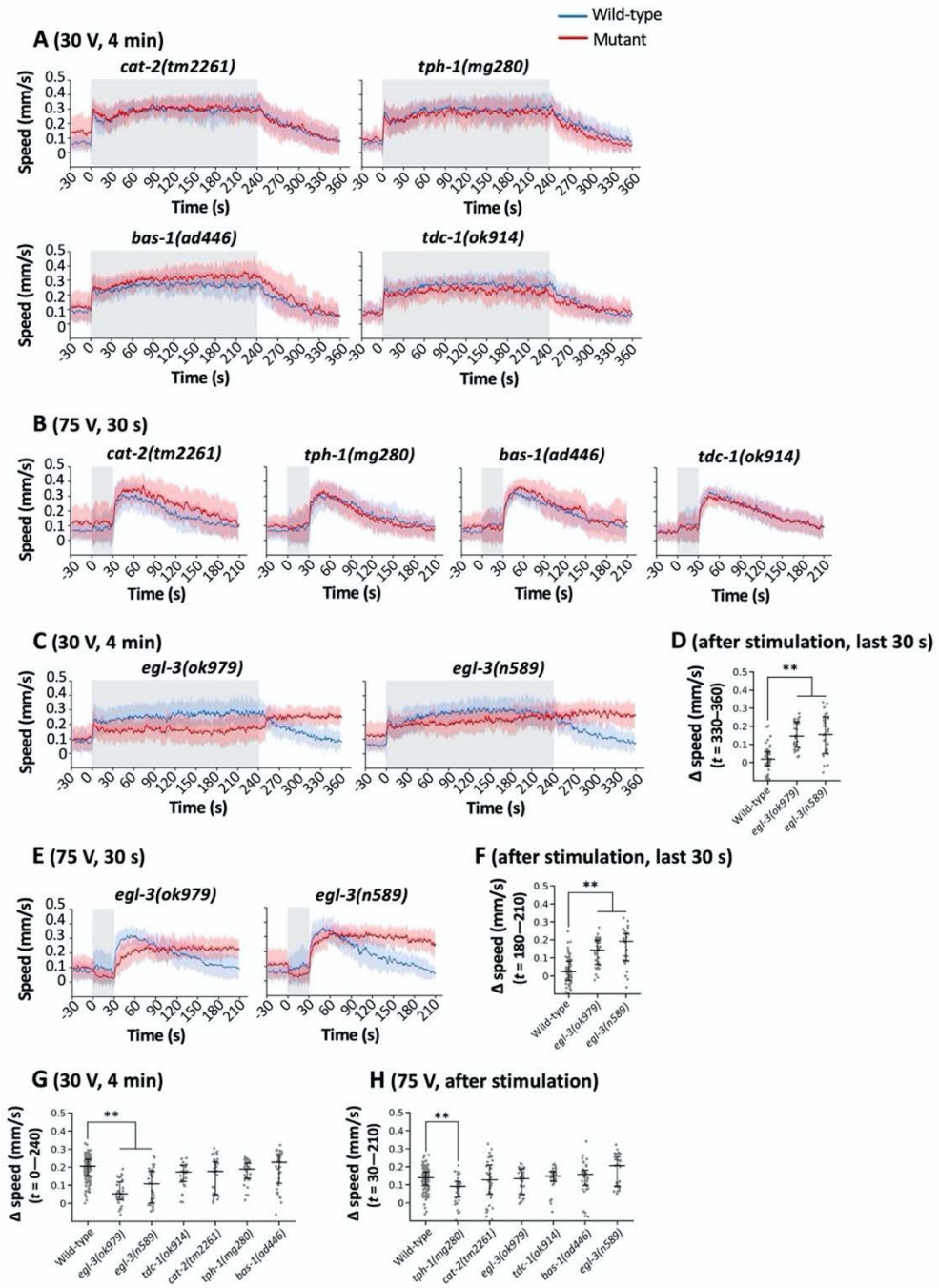

Figure 14. Neuropeptides, but not other neuromodulators, are involved in the regulation of response persistence. **A** and **B,** Speed-time graphs of ON response with 30-V stimulation of 4 minutes (**A**) or OFF response with 75-V stimulation of 30 seconds (**B**) on mutants of biogenic amine biosynthesis. **C** and **D,** Speed-time graphs (**C**) and scatter plot showing Δspeed (**D**) of ON response with 30-V stimulation of 4 minutes on 2 alleles of *egl-3* mutants. **E** and **F,** Speed-time graphs (**E**) and scatter plot showing Δspeed (**F**) of OFF response with 75-V stimulation of 30 seconds on 2 alleles of *egl-3* mutants. The time used for scatter plot was t = 330–360 seconds (**D**) or 180–210 seconds (**F**). **G** and **H**, Scatter plot showing Δspeed of individual animals during 4 minutes of (**G**) or 3 minutes after (**H**) the stimulation (i.e. t = 0–240 seconds or t = 30–210 seconds, respectively). Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. $^{**}P < 0.001$. Sample numbers were 29–36 per mutant strain.

I then further tested the involvement of neuropeptides by using loss- or reduction-of-function mutations of *egl-3*, a gene required for maturation of pro-neuropeptides (Kass et al., 2001). Unexpectedly, mutations in both alleles of *egl-3*, *n589* and *ok979*, caused weaker 30-V ON response and, moreover, much longer persistence of the speed increase after the electric shock in ON and OFF responses (Figure 14C–H), indicating that the persistent activity in the neural circuit for speed increase is down-regulated by neuropeptide signaling in the wild-type animals.

Chapter 4: Discussion

In the present study, I revealed that *C. elegans* exhibits a persistent speed increase in response to AC stimuli. This behavioral response appears characterized by persistence, scalability, and valence, suggesting that it may reflect an emotional state of *C. elegans*, which has never been reported. In addition, genetic analysis revealed that genes involved in electro-sensation in cartilaginous fishes and a neuropeptide biosynthesis gene are required for the response, demonstrating that this behavior is an ideal paradigm for genetic dissection of both electro-sensation and persistent behavioral states.

4.1 Response to electric stimulus and its mechanisms in *C. elegans* and other animal species

