

Title: Electric shock causes a fear-like persistent behavioral response in the nematode

Caenorhabditis elegans

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ABSTRACT

Electricity is utilized as an environmental stimulus by many animal species. Despite its importance, however, molecular and physiological mechanisms for responding to electric stimuli are poorly understood compared to other sensory stimuli. Here we report novel behavioral responses to electric stimuli in the *Caenorhabditis elegans*. When the animals on food are stimulated by alternating current, their movement speed suddenly increases more than 2-fold, which persists for minutes even after the electric stimulation is terminated. Genetic analyses reveal that multiple types of voltage-gated channels are required for the response, possibly as the sensors, and neuropeptide signaling regulates the duration of persistent response. Additional behavioral analyses indicate 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 the animal's response to electric shock may express a form of emotion, such as fear.

INTRODUCTION

In order to survive and reproduce, animals respond to various environmental sensory stimuli by perceiving and processing these cues within a neural circuit, and converting them into behavioral outputs. In addition to well-known stimulus cues, such as light, sound, chemicals and temperature, some animal species respond to other stimuli, such as magnetic fields and electricity (Collin, 2019; Wiltschko & Wiltschko, 2005).

In neuroscience research, electricity is used as an unconditioned stimulus with negative valence to cause associative learning in rodents and in 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 (Clarke et al., 2013; 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, platypus (*Ornithorhynchus anatinus*), blind cave salamander (*Proteus anguinus*), and bumblebees (*Bombus terrestris*) are also known to sense electric signals for navigation and/or foraging (Clarke et al., 2013; Roth & Schlegel, 1988; Scheich et al., 1986). Such wide use of electric signals in the animal kingdom suggests that the molecular mechanisms of electricity perception as well as the neural circuits to utilize the perceived information have independently emerged or diverged during evolution. Despite their importance, the molecules required for responses to electric signals have only been revealed in sharks and skates: Bellono et al. reported that electrosensory cells in little skate and chain catshark use L-type voltage-gated calcium channels (VGCC) (Bellono et al., 2017, 2018).

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). 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 and ASJ) were found to be required for their ability to align themselves according to the DC field (Gabel et al., 2007). The animals are also reported to respond to strong alternating current (AC)—they exhibit a "convulsant" phenotype (paralysis and elongation) upon delivery of a brief electric shock (200 Hz, 3.5 ms, 47 V) and recover rapidly after removal of the electric shock (Risley et al., 2016). However, other behavioral responses as well as molecular mechanisms for electric signals have not been revealed.

In this study, we report that *C. elegans* responds to AC electric stimulus by immediately increasing their speed. The speed increase lasts for minutes even after an electric stimulus of seconds is terminated, suggesting that the response is caused not by direct stimulation of the motor system for rapid movement but possibly by persistent activity of a specific set of neurons to generate the behavioral response. Further behavioral analysis revealed that the effect of electric shock increases when the shock amplitude is increased (i.e., scalable). In addition, the increased speed is not significantly affected by the presence or absence of food, which is the most important environmental stimulus for the animals and is known to affect movement speed of animals, suggesting the electric shock has a negative valence that is stronger than the positive valence of food signal. 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 three of four key features of animal emotions

proposed by Anderson and Adolphs (Anderson & Adolphs, 2014). A series of candidate genetic analyses reveal that the response is not mediated by well-known chemo- or mechanosensory mechanisms. Instead, it requires both L-type VGCC, as in the shark and skate, and N-type VGCC, which have not previously been implicated in animal electrical responses. Furthermore, we find that neuropeptide signaling regulates the duration of persistence. These results indicate that the animals' response to electric shock can be a suitable paradigm to reveal molecular and physiological mechanisms of electrosensation as well as emotion, such as fear.

RESULTS

Worms' speed increases by AC stimulation

In order to study *C. elegans*' response to electric shock, we established a setup (Fig. 1), where several adult wild-type animals were placed onto 9 cm NGM 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 based on the *x-y* coordinates of animals' centroids in each image frame.

We first studied the response to AC stimulation covering a range between 15 - 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 (Supplementary Fig. 1). We then conducted a series of systematic analyses with different voltages and frequencies at 30–75 V and 0.25–256 Hz (Supplementary Fig. 2). After the analysis, we noticed that an interesting characteristic of this behavioral phenotype is most apparent when using 4 Hz stimuli: When worms were stimulated with 30 V, their average speed of movement suddenly increased more than 2-fold, and this persisted during the electric admission. We named this

