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).

53 The nematode *Caenorhabditis elegans* has been widely used in neurobiological research
54 because of the feasibility of molecular, physiological, and behavioral analyses of neural
55 functions (Bargmann, 2006; de Bono & Maricq, 2005; Sasakura & Mori, 2013). The animals
56 are known to respond to direct current (DC), migrating along the electric field from the
57 positive end to the negative end (Sukul & Croll, 1978), and a few classes of chemosensory
58 neurons (ASH and ASJ) were found to be required for their ability to align themselves
59 according to the DC field (Gabel et al., 2007). The animals are also reported to respond to
60 strong alternating current (AC)—they exhibit a "convulsant" phenotype (paralysis and
61 elongation) upon delivery of a brief electric shock (200 Hz, 3.5 ms, 47 V) and recover rapidly
62 after removal of the electric shock (Risley et al., 2016). However, other behavioral responses
63 as well as molecular mechanisms for electric signals have not been revealed.
64
65 In this study, we report that *C. elegans* responds to AC electric stimulus by immediately
66 increasing their speed. The speed increase lasts for minutes even after an electric stimulus of
67 seconds is terminated, suggesting that the response is caused not by direct stimulation of the
68 motor system for rapid movement but possibly by persistent activity of a specific set of
69 neurons to generate the behavioral response. Further behavioral analysis revealed that the
70 effect of electric shock increases when the shock amplitude is increased (i.e., scalable). In
71 addition, the increased speed is not significantly affected by the presence or absence of food,
72 which is the most important environmental stimulus for the animals and is known to affect
73 movement speed of animals, suggesting the electric shock has a negative valence that is
74 stronger than the positive valence of food signal. Persistent behavioral response is one of the
75 most prominent characteristics of emotions of animals (Abbott, 2020; Anderson & Adolphs,
76 2014; Nettle & Bateson, 2012; Paul & Mendl, 2018; Perry & Baciadonna, 2017) , and
77 persistency, scalability and valence are three of four key features of animal emotions

78 proposed by Anderson and Adolphs (Anderson & Adolphs, 2014). A series of candidate
79 genetic analyses reveal that the response is not mediated by well-known chemo- or mechano-
80 sensory mechanisms. Instead, it requires both L-type VGCC, as in the shark and skate, and
81 N-type VGCC, which have not previously been implicated in animal electrical responses.
82 Furthermore, we find that neuropeptide signaling regulates the duration of persistence. These
83 results indicate that the animals' response to electric shock can be a suitable paradigm to
84 reveal molecular and physiological mechanisms of electrosensation as well as emotion, such
85 as fear.
86
87 **RESULTS**
88 **Worms' speed increases by AC stimulation**
89 In order to study *C. elegans*' response to electric shock, we established a setup (Fig. 1), where
90 several adult wild-type animals were placed onto 9 cm NGM agar plates seeded with a small
91 bacterial food patch and subjected to AC stimulation. The complete trajectories produced by
92 the animals were video-recorded, and their speed was calculated based on the *x-y* coordinates
93 of animals' centroids in each image frame.
94
95 We first studied the response to AC stimulation covering a range between 15 - 105 V at 60
96 Hz (the commercial power frequency in Japan), and found that the animals increased their
97 average speed during electric stimulation by varying amounts (Supplementary Fig. 1). We
98 then conducted a series of systematic analyses with different voltages and frequencies at 30–
99 75 V and 0.25–256 Hz (Supplementary Fig. 2). After the analysis, we noticed that an
100 interesting characteristic of this behavioral phenotype is most apparent when using 4 Hz
101 stimuli: When worms were stimulated with 30 V, their average speed of movement suddenly
102 increased more than 2-fold, and this persisted during the electric admission. We named this

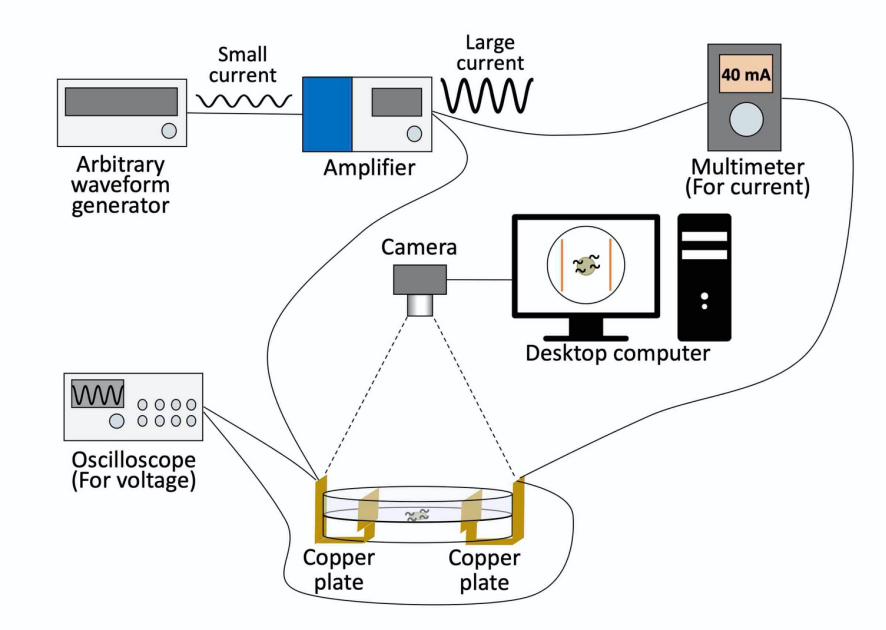


Fig. 1. Experimental setup f or electric shock experiment. This setup consists of an arbitrary waveform generator, amplifier, multimeter, camera, desktop computer and oscilloscope.