In neuroscience research, electricity is used as an unconditioned stimulus with negative valence to cause associative learning in rodents and flies (Quinn et al., 1974; Rescorla, 1968). In nature, however, multiple animal species are known to respond to electricity for survival purposes, such as communication, navigation and/or prey detection (Crampton, 2019; Pettigrew, 1999). For example, weakly electric African fish (*Gnathonemus petersii*) utilize their epidermal electroreceptors to receive self-produced electric signals, allowing the fish to identify, locate, and examine nearby objects (von der Emde et al., 2008). In addition, platypuses (*Ornithorhynchus anatinus*) detect electric signals via their duck-like bills to locate and avoid objects when navigating in the water (Scheich et al., 1986). Blind cave salamanders (*Proteus anguinus*) perceive a moving back-and-forth direct-current field and its polarity via ampullary organs to survive and navigate in their environment, which is in complete darkness as their eyes are undeveloped (Istenič & Bulog, 1984; Roth & Schlegel, 1988). In invertebrates, bumblebees (*Bombus terrestris*) sense environmental electric fields

via sensory hairs to make foraging decisions (Clarke et al., 2013; Sutton et al., 2016). In a recent study, *C. elegans* is also shown to exhibit phoretic attachment to other insects by nictating and transferring across DC electric fields (Chiba et al. 2023). Such diverse usage of electric signals, across a range of animal taxa, suggests that detecting and responding to electric signals is of broad importance, yet the underlying molecular mechanisms remain poorly understood.

In this study, I established an original experimental paradigm and found that *C. elegans* responds to an AC electric stimulus: The animals significantly increase their movement speed during and after the stimulus for minutes. Although the animals have also been reported to respond to and utilize DC (Chiba et al. 2023; Chrisman et al., 2016; Gabel et al., 2007), I consider that the responses to AC and DC are substantially different for the following reasons. (1) In the DC field, the animals moved at a certain angle (~4° per 1 V/cm), which was not observed in this AC stimulus (Figure 5). (2) Movement speed did not change with the DC stimulus (Gabel et al., 2007). (3) Another DC response of worms, described as "nictating-and-leaping", involves worms being moved passively by the electricity (Chiba et al. 2023), whereas the AC response I report is actively directed by the worm.