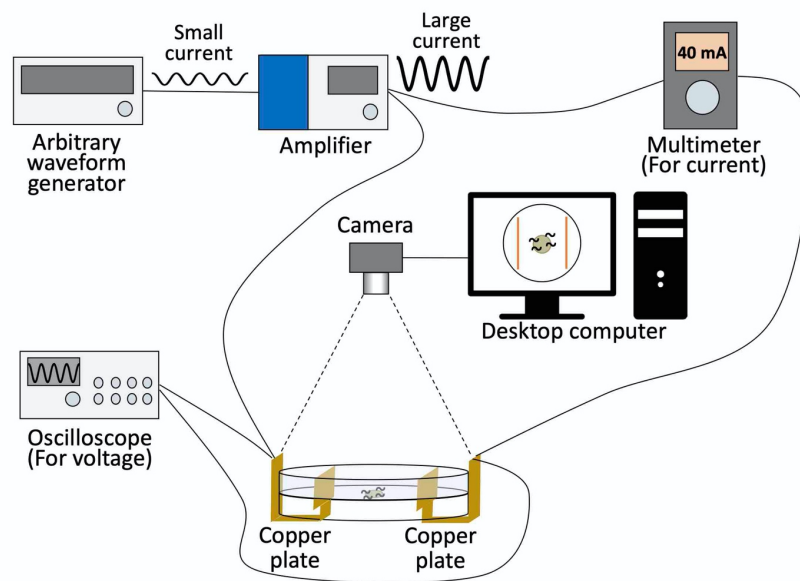


Fig. 1. Experimental setup for electric shock experiment. This setup consists of an arbitrary waveform generator, amplifier, multimeter, camera, desktop computer and oscilloscope.

behavior the “ON response” (Fig. 2A and C). During this running behavior, the worms engage in rapid body bends as well as rapid head movements (Supplementary Videos 1 and 2). In the ON response, we did not detect a statistical bias in any direction (Supplementary Fig. 3). Moreover, 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 we named the “OFF response” (Fig. 2B). 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 (Supplementary Fig. 4 and Videos 3 and 4). With other frequencies, ON and OFF responses

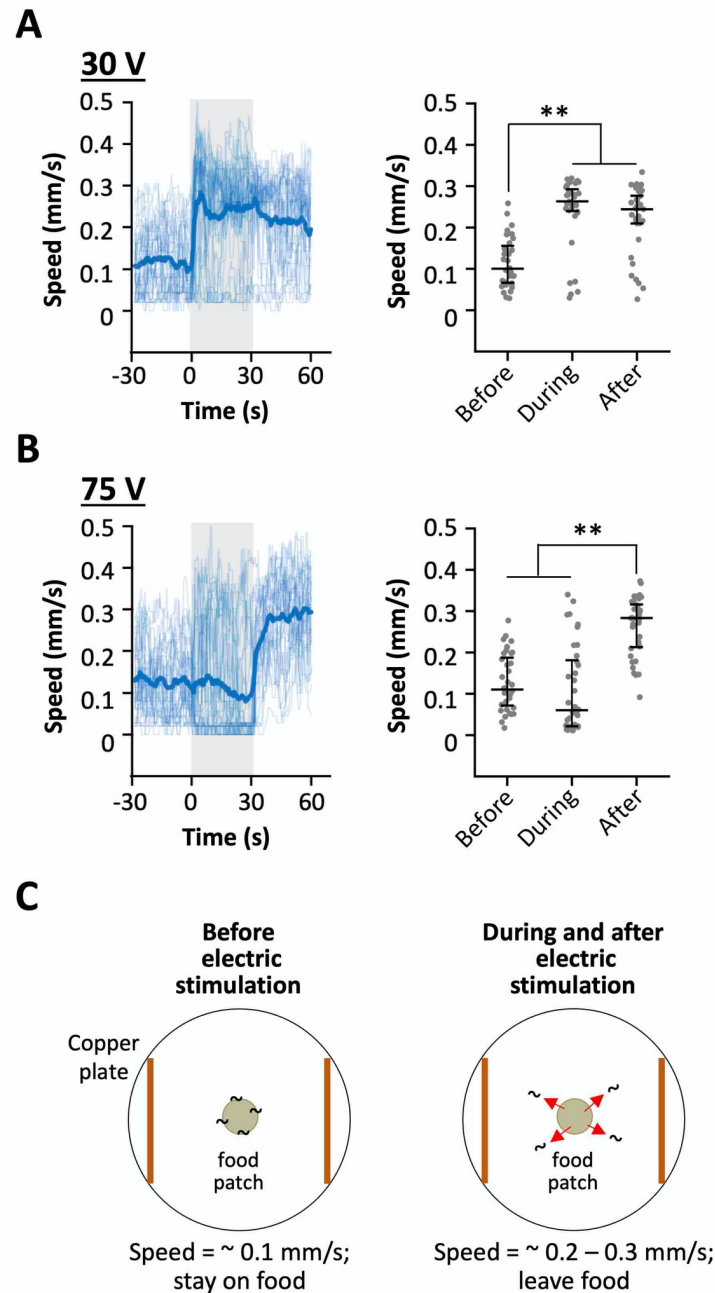


Fig. 2. Animals' speed is increased by AC stimulation. **A**, (Left) Speed-time graph with 30 V stimulation at 4 Hz. Thin and thick lines are for individual and average values, respectively. Gray indicates the duration of electric stimulation (0-30 s). (Right) Scatter plot showing average speed of individual animals before, during and after electric stimulation. Each period is 30 s. $n = 35$. **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 worms stay on food patch and maintain their speed at around 0.1 mm/s. (Right) During electric stimulation is delivered, the worms increase speed to around 0.2 - 0.3 mm/s and leave the food patch which persists even after the stimulus is terminated. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. $** p < 0.001$.

were also observed, but were less clear compared to those with 4 Hz (Supplementary Fig. 2). The range of voltage per length ($30\text{--}75\text{ V}/6\text{ cm} = 5\text{--}12\text{ V/cm}$) is similar to the ones previously observed in the worms' DC response ($3\text{--}12\text{ V/cm}$) (Gabel et al., 2007). The fact that ON and OFF responses at 4 Hz were completely different with only a 2.5-fold difference in the voltage at the same frequency is interesting because different behavioral responses generally require much larger differences in stimulus intensity with other stimuli, such as odor (Bargmann et al., 1993).