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

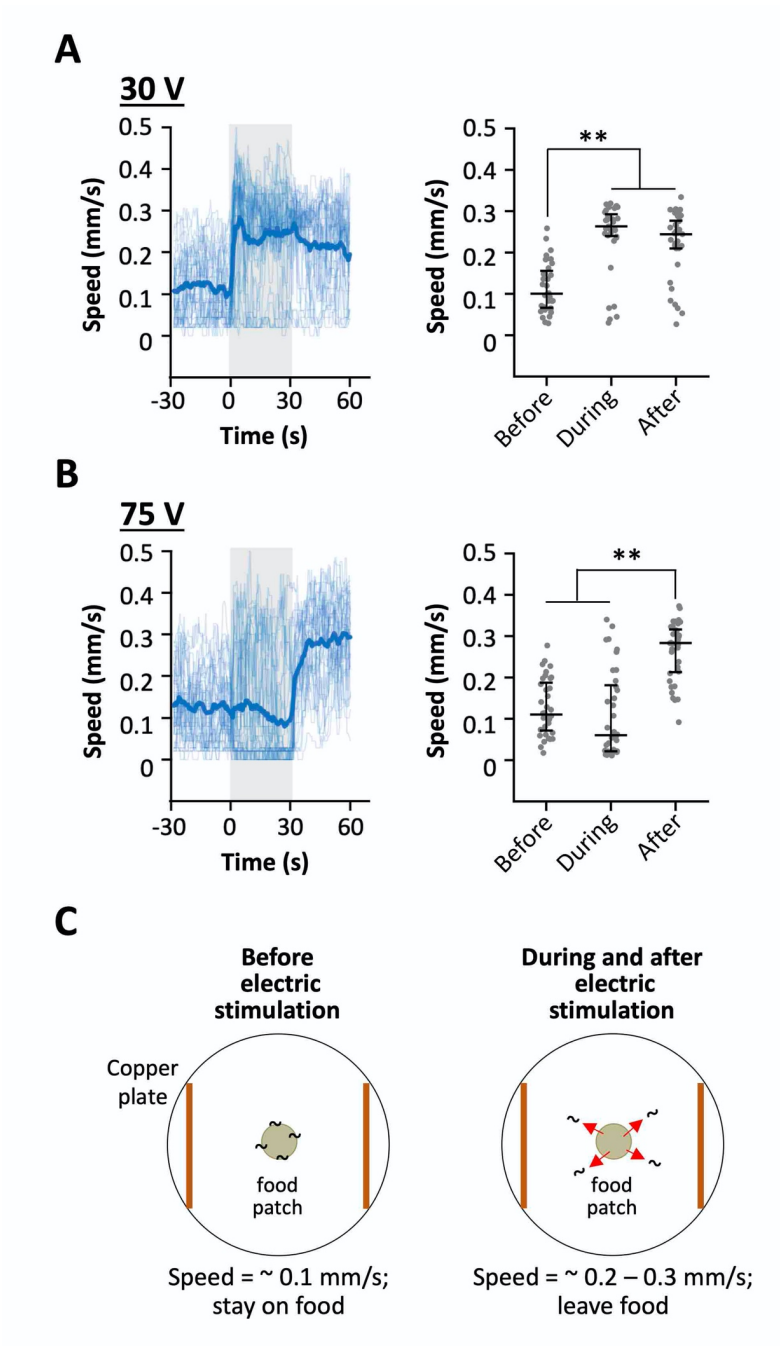


Fig. 2. Animals' speed is increased by AC stimulation. **A,** (Left) Speed-time graph with 30 V stimulation at 4 Hz. Thin and thick lines are for individual and average values, respectively. Gray indicates the duration of electric stimulation (0-30 s). (Right) Scatter plot showing average speed of individual 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. ** $p < 0.001$.

113

114 were also observed, but were less clear compared to those with 4 Hz (Supplementary Fig. 2).
115 The range of voltage per length (30–75 V/6 cm = 5–12 V/cm) is similar to the ones
116 previously observed in the worms' DC response (3–12 V/cm) (Gabel et al., 2007). The fact
117 that ON and OFF responses at 4 Hz were completely different with only a 2.5-fold difference
118 in the voltage at the same frequency is interesting because different behavioral responses
119 generally require much larger differences in stimulus intensity with other stimuli, such as
120 odor (Bargmann et al., 1993).
121
122 We then analyzed whether this response depends on voltage or current by manipulating the
123 salt concentration in the assay plate: The higher salt concentration should result in a larger
124 current when the same strength of voltage is applied. As shown in Fig. 3, 30 V and 75 V
125 stimulus caused ON and OFF responses, respectively, regardless of the current value,
126 indicating that the behavioral response depends on voltage.
127
128 **Speed increase lasts for several minutes**
129 Next, we examined how long the increased speed persists during and after the stimulus.
130 When the duration of applied electric shock was 1-2 minutes, significant speed increases
131 were maintained during the stimulus, lasted for ~1 min after the stimulus, and went back to
132 the baseline level (Fig. 4A). Interestingly, when the animals were stimulated only for 5 sec,
133 the speed increase still lasted for 1.5 min. When 4 min stimulus was applied, the increase was
134 maintained during the stimulus but went back to the baseline level 30 sec after the stimulus.
135 During 10 min stimulation, the significant speed increase was observed only for 5.5 min.
136 Thus, we concluded that the ON response caused by 30 V stimulation persists ~5 min at
137 most.