In addition, although 3 pairs of amphid sensory neurons play important roles in the DC response (Chrisman et al., 2016; Gabel et al., 2007), mutations in genes required for sensory signaling in amphid sensory neurons (*tax-4*, *osm-9*, *ocr-2*, and *che-2*) did not affect the AC response substantially in our study (Figure 10 and 11), indicating that DC and AC responses utilize different sensory mechanisms. This result also rules out the possibility that the animals respond to increased agar temperature due to the AC stimulus, because *tax-4* is essential for temperature sensation (Komatsu et al., 1996). The genes required for mechanosensation

(*mec-4, mec-10*, and *trp-4*) do not seem to play a critical role in the AC response either. Still, it is possible that the AC stimulus is sensed by multiple types of sensory neurons redundantly.

I found that the VGCC and possibly the BK channel, the voltage-gated calcium and potassium channels for electro-sensation in cartilaginous fishes, are involved in the AC response of *C. elegans*. The involvement of multiple types of voltage-gated channels, homologous across distantly related species, in the sensation of electricity suggests that this mechanism is evolutionarily conserved. It also suggests that EGL-19 and SLO-1 may function coordinately in a subset of neurons that sense electricity. Indeed, our data indicate that *egl-19* functions in neurons instead of muscles in this behavioral response (Figure 10D and E). Since *egl-19* and *slo-1* are widely expressed in most neurons (Davies et al., 2003; Lee et al., 1997; Wang et al., 2001), it would be interesting to identify the neurons where these genes function to sense the electric signals. It should be noted that the N-type VGCC UNC-2 is also essential for the response to electric shock (Figure 10B and 11B), suggesting that mechanisms of electric sensation may be more diverse among animal species.

4.2 Electric stimulus causes persistent behavioral response

Persistent neural activity, a sustained neural activity caused by a short-term stimulus, plays critical roles in brain function, such as controlling motivation, arousal, and emotion as well as working memory and decision-making, although its detailed mechanisms have not been sufficiently elucidated (Anderson, 2016; Berridge, 2004; Curtis & Lee, 2010; Major & Tank, 2004). Persistent behavioral state is caused by persistent neural activity, suggesting that genetic analysis of persistent behavioral state can reveal molecular mechanism(s) of persistent neural activity that underlies brain functions.

I unexpectedly found that *C. elegans*' high speed response persists after electric shock. In *C. elegans*, 2 other types of persistent behavioral responses related to speed change have been reported. The first is that the animal's movement speed is elevated at high O_2 concentration in *npr-1(lf)* and in the Hawaiian wild isolate CB4856, which has the same amino acid variation in *npr-1* gene (Cheung et al., 2005). In this behavioral response, (1) the elevated speed returns rapidly to the basal speed when the high O_2 is terminated, (2) the animals still recognize and aggregate at the edge of a food lawn, and (3) a mutation in the *tax-4* CNGC homolog for sensory depolarization abolishes the response (Coates & de Bono, 2002). Another type of persistent behavioral response is roaming (Flavell et al., 2020; Fujiwara et al., 2002). Roaming is a behavioral state with high movement speed, although it is only exhibited when the animals are on food and requires serotonin signaling. Because the behavioral response to electric shock persists more than 2 minutes after 30–45 seconds stimulus with 75 V and more than 1.5 minutes after only 5-second stimulus, is not affected by food stimulus, and does not require CNGC activity or serotonin signaling, electric shock response is likely different from the two above-mentioned behavioral responses, and its analysis may provide a unique opportunity for genetic dissection of a persistent behavioral state and neural activity.