We then analyzed whether this response depends on voltage or current by manipulating the salt concentration in the assay plate: The higher salt concentration should result in a larger current when the same strength of voltage is applied. As shown in Fig. 3, 30 V and 75 V stimulus caused ON and OFF responses, respectively, regardless of the current value, indicating that the behavioral response depends on voltage.

Speed increase lasts for several minutes

Next, we examined how long the increased speed persists during and after the stimulus. When the duration of applied electric shock was 1-2 minutes, significant speed increases were maintained during the stimulus, lasted for ~1 min after the stimulus, and went back to the baseline level (Fig. 4A). Interestingly, when the animals were stimulated only for 5 sec, the speed increase still lasted for 1.5 min. When 4 min stimulus was applied, the increase was maintained during the stimulus but went back to the baseline level 30 sec after the stimulus. During 10 min stimulation, the significant speed increase was observed only for 5.5 min. Thus, we concluded that the ON response caused by 30 V stimulation persists ~5 min at most.

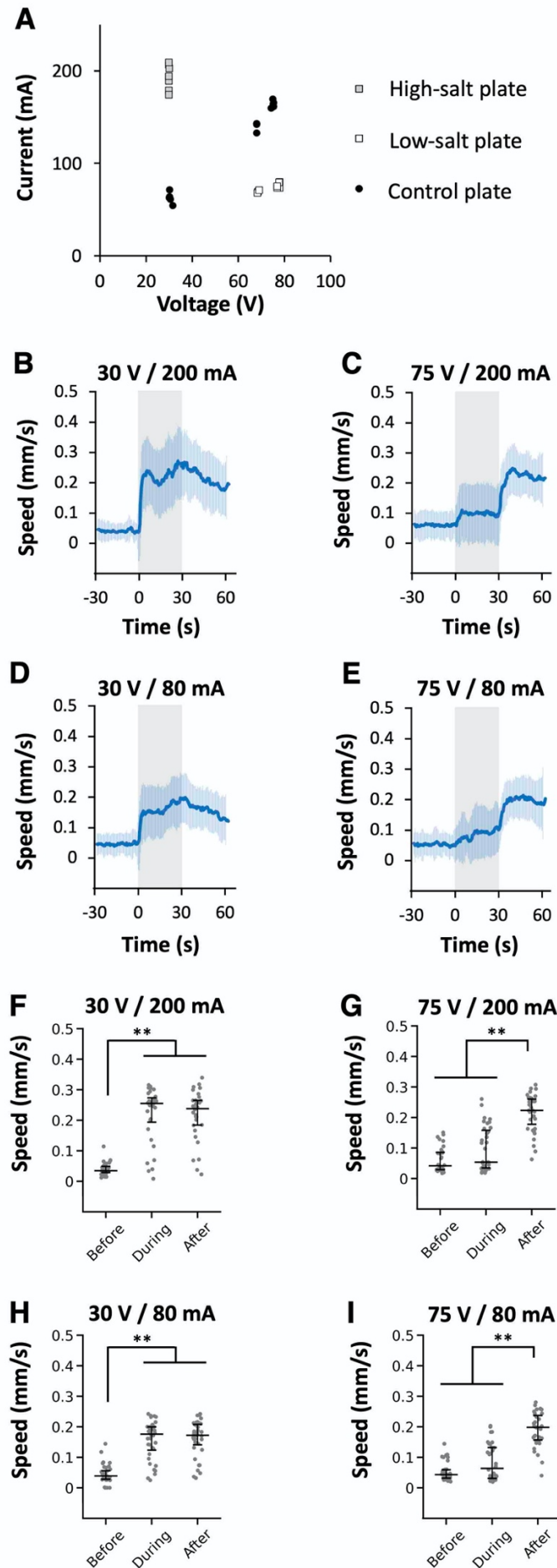


Fig. 3. Speed increase is dependent on voltage, not on current. **A**, Voltage-current graph with different salt concentrations (indicated by different symbols). Each dot represents the measured value on the day of the experiment. The final total osmolarity for sodium chloride (Na^+ and Cl^-) and sucrose for all the plates was 400 mOsm. **B–E**, Behavioral responses of animals assayed on high-salt plate with 30 V (**B**; $n = 32$), on control plate with 75 V (**C**; $n = 35$) or 30 V (**D**; $n = 36$), or on low-salt plate with 75 V (**E**; $n = 34$). Stimulation period is indicated by a shaded grey box. **F–I**, Scatter plot showing average speed of individual animals before, during and after electric stimulation, corresponding to the panels **B–E**, respectively. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. ** $p < 0.001$.

This result suggested the possibility that the speed increase persisted for ~5 min 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 (Fig. 4B versus "10 minutes" in A). This result suggests 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).

We then tested the persistence of speed increase in the OFF response with 75 V. Interestingly, 5 and 30 sec stimuli caused similar or longer persistent responses after the stimulus than 30 V did (Fig. 4C). 45 sec stimulus caused >2 min persistent response, which is the longest among the responses to 30 and 75 V stimuli after the stimulus. When animals were stimulated for 1 min, 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).