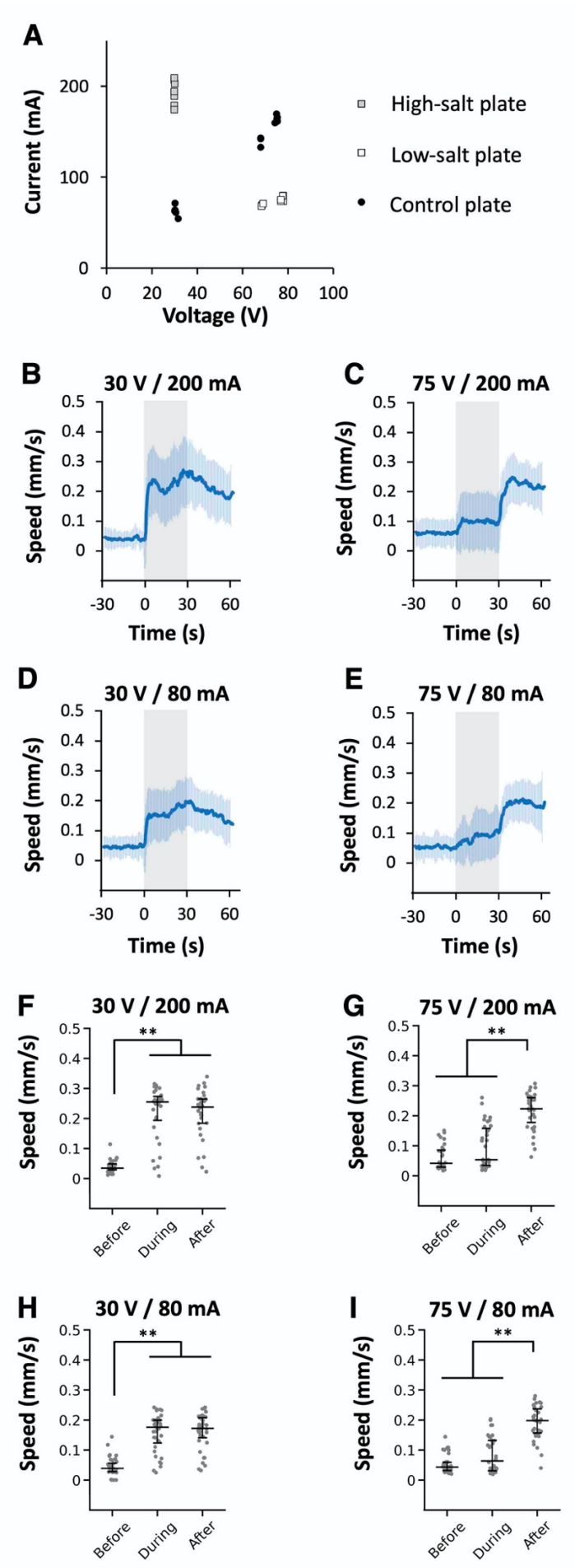
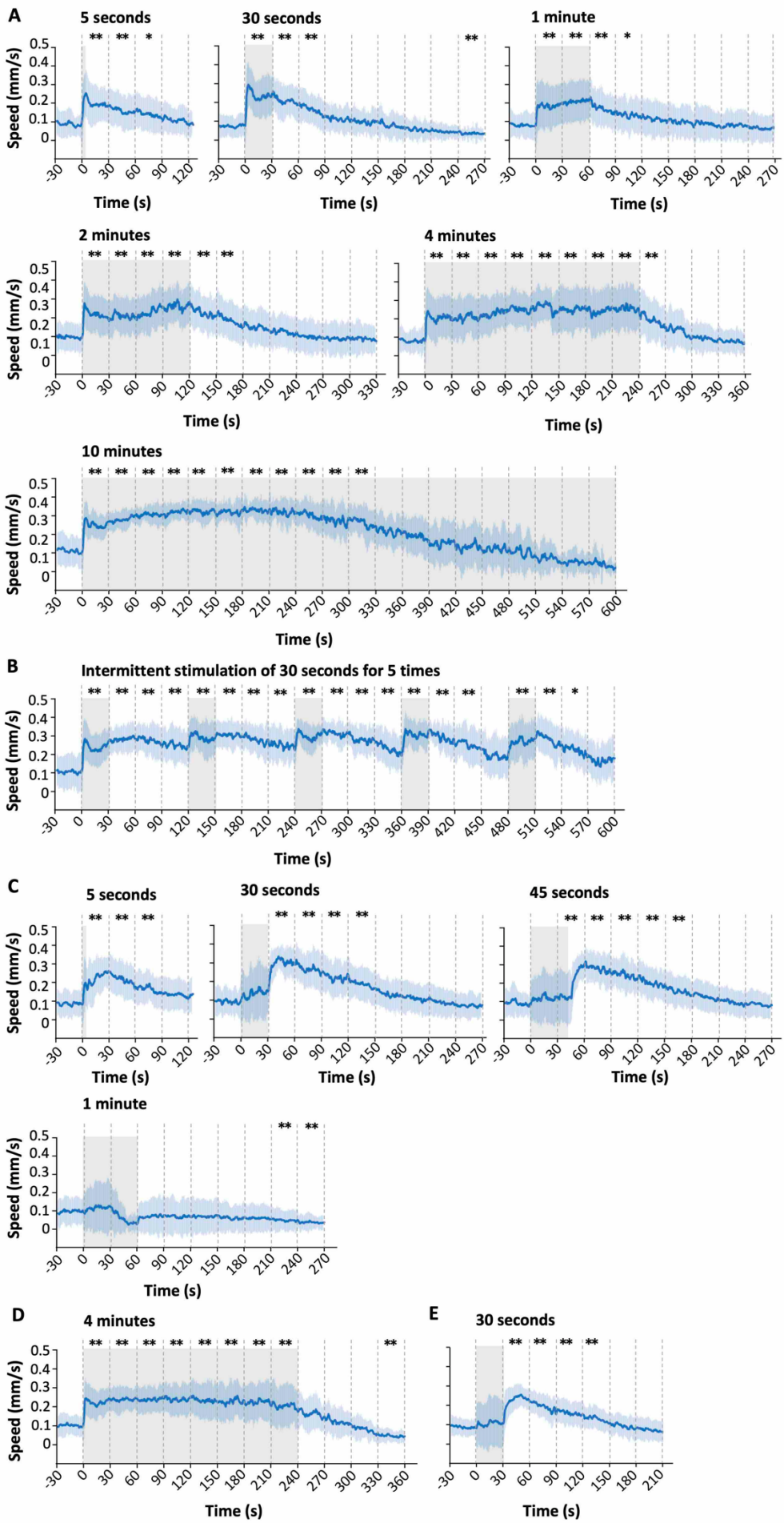


Fig. 3. Speed increase is dependent on voltage, not on current. **A,** Voltage-current graph with different salt concentrations (indicated by different symbols). Each dot represents the measured value on the day of the experiment. The final total osmolarity for sodium chloride (Na^+ and Cl^-) and sucrose 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. ** $p < 0.001$.