Interestingly, I revealed that the persistent aspect of the behavioral response is down-regulated by *egl-3*, a gene required for maturation of pro-neuropeptides (Kass et al., 2001), which affects biosynthesis of FMRFamide-like peptide (FLP) and neuropeptide-like protein (NLP), but not insulin-like peptides (ILP) (Husson et al., 2007). Because the requirement of neuropeptide signaling is reminiscent of neuropeptide regulation of fear in mammals including humans (Bowers et al., 2012; Comeras et al., 2019; van den Burg & Stoop, 2019), the fear-like brain state may be regulated by evolutionarily conserved molecular mechanisms.

Electric shock is widely utilized as an unconditional stimulus in fear conditioning paradigms, especially in rodents, where less than 1 mA of current for a few seconds is generally used (Korte et al., 1999; Toth et al., 2012). Thus, the conditions used in this study (80-200 mA in current) may appear artificial. However, I consider that the responses of *C. elegans* to these stimuli reflect physiologically meaningful biological mechanisms for the following reasons: (1) The range of voltage per length (30–75 V/6 cm = 5–12.5 V/cm) is similar to the one used to study the animal's DC response (3–12 V/cm) (Gabel et al., 2007). (2) The electric current flowing inside the worm's body could be weak because it depends on the resistance of its body and cuticle. (3) Only a 5-second stimulus causes a persistent response that lasts more than a minute, meaning that the electric shock itself is just a trigger and what I observe is a physiological response to that trigger. The speed increase behavior that is observed may resemble fleeing, one of the most common responses caused by fear in higher animals and humans (Adolphs, 2013; Bliss-Moreau, 2017; Mobbs & Kim, 2015).

4.3 Response to the electric stimulus may reflect a form of emotion

Emotions are internal brain states triggered by certain types of environmental stimuli, which are associated with cognitive, behavioral, and physiological responses (Abbott, 2020; Anderson & Adolphs, 2014; Nettle & Bateson, 2012; Perry & Baciadonna, 2017). Recently, multiple species of invertebrates are considered to possess internal brain states that resemble what is considered to be emotions (Bacqué-Cazenave et al., 2017; Fossat et al., 2014; Gibson et al., 2015; Hamilton et al., 2016; Mohammad et al., 2016; Solvi et al., 2016). One of the most prominent characteristics of emotion across animal species is its persistence: For example, even a transient environmental stimulus can cause a persistent behavioral response, such as courtship, aggressive, and defensive behavior (Abbott, 2020; Anderson & Adolphs, 2014; Nettle & Bateson, 2012; Paul & Mendl, 2018; Perry & Baciadonna, 2017).

Anderson and Adolphs proposed a new framework to study emotions across animal species, wherein hallmarks of an emotional state are persistence, scalability, valence, and generalization. In addition to persistence (Figure 8A–C), I consider that the electric response has negative valence. This is because the animals ignore food during the electric shock response (Figure 8D and E, and Figure 9), despite the fact that food is one of the most influential signals for *C. elegans*, affecting many aspects of their behavior. For example, during the high speed state caused by high O_2, animals still recognize and stay at the edge of a food lawn (Cheung et al., 2005; Coates & de Bono, 2002), suggesting that the electric shock signal has a strong negative valence that overrides the strong positive valence of food. The third point is the scalability—stronger stimulus causes stronger behavioral response. Compared to the 30-V stimulus, the 75-V stimulus results in a longer-lasting high speed response after the stimulus (Figure 8A and C). The fourth point is generalization – the same emotional state can be triggered by different stimuli and, in turn, the emotional state triggered by one stimulus can then affect responses to other stimuli. The lack of response to food during and following the electric stimulus might support this point as well, as the emotional state induced by electricity influences the response to food, an entirely different stimulus. Taken together, these results may suggest that the animal's response to electric shock represents a form of emotion, possibly akin to fear.

Chapter 5: Conclusions and Future Prospects

In summary, I found that *C. elegans* persistently responds to electric shock, which is regulated by voltage-gated ion channels and neuropeptide signaling. Our findings suggest the following model (Figure 15). When the animal sense 30- or 75-V AC stimulus at 4 Hz, the stimulus is sensed with the VGCC and BK channel and their internal state transits from basal speed state to persistent high speed state. The persistent high speed state eventually returns to the basal speed state, which requires neuropeptide signaling.