We then 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 we used a small food lawn to localize the animal's initial positions to the center of the plate (Fig. 1 and 2C), 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 the hypothesis, we compared the time-course of speed

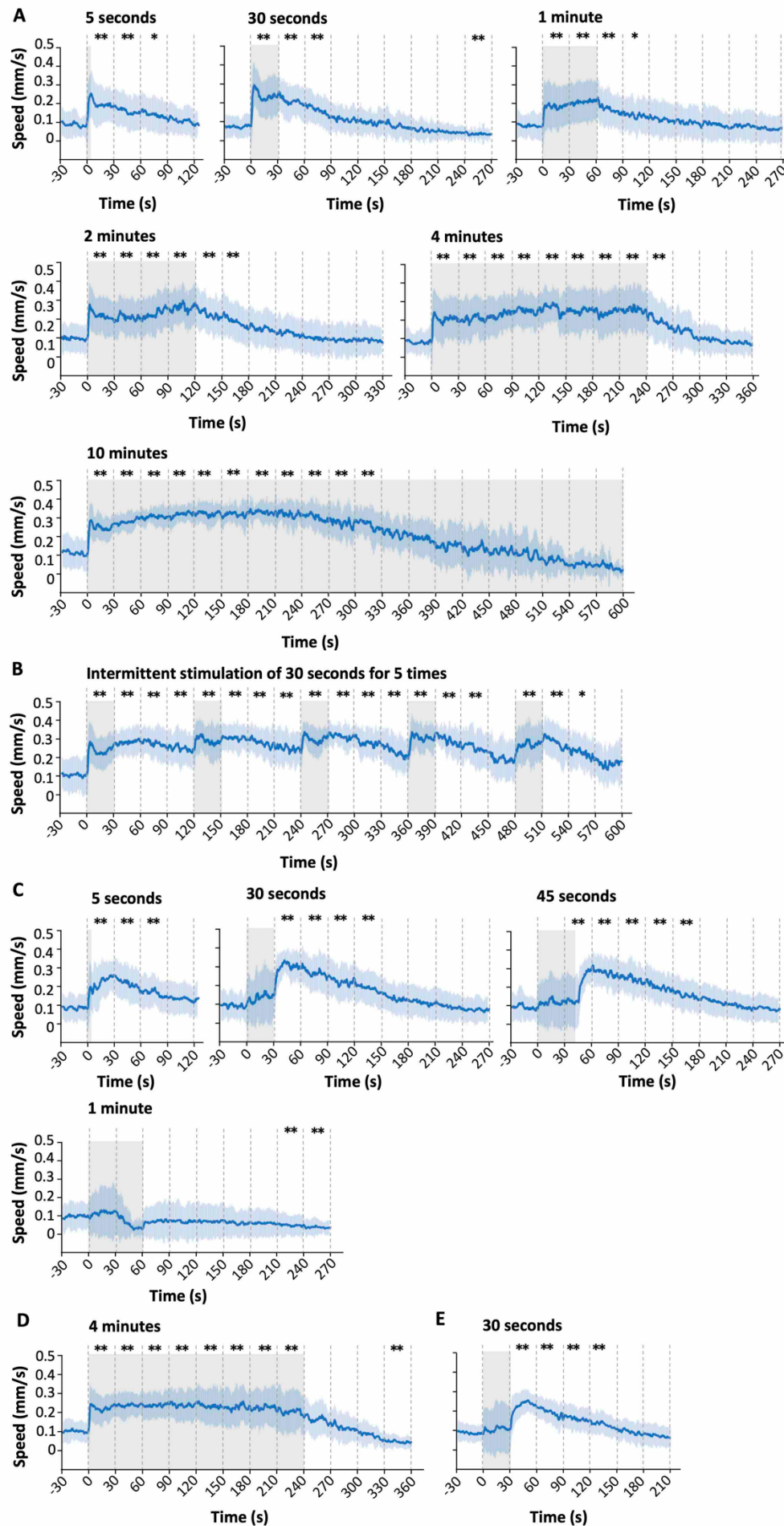


Fig. 4. Speed increase persisted for minutes even after the stimulation. **A**, Speed-time graphs of ON response with 30 V stimulation of different time periods, ranging from 5 seconds to 10 minutes. **B**, Speed-time graph for intermittent electric stimulation of 30 seconds, 5 times with 90 s-intervals. **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 s (**E**) with worms placed on full food lawn. Shaded regions around the lines represent standard deviation. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. * $p < 0.01$, ** $p < 0.001$. Sample numbers were 32–46 per condition, and the details are described in Supplementary Table.

changes on plates with a small patch of food lawn and with a full food lawn. As shown in Fig. 4D and E (compare Fig. 4A "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 the result, we analyzed the animals' speed on a stripe-like food pattern (Supplementary Fig. 5A). We did not observe a significant difference in speeds when the animals moved into or out of the food area (Supplementary Fig. 5B). This result indicates that the animals' migratory speed is not affected by the presence or absence of food, which is one the most influential environmental signals for the animals. It may further suggest that animals prioritize moving away from a harmful condition, such as strong electric shock, to protect themselves.

Two types of voltage-gated calcium channels, but not chemo- or mechano-sensory molecules, are required for the sensation of electric shock.