138

139 This result suggested the possibility that the speed increase persisted for ~5 min because of
140 fatigue in motor systems. However, animals stimulated intermittently 5 times for 30 seconds
141 per stimulation maintained a speed increase for a much longer time than those under the
142 continuous stimulus (Fig. 4B versus "10 minutes" in A). This result suggests that the decrease
143 in speed during the long ON stimulation period is not caused by fatigue in the motor system,
144 but possibly by sensory adaptation, which is widely known to adjust the animal's sensory
145 response to new environments (Wark et al., 2007).
146
147 We then tested the persistence of speed increase in the OFF response with 75 V.
148 Interestingly, 5 and 30 sec stimuli caused similar or longer persistent responses after the
149 stimulus than 30 V did (Fig. 4C). 45 sec stimulus caused >2 min persistent response, which is
150 the longest among the responses to 30 and 75 V stimuli after the stimulus. When animals
151 were stimulated for 1 min, no ON or OFF responses were observed. The fact that the larger
152 stimulus (75 V) caused longer persistent responses than the smaller one (30 V) suggests that
153 the response to electric shock is "scalable" (i.e., different strength of stimulus causes different
154 strength of behavioral response), one of the critical "emotion primitives" together with
155 persistence (Anderson & Adolphs, 2014).
156
157 We then tested the effect of food presence on the speed increase. *C. elegans* move slowly on
158 the bacterial food lawn and faster out of the lawn (Sawin et al., 2000). As we used a small
159 food lawn to localize the animal's initial positions to the center of the plate (Fig. 1 and 2C), it
160 was possible that the electric stimulus caused the animals to move away from the food lawn,
161 which then caused increased speed due to the absence of food. If this is the case, the animal's
162 speed would be considerably lower with the electric stimulus when the plates were fully
163 covered with a bacterial lawn. To test the hypothesis, we compared the time-course of speed


A
5 seconds
30 seconds
1 minute
2 minutes
4 minutes
10 minutes
B
Intermittent stimulation of 30 seconds for 5 times
C
5 seconds
30 seconds
45 seconds
1 minute
D
4 minutes
E
30 seconds
Speed (mm/s)
Time (s)

164

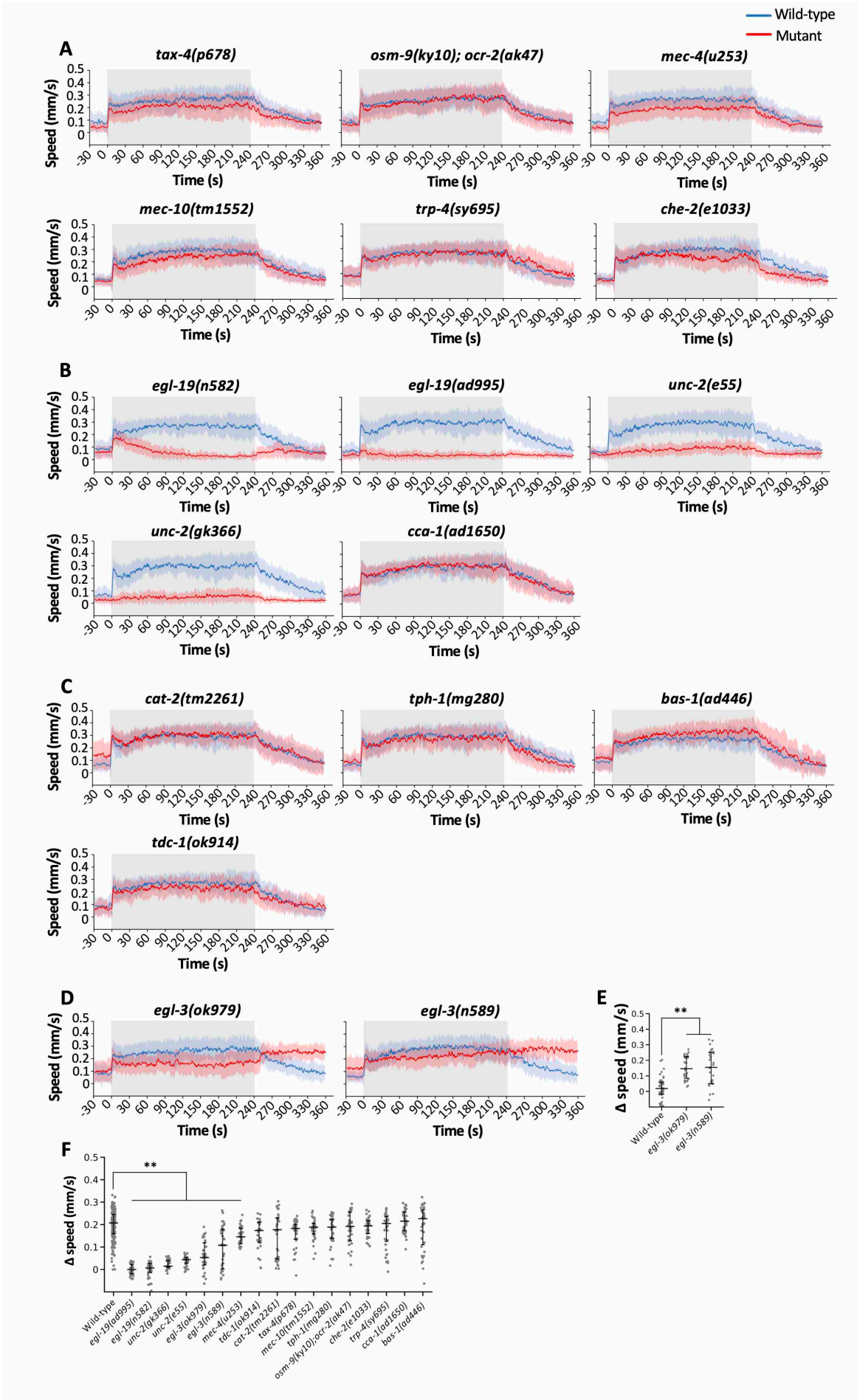
Fig. 4. Speed increase persisted for minutes even after the stimulation. **A,** Speed-time graphs of ON response with 30 V stimulation of different time periods, ranging from 5 seconds to 10 minutes. **B,** Speed-time graph for intermittent electric stimulation of 30 seconds, 5 times with 90 s–intervals. **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.