By taking advantage of connectome information and the methods for imaging whole brain activity of identified neurons (Randi & Leifer, 2020; Wen et al., 2021; White et al., 1986; Yemini et al., 2021), *C. elegans* may become one of the ideal models for revealing the dynamic information processing involved in the entire neural circuit related to emotion.

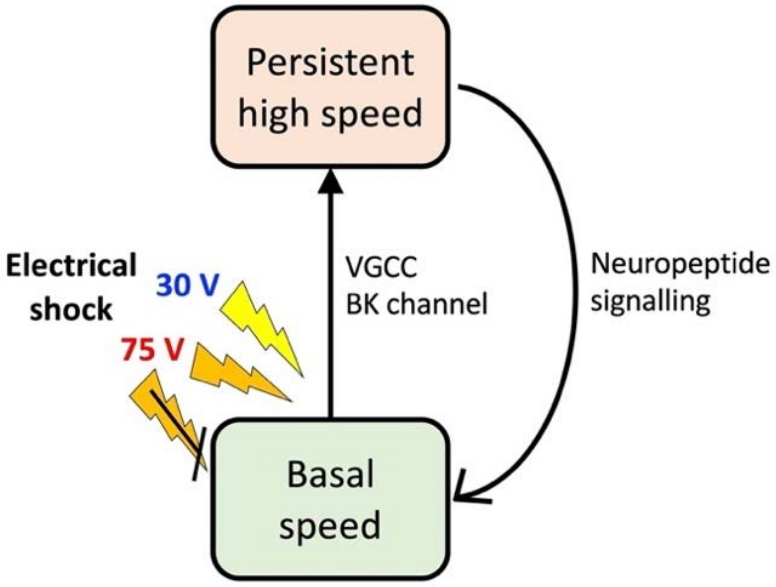

Figure 15. Model for mechanism of speed increase caused by electric shock.

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List of Scientific Publications

Tee, L. F., Young, J. J., Maruyama, K., Kimura, S., Suzuki, R., Endo, Y. & Kimura, K. D. (2023). Electric shock causes a fleeing-like persistent behavioral response in the nematode *Caenorhabditis elegans*. *Genetics*, iyad148.

List of Conference Presentations

Tee, L. F., Young, J. J., Suzuki, R., Maruyama, K., Endo, Y. & Kimura, K. D. Electric shock causes a fear-like persistent behavioral response in the nematode Caenorhabditis elegans. 9th Asia-Pacific Worm Meeting 2022. 20 July 2022, online meeting (Oral presentation).

Tee, L. F., Young, J. J., Maruyama, K., Suzuki, R. & Kimura, K. D. Toward the understanding of molecular mechanism of electrical sensation and response. 23rd International C. elegans conference. 22 June 2021, online meeting (Oral presentation).

Tee, L. F., Young, J. J., Maruyama, K., Suzuki, R. & Kimura, K. D. Electric shock causes a fear-like persistent behavioral response in the nematode Caenorhabditis elegans. Kansai Worm Meeting. 10 March 2022, online meeting (Oral presentation).

Tee, L. F., Young, J. J., Suzuki, R., Maruyama, K. & Kimura, K. D. Toward the understanding of molecular mechanism of electrical sensation and response. Kansai Worm Meeting. 9 January 2021, online meeting (Oral presentation).

Tee, L. F., Young, J. J., Maruyama, K., Suzuki, R. & Kimura, K. D. Toward the understanding of molecular mechanism of electrical sensation and response using the nematode C. elegans. The 44th Annual Meeting of the Japan Neuroscience Society. 29 July 2021, online meeting (Oral presentation).

Tee L. F., Young, J. J., & Kimura. K. D. Toward the understanding of mechanism of electrical sensation using the roundworm *C. elegans* as a model animal. NCU Contact Points in Asia Symposium 2019. 6 December 2019, Nagoya City University Hospital, Aichi, Japan (Oral & poster presentation).