To identify gene(s) required for the response to electric shock, we analyzed a series of mutant strains of candidate genes. We tested mutants of genes involved in the animals' chemo- and mechano-sensation, the homologues of genes involved in electroreception in shark and skate, and genes involved in the biosynthesis of neuromodulators.

C. elegans' chemo-sensation is largely mediated by the 12 pairs of amphid sensory neurons in the head, which are 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, we tested mutants for *che-2*, a gene required for the proper formation and function of the sensory cilia (Fujiwara et al., 1999). For mechano-sensation, we 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 form a mechanosensory ion channel complex for transduction of gentle touch (Driscoll & Chalfie, 1991; Huang & Chalfie, 1994), while *trp-4* encodes TRPN (NOMPC) for harsh touch response (Kang et al., 2010). All the mutant strains exhibited wild-type-like responses in ON as well as OFF responses (panel A in Fig. 5 and 6 for ON and OFF responses, respectively). Some mutants (*osm-9;ocr-2*, *che-2*, *mec-10*, and *tph-1*) exhibited statistical differences in the OFF response (Fig. 5F and 6F), suggesting the partial involvement of these genes, although the defects in speed increase (i.e. Δ Speed) were not as severe as the ones of VGCC mutants (see below). The non-involvement of *tax-4* also indicates that temperature increase caused by the electric stimulus is not responsible for the response (Coates & de Bono, 2002) (see Discussion for details).

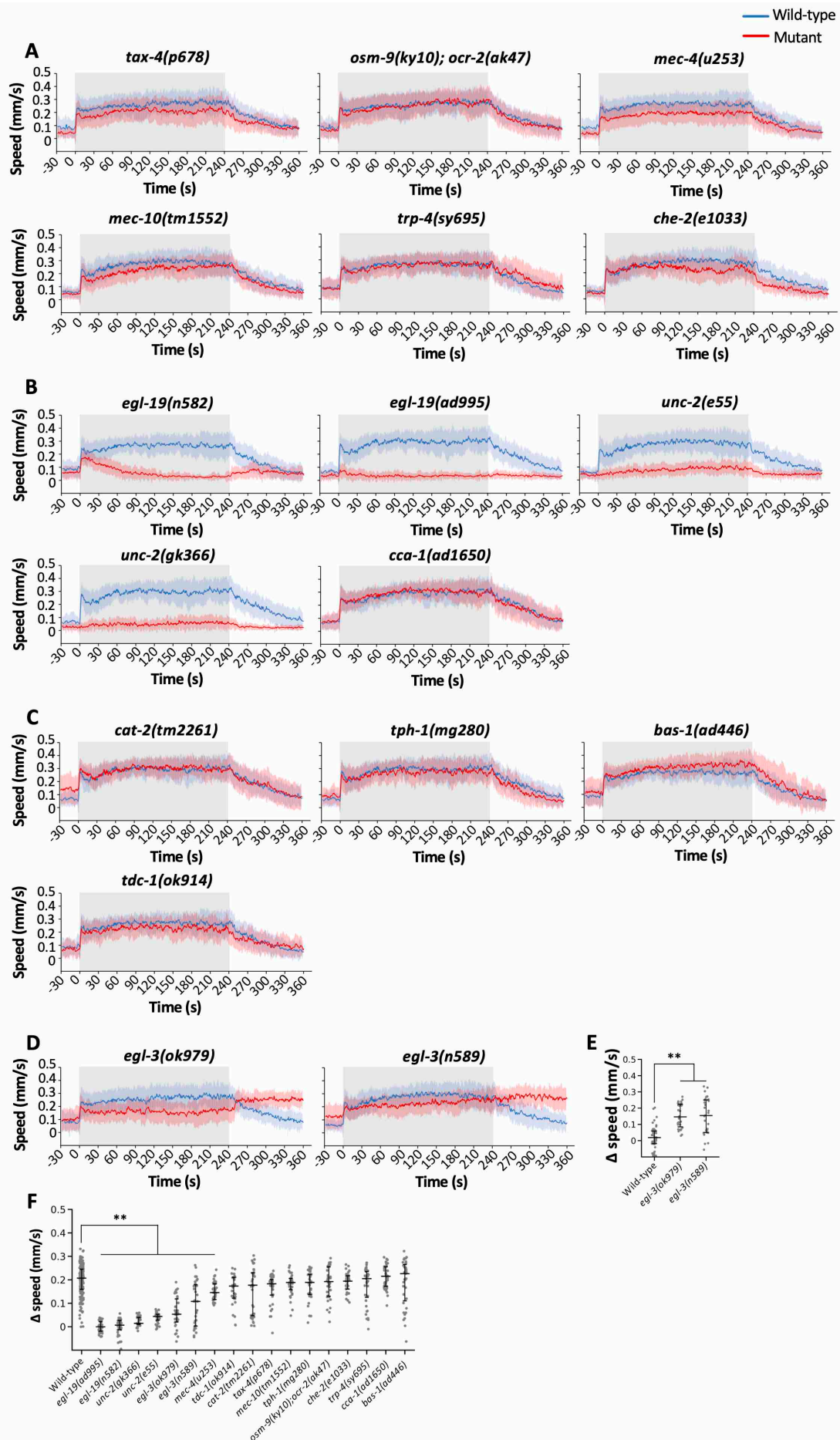


Fig. 5. Genetic analysis of ON response. A–D, Speed-time graphs of ON response with 30 V stimulation of 4 min on mutants of sensory signaling (A), VGCC (B), biogenic amine biosynthesis (C), and neuropeptide biosynthesis (D). E, Scatter plot showing Δ speed of individual animals during $t = 330$ – 360 s in D. F, Scatter plot showing Δ speed of individual animals during the stimulation. In a series of daily experiments, wild-type N2 and three to five mutant strains were analyzed in parallel, and all the N2 data are combined in F. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. ** $p < 0.001$. The mutant strains are arranged in ascending order of median values. Sample numbers were 30–36 per mutant strain, and the details are described in the Supplementary Table.