182 To identify gene(s) required for the response to electric shock, we analyzed a series of mutant
183 strains of candidate genes. We tested mutants of genes involved in the animals' chemo- and
184 mechano-sensation, the homologues of genes involved in electroreception in shark and skate,
185 and genes involved in the biosynthesis of neuromodulators.
186
187 *C. elegans'* chemo-sensation is largely mediated by the 12 pairs of amphid sensory neurons in
188 the head, which are classified into the ones using TAX-2 and TAX-4 cyclic nucleotide-gated
189 channel (CNGC) subunits or the others using OSM-9 and OCR-2 transient receptor potential
190 (TRP) channel subunits for depolarization (Coburn & Bargmann, 1996; Colbert et al., 1997;
191 Komatsu et al., 1996; Tobin et al., 2002). In addition to loss-of-function mutants for the
192 above-mentioned genes, we tested mutants for *che-2*, a gene required for the proper
193 formation and function of the sensory cilia (Fujiwara et al., 1999). For mechano-sensation,
194 we analyzed loss- or reduction-of-function alleles of *mec-4, mec-10*, and *trp-4*. *mec-4* and
195 *mec-10* genes encode DEG/ENaC proteins and form a mechanosensory ion channel complex
196 for transduction of gentle touch (Driscoll & Chalfie, 1991; Huang & Chalfie, 1994), while
197 *trp-4* encodes TRPN (NOMPC) for harsh touch response (Kang et al., 2010). All the mutant
198 strains exhibited wild-type-like responses in ON as well as OFF responses (panel A in Fig. 5
199 and 6 for ON and OFF responses, respectively). Some mutants (*osm-9;ocr-2*, *che-2*, *mec-10*,
200 and *tph-1*) exhibited statistical differences in the OFF response (Fig. 5F and 6F), suggesting
201 the partial involvement of these genes, although the defects in speed increase (i.e. ΔSpeed)
202 were not as severe as the ones of VGCC mutants (see below). The non-involvement of *tax-4*
203 also indicates that temperature increase caused by the electric stimulus is not responsible for
204 the response (Coates & de Bono, 2002) (see Discussion for details).
205


Wild-type
Mutant
A
tax-4(p678)
osm-9(ky10); ocr-2(ak47)
mec-4(u253)
mec-10(tm1552)
trp-4(sy695)
che-2(e1033)
B
egl-19(n582)
egl-19(ad995)
unc-2(e55)
unc-2(gk366)
cca-1(ad1650)
C
cat-2(tm2261)
tph-1(mg280)
bas-1(ad446)
tdc-1(ok914)
D
egl-3(ok979)
egl-3(n589)
E
F
Speed (mm/s)
Time (s)
Δ speed (mm/s)
**
Wild-type
egl-19(ad995)
egl-19(n582)
unc-2(gk366)
unc-2(e55)
egl-3(ok979)
egl-3(n589)
mec-4(u253)
tdc-1(ok914)
cat-2(tm2261)
tax-4(p678)
mec-10(tm1552)
tph-1(mg280)
osm-9(ky10);ocr-2(ak47)
che-2(e1033)
trp-4(sy695)
cca-1(ad1650)
bas-1(ad446)

206

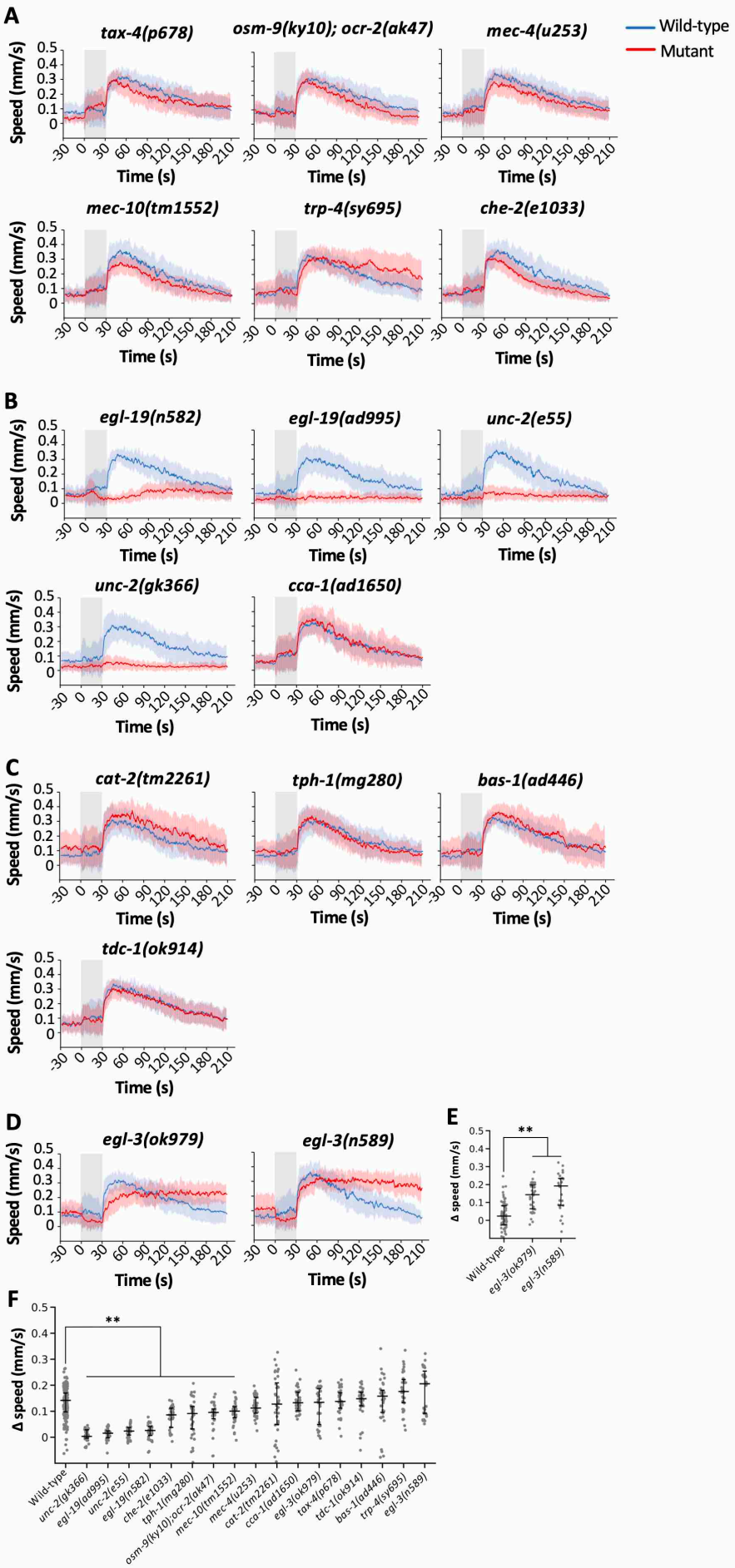
Fig. 5. Genetic analysis of ON response. A–D, Speed-time graphs of ON response with 30 V stimulation of 4 min on mutants of sensory signaling (**A**), VGCC (**B**), biogenic amine biosynthesis (**C**), and neuropeptide biosynthesis (**D**). **E**, Scatter plot showing Δspeed of individual animals during t = 330-360 s in **D**. **F**, Scatter plot showing Δspeed 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.

207

208 We then tested *egl-19*, the orthologue of the L-type VGCC alpha subunit, which functions in
209 the sensory organ for environmental electric signals for shark and skate (Bellono et al., 2017,
210 2018). We found that two reduction-of-function alleles of *egl-19* mutants exhibited strong
211 defects in ON and OFF responses (Fig. 5 and 6, panels B and F). While *egl-19* is expressed
212 widely (Lee et al., 1997), at least one allele of *egl-19* mutants exhibited movement speed
213 comparable to wild-type animals before stimulation (Supplementary Fig. 6), indicating that
214 the defect in the response is not caused by a problem in the basal locomotory system. These
215 results suggests that the VGCC may be an evolutionarily conserved sensor for environmental
216 electricity.

217

218 This finding further motivated us to test two other types of voltage-gated calcium channels,
219 i.e. N-type voltage-gated calcium channel (UNC-2) and T-type voltage-gated calcium
220 channel (CCA-1) (Schafer & Kenyon, 1995; Steger et al., 2005), although only L-type VGCC
221 had been found to be involved in electrical responses in other animals. Unexpectedly,
222 mutants for two alleles of *unc-2* were defective in both ON and OFF responses, while *cca-*
223 *1(ad1650)* mutants behaved similar to the wild-type controls (Fig. 5 and 6, panels B and F).
224 These results demonstrate that UNC-2, the N-type VGCC, is also required for the electric


A
tax-4(p678)
osm-9(ky10); ocr-2(ak47)
mec-4(u253)
Wild-type
Mutant
mec-10(tm1552)
trp-4(sy695)
che-2(e1033)
B
egl-19(n582)
egl-19(ad995)
unc-2(e55)
unc-2(gk366)
cca-1(ad1650)
C
cat-2(tm2261)
tph-1(mg280)
bas-1(ad446)
tdc-1(ok914)
D
egl-3(ok979)
egl-3(n589)
E
F
Speed (mm/s)
Time (s)
Δ speed (mm/s)
**
Wild-type
unc-2(gk366)
egl-19(ad995)
unc-2(e55)
egl-19(n582)
che-2(e1033)
tph-1(mg280)
osm-9(ky10);ocr-2(ok47)
mec-10(tm1552)
mec-4(u253)
cat-2(tm2261)
cca-1(ad1650)
egl-3(ok979)
tax-4(p678)
tdc-1(ok914)
bas-1(ad446)
trp-4(sy695)
egl-3(n589)

225

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 Δspeed of individual animals during t = 180-210 s in **D**. **F**, Scatter plot showing Δspeed 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.

226

227 response, and also suggest that the worms may utilize similar but substantially different

228 molecular mechanisms for electric sensation than sharks and skates.

229

230 Lastly, we analyzed the genes required for the biosynthesis of neuromodulators, such as

231 serotonin, dopamine, octopamine and tyramine and tested *cat-2(tm2261)*, *tph-1(mg280)*, *bas-*

232 *1(ad446)* and *tdc-1(ok914)* (Alkema et al., 2005; Lints & Emmons, 1999; Loer & Kenyon,

233 1993; Sze et al., 2000). As shown in panel C in Fig. 5 and 6, these mutants also exhibited

234 wild-type-like responses, indicating that these neuromodulators are not involved. Because

235 dopamine and serotonin signaling are known to be required for the feeding status-dependent

236 modulation of migratory speed, these results are also consistent with the fact that feeding

237 status is not the causal reason for the speed increase (Fig. 4D and E, and Supplementary Fig.