We then tested *egl-19*, the orthologue of the L-type VGCC alpha subunit, which functions in the sensory organ for environmental electric signals for shark and skate (Bellono et al., 2017, 2018). We found that two reduction-of-function alleles of *egl-19* mutants exhibited strong defects in ON and OFF responses (Fig. 5 and 6, panels B and F). While *egl-19* is expressed widely (Lee et al., 1997), at least one allele of *egl-19* mutants exhibited movement speed comparable to wild-type animals before stimulation (Supplementary Fig. 6), indicating that the defect in the response is not caused by a problem in the basal locomotory system. These results suggests that the VGCC may be an evolutionarily conserved sensor for environmental electricity.

This finding further motivated us to test two other types of voltage-gated calcium channels, i.e. N-type voltage-gated calcium channel (UNC-2) and T-type voltage-gated calcium channel (CCA-1) (Schafer & Kenyon, 1995; Steger et al., 2005), although only L-type VGCC had been found to be involved in electrical responses in other animals. Unexpectedly, mutants for two alleles of *unc-2* were defective in both ON and OFF responses, while *cca-1(ad1650)* mutants behaved similar to the wild-type controls (Fig. 5 and 6, panels B and F). These results demonstrate that UNC-2, the N-type VGCC, is also required for the electric

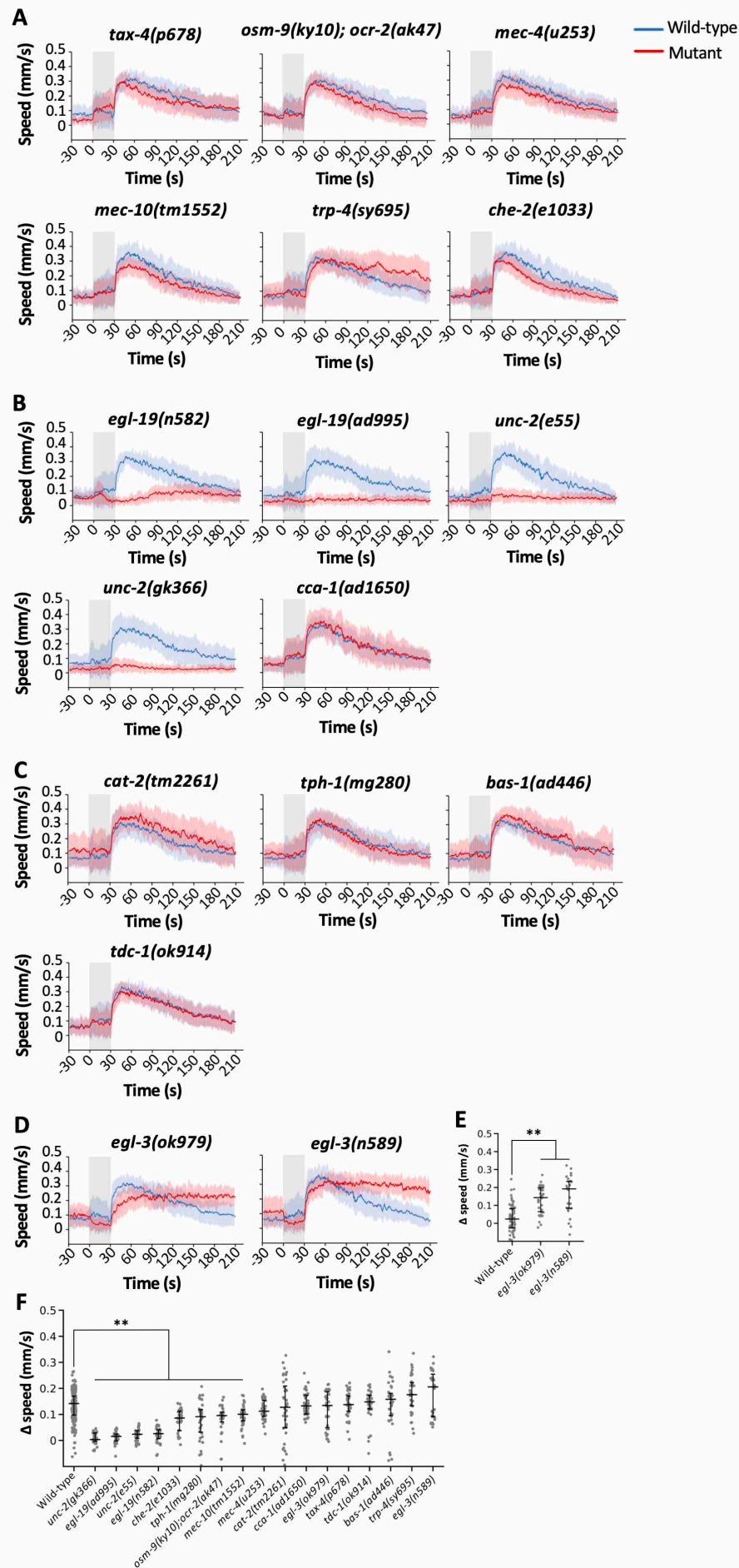


Fig. 6. Genetic analysis of OFF response. A–D, Speed-time graph of OFF response with 75 V stimulation of 30 s on mutants of sensory signaling (A), VGCC (B), biogenic amine biosynthesis (C), and neuropeptide biosynthesis (D). E, Scatter plot showing Δ speed of individual animals during $t = 180$ –210 s in D. F, Scatter plot showing Δ speed of individual animals during the stimulation. In a set of daily experiments, wild-type and three to five mutant strains were analyzed in parallel, and all the wild-type data are combined in F. Statistical values were calculated using Kruskal-Wallis test with Bonferroni correction. ** $p < 0.001$. The mutant strains are arranged in ascending order of median values. Sample numbers were 30–36 per mutant strain, and the details are described in the Supplementary Table.

response, and also suggest that the worms may utilize similar but substantially different molecular mechanisms for electric sensation than sharks and skates.

Lastly, we analyzed the genes required for the biosynthesis of neuromodulators, such as serotonin, dopamine, octopamine and tyramine and tested *cat-2(tm2261)*, *tph-1(mg280)*, *bas-1(ad446)* and *tdc-1(ok914)* (Alkema et al., 2005; Lints & Emmons, 1999; Loer & Kenyon, 1993; Sze et al., 2000). As shown in panel C in Fig. 5 and 6, these mutants also exhibited wild-type-like responses, indicating that these neuromodulators are not involved. 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 (Fig. 4D and E, and Supplementary Fig. 5).

We also 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 much longer persistence of the speed increase after the electric shock (Fig. 5 and 6, panels D-F), indicating

that the persistent activity in the neural circuit for speed increase is down-regulated by neuropeptide signaling in the wild-type animals.

DISCUSSION

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

Multiple vertebrate and invertebrate species are known to sense electric signals for navigation and/or foraging. For example, in addition to the electric fish, platypus (*Ornithorhynchus anatinus*) detects electric signals via their duck-like bills to locate and avoid objects when navigating in the water (Scheich et al., 1986). Blind cave salamander (*Proteus anguinus*) perceives 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). These results suggest that sensation of electric signals are essential for survival and reproduction of some animals in the wild.