238 5).

239

240 We also tested the involvement of neuropeptides by using loss- or reduction-of-function

241 mutations of *egl-3*, a gene required for maturation of pro-neuropeptides (Kass et al., 2001).

242 Unexpectedly, mutations in both alleles of *egl-3*, *n589* and *ok979*, caused much longer

243 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 (~4° 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).

268 In addition, five pairs of amphid sensory neurons were involved in the DC response (Gabel et
269 al., 2007), while mutations in genes required for sensory signaling in the amphid sensory
270 neurons (*tax-4*, *osm-9*, *ocr-2*, and *che-2*) did not affect the AC response in our study (Fig. 5
271 and 6), indicating that DC and AC responses utilize different sensory mechanisms. Our result
272 also rules out the possibility that the animals respond to increased agar temperature due to the
273 AC stimulus, because the mutation in *tax-4*, the gene essential for temperature response
274 (Komatsu et al., 1996) did not affect the response. In addition, the genes required for
275 mechano-sensation (*mec-4*, *mec-10*, and *trp-4*) are not required for the AC response either.
276
277 We found that L-type as well as N-type VGCC, EGL-19 and UNC-2, respectively, are
278 required for the AC response. L-type VGCC has been found to function in the electrosensory
279 organs in the shark and skate, but not N-type, suggesting that *C. elegans* utilizes similar but
280 different molecular mechanisms. Since EGL-19 is expressed in most if not all the neurons
281 (Lee et al., 1997; Taylor et al., 2021), it will be interesting to identify the neurons in which
282 the channel functions, whether they are the same or different from the neurons that utilize the
283 N-type channels, and how they contribute to the increase in the movement speed. As
284 mentioned above, various organs in different animal species are known to sense electric
285 stimuli. Therefore, it would be interesting to investigate whether L-type as well as N-type
286 VGCCs also function in the organs of these animals to sense electric signals.
287
288 **Electric stimulus causes persistent behavioral response**
289 Persistent neural activity, a sustained neural activity caused by a short-term stimulus, plays
290 critical roles in brain function, such as controlling the oculo-motor system, working memory,
291 and decision making, although its detailed mechanisms have not been sufficiently elucidated
292 (Curtis & Lee, 2010; Major & Tank, 2004). Persistent behavioral state is likely caused by

293 persistent neural activity, suggesting that genetic analysis of persistent behavioral state may
294 reveal molecular mechanism(s) of persistent neural activity.
295
296 We unexpectedly found that *C. elegans*' high speed response persists after electric shock. In
297 *C. elegans*, two other types of persistent behavioral responses have been reported. The first is
298 that the animal's movement speed is elevated at high O_2 concentration in *npr-1(lf)* and in the
299 Hawaiian wild isolate CB4856, which has the same amino acid variation in *npr-1* (Cheung et
300 al., 2005). In this behavioral response, the elevated speed returns rapidly to the basal speed
301 when the high O_2 is terminated, the animals still recognize and aggregate at the edge of a
302 food lawn, and a mutation in the *tax-4* CNGC homolog for sensory depolarization abolishes
303 the response (Coates & de Bono, 2002). Another type of persistent behavioral response is
304 roaming (Flavell et al., 2020; Fujiwara et al., 2002). Roaming is a behavioral state with high
305 movement speed, although it is only exhibited when the animals are on food and requires
306 serotonin signaling. Because the behavioral response to electric shock persists more than 2
307 min after 30-45 sec stimulus with 75 V and more than 1.5 min after only 5 sec stimulus, is
308 not affected by food stimulus, and does not require CNGC activity or serotonin signaling, the
309 analysis of electric shock response is likely different from the above-mentioned two
310 behavioral responses and may provide a unique opportunity for genetic dissection of a
311 persistent behavioral state and neural activity.
312
313 The 30 or 75 V of voltage used in this study may appear artificial. However, we consider the
314 responses of *C. elegans* to these stimuli to reflect physiologically meaningful biological
315 mechanisms because of the following reasons: (1) The range of voltage per length (30–75
316 V/6 cm = 5–12.5 V/cm) is similar to the one used for the animal's DC response (3–12 V/cm)
317 (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

343 aspects of their behavior. For example, during the high speed state caused by high O_2,
344 animals still recognize and stay at the edge of a food lawn (Cheung et al., 2005; Coates & de
345 Bono, 2002), suggesting that the electric shock signal has a strong negative valence that
346 overrides the strong positive valence of food. The third point is the scalability—stronger
347 stimulus causes stronger behavioral response. Compared to the 30 V stimulus, the 75 V
348 stimulus results in a larger number of immobile animals during the stimulus period (right
349 panels in Fig. 2A and B) as well as a longer high speed response after the stimulus (compare
350 the panels for responses to 30 and 45 second stimulus in Fig. 4C). The fourth point is
351 generalization – the same emotional state can be triggered by different stimuli and, in turn,
352 the emotional state triggered by one stimulus can then affect responses to other stimuli. The
353 lack of response to food during and following our electric stimulus might supports this point
354 as well, as the emotional state induced by electricity influences the response to food, an
355 entirely different stimulus.
356
357 Taken together, these results suggest that the animal's response to electric shock represent a
358 form of emotion, probably fear. As we revealed that the persistent aspect of the behavioral
359 response is regulated by neuropeptide signaling, which may resemble the neuropeptide
360 regulation of fear in mammals including humans (Bowers et al., 2012; Comeras et al., 2019;
361 van den Burg & Stoop, 2019), the fear-like brain state may be regulated by evolutionarily
362 conserved molecular mechanisms.
363
364 In summary, we found that *C. elegans* responds to electric shock, which is regulated by
365 VGCCs and neuropeptide signaling. Our findings may suggest the following model (Fig. 7).
366 When the animals sense 30 or 75V AC stimulus at 4 Hz, the stimulus is sensed with the L-
367 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.

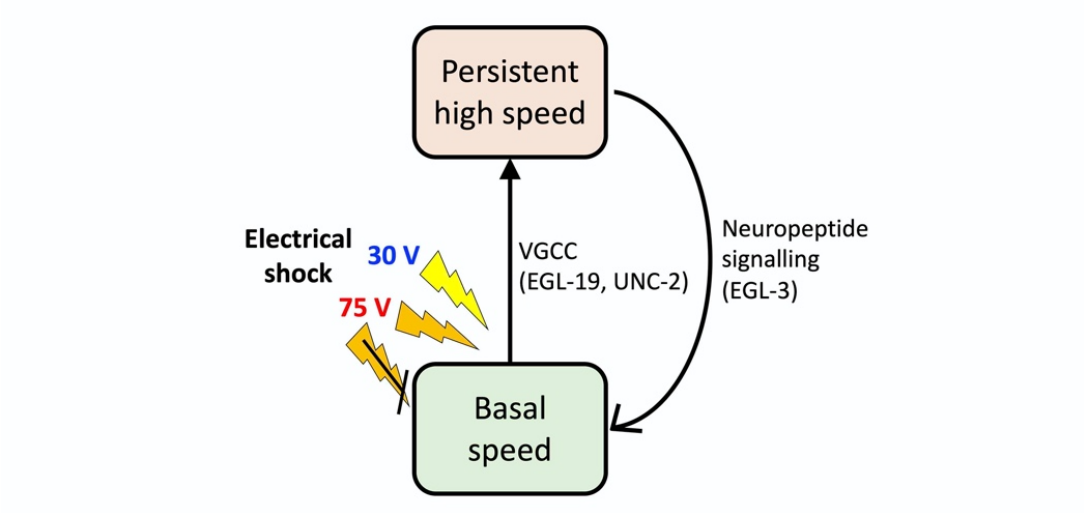


Fig. 7. Model for mechanism of speed increase caused by electric shock.

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

386 *bas-1(ad446)*, GR1321 *tph-1(mg280),* RB993 *tdc-1(ok914),* VC671 *egl-3(ok979)* and
387 MT1219 *egl-3(n589)*.
388
389 ***C. elegans* cultivation for electric shock behavioral assay**
390 Before the behavioral assay, animals were cultivated as described previously (Kimura et al.,
391 2010). In brief, four adult wild-type animals were placed onto NGM agar plates with OP50
392 and kept at 19.5°C for 7.5 hours before being removed. After removal, these plates were
393 incubated at 19.0–19.5 °C for 3 days until the assay day. On the assay day, about 100
394 synchronized young adult animals were grown on each plate. As some mutant animals had
395 slower growth or laid fewer eggs than wild-type animals did, the incubation temperature and
396 number of these mutant animals were adjusted and increased accordingly in order to obtain a
397 comparable developmental stage (i.e. young adult) and worm number with the wild-type
398 animals. All behavioral assays were carried out with young adult hermaphrodites.
399
400 **Experimental instruments for electric shock behavioral assay**
401 The following electric instruments (Fig. 1) were utilized for the electric shock behavioral
402 assay. A 50 MHz Arbitrary Waveform Generator (FGX-295, Texio Technology Corporation)
403 was used to generate different types of electric waveforms over a wide range of frequencies.
404 This waveform generator has an output limit of 10 V. Thus, an AC Power Supply
405 (PCR500MA, Kikusui Electronics Corp.) was used to amplify the voltage supply. We also
406 used an Digital Storage Oscilloscope (DCS-1054B, Texio Technology Corporation) in
407 parallel to measure the voltage and observe the electric waveforms produced as well as a
408 Digital Multimeter (PC720M, Sanwa Electric Instrument Co., Ltd.) to measure current. A
409 USB camera (DMK72AUC02, The Imaging Source Co., Ltd.) with a lens (LM16JC5M2,
410 Kowa) was used to record trajectories produced by the animals.

411

412 **Electric shock behavioral assay with small OP50 bacterial food patch**

413 Most of the behavioral assays were conducted on 9 cm NGM agar plates seeded with a small
414 food patch unless indicated otherwise. For the food patch, the bacteria OP50 was grown in
415 100 mL of LB culture overnight at 37°C, spun down and resuspended in 10 volumes of NGM
416 buffer, and 5 μL of the suspension was applied at the center of the plate to create a food patch
417 3 × 10 mm in size on the assay day. This process was used to minimize the thickness of the
418 food patch as it prevents clear images of worms in the patch. Four animals per plate were
419 placed in the food patch one hour before the assay to accustom the animals to the
420 environment and to reduce their movement speed to the basal level. The assay plates were
421 then inverted and placed onto a custom-made copper plate bridge, whose distance is 6 cm
422 (Fig. 1). The images were acquired 2 frames per s, and electric shock was delivered with the
423 conditions described in each figure. Move-tr/2D software (Library Inc., Japan) was used to
424 calculate the *x*-*y* coordinates of the animal centroids in each image frame, which were then
425 analyzed using Excel (Microsoft) or R (The R Project). Baseline speed was calculated from
426 the average speed over 30 s before the stimulation, and ΔSpeed was calculated by subtracting
427 the baseline value from each animal's speed during or after the stimulus.

428

429 **Electric shock behavioral assay with full OP50 bacterial food lawn**

430 For the assays conducted with full food lawn, the area of assay plates between the copper
431 plates were fully seeded with OP50 and kept on the bench overnight until the assay began.
432 Animals grown in regular cultivation plates were washed in two droplets of NGM buffer and
433 then transferred to the center of the assay plate and left for 5 minutes. The rest of the
434 procedures were the same as for assays conducted with small food patch.

435

436 **Investigation of relationship between speed increase, current and voltage**

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

444

445 **Data analysis and statistics**

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

452

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COMPETING INTERESTS

The authors declare no competing interests.

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