In this study, we established an original experimental paradigm and found that *C. elegans* responds to 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 DC (Gabel et al., 2007), we consider that the responses to AC and DC are different for the following reasons. (1) In the DC field, the animals moved at a certain angle ($\sim 4^\circ$ per 1 V/cm), which was not observed in our AC stimulus (Supplementary Fig. 3). (2) Movement speed did not change with the DC stimulus (Gabel et al., 2007).

In addition, five pairs of amphid sensory neurons were involved in the DC response (Gabel et al., 2007), while mutations in genes required for sensory signaling in the amphid sensory neurons (*tax-4*, *osm-9*, *ocr-2*, and *che-2*) did not affect the AC response in our study (Fig. 5 and 6), indicating that DC and AC responses utilize different sensory mechanisms. Our result also rules out the possibility that the animals respond to increased agar temperature due to the AC stimulus, because the mutation in *tax-4*, the gene essential for temperature response (Komatsu et al., 1996) did not affect the response. In addition, the genes required for mechano-sensation (*mec-4*, *mec-10*, and *trp-4*) are not required for the AC response either.

We found that L-type as well as N-type VGCC, EGL-19 and UNC-2, respectively, are required for the AC response. L-type VGCC has been found to function in the electrosensory organs in the shark and skate, but not N-type, suggesting that *C. elegans* utilizes similar but different molecular mechanisms. Since EGL-19 is expressed in most if not all the neurons (Lee et al., 1997; Taylor et al., 2021), it will be interesting to identify the neurons in which the channel functions, whether they are the same or different from the neurons that utilize the N-type channels, and how they contribute to the increase in the movement speed. As mentioned above, various organs in different animal species are known to sense electric stimuli. Therefore, it would be interesting to investigate whether L-type as well as N-type VGCCs also function in the organs of these animals to sense electric signals.

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 the oculo-motor system, working memory, and decision making, although its detailed mechanisms have not been sufficiently elucidated (Curtis & Lee, 2010; Major & Tank, 2004). Persistent behavioral state is likely caused by

persistent neural activity, suggesting that genetic analysis of persistent behavioral state may reveal molecular mechanism(s) of persistent neural activity.

We unexpectedly found that *C. elegans*' high speed response persists after electric shock. In *C. elegans*, two other types of persistent behavioral responses have been reported. The first is that the animal's movement speed is elevated at high O₂ concentration in *npr-1(lf)* and in the Hawaiian wild isolate CB4856, which has the same amino acid variation in *npr-1* (Cheung et al., 2005). In this behavioral response, the elevated speed returns rapidly to the basal speed when the high O₂ is terminated, the animals still recognize and aggregate at the edge of a food lawn, and 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 min after 30-45 sec stimulus with 75 V and more than 1.5 min after only 5 sec stimulus, is not affected by food stimulus, and does not require CNGC activity or serotonin signaling, the analysis of electric shock response is likely different from the above-mentioned two behavioral responses and may provide a unique opportunity for genetic dissection of a persistent behavioral state and neural activity.

The 30 or 75 V of voltage used in this study may appear artificial. However, we consider the responses of *C. elegans* to these stimuli to reflect physiologically meaningful biological mechanisms because of 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 for 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 we observe is a physiological response to that trigger. (4) Fear conditioning in rodents is also triggered by electrical shock. The speed increase behavior we 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).

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). One of the most prominent characteristics of emotion 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). Recently, multiple species of invertebrates are considered to possess internal brain states that resemble what we consider to be emotions (Bacqué-Cazenave et al., 2017; Cwyn et al., 2016; Gibson et al., 2015; Hamilton et al., 2016; Mohammad et al., 2016; Pascal et al., 2014).

Anderson and Adolph 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, we also consider that the electrical response has negative valence. This is because the animals ignore food during the electric shock response, 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 O₂, 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 larger number of immobile animals during the stimulus period (right panels in Fig. 2A and B) as well as a longer high speed response after the stimulus (compare the panels for responses to 30 and 45 second stimulus in Fig. 4C). 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 our electric stimulus might supports this point as well, as the emotional state induced by electricity influences the response to food, an entirely different stimulus.

Taken together, these results suggest that the animal's response to electric shock represent a form of emotion, probably fear. As we revealed that the persistent aspect of the behavioral response is regulated by neuropeptide signaling, which may resemble the 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.

In summary, we found that *C. elegans* responds to electric shock, which is regulated by VGCCs and neuropeptide signaling. Our findings may suggest the following model (Fig. 7). When the animals sense 30 or 75V AC stimulus at 4 Hz, the stimulus is sensed with the L- and N-type VGCCs 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 that regulates emotion.

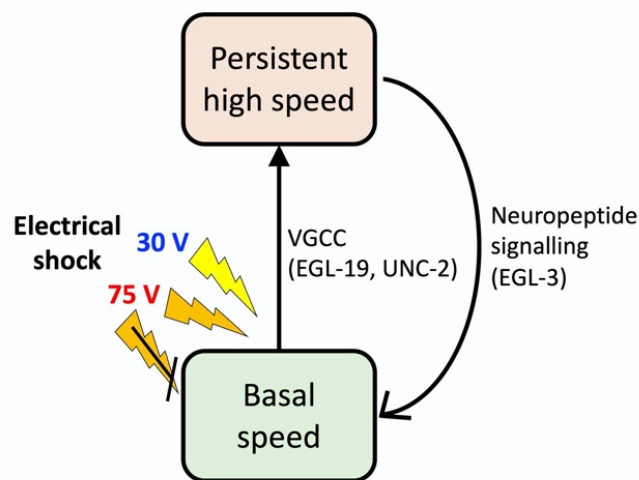


Fig. 7. Model for mechanism of speed increase caused by electric shock.

MATERIALS AND METHODS

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 which had been spread with *E. coli* strain OP50 and incubated at 19.0-19.5 °C. Strains used were the wild-type strain Bristol N2 and 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)*, KDK11 *cat-2(tm2261)*, MT7988

bas-1(ad446), GR1321 *tph-1(mg280)*, RB993 *tdc-1(ok914)*, VC671 *egl-3(ok979)* and MT1219 *egl-3(n589)*.

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

Before the behavioral assay, animals were cultivated as described previously (Kimura et al., 2010). In brief, four 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 comparable developmental stage (i.e. young adult) and worm number with the wild-type animals. All behavioral assays were carried out with young adult hermaphrodites.

Experimental instruments for electric shock behavioral assay

The following electric instruments (Fig. 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. We also used an Digital Storage Oscilloscope (DCS-1054B, Texio Technology Corporation) 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.

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 indicated otherwise. 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 one hour 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 (Fig. 1). The images were acquired 2 frames per s, and electric shock was delivered with the conditions described in each figure. 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). Baseline speed was calculated from the average speed over 30 s before the stimulation, and ΔSpeed was calculated by subtracting the baseline value from each animal's speed during or after the stimulus.

Electric shock behavioral assay with full OP50 bacterial food lawn

For the assays conducted with full food lawn, the area of assay plates between the copper plates were fully seeded with OP50 and kept on the bench overnight until the assay began. Animals grown in regular cultivation plates were washed in two 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.

Investigation of relationship between speed increase, current and voltage

Three different types of NGM agar plates were prepared with varying salt concentration and similar osmolarity: High-salt plates had 200 mM sodium chloride; low-salt plates had 10 mM sodium chloride and 380 mM sucrose; control plates had 50 mM sodium chloride and 300 mM sucrose. The purpose of adding sucrose into the plates was to adjust and balance the osmolarity. The final total osmolarity for sodium chloride (Na^+ and Cl^-) and sucrose for all the plates was 400 mOsm. The rest of the procedures were the same as for assays conducted with small food patch.

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. We chose this sample number based on a large scale behavioral analysis of *C. elegans* (Yemini et al., 2013). Data is presented as means \pm SD unless otherwise specified. Experimental conditions, such as the electric stimulation or different strains were randomized on a daily basis.

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COMPETING INTERESTS

The authors declare no competing interests.

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