Parallel Mechanosensory Systems are Required for Negative Gravitaxis in *C. elegans*

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Abstract

The ability to sense Earth's gravitational pull is essential for orientation, navigation, and proprioception in most organisms. Here, we demonstrate that *C. elegans* adults and dauers exhibit negative gravitaxis and that this behavior is modulated by other environmental inputs such as light and electromagnetic fields. We found that components of the DEG/ENaC pathway involved in gentle touch sensation – specifically the neuronally-enriched microtubule subunits MEC-7 and MEC-12, but not the related MEC-4 and MEC-10 mechanosensory subunits – are required for negative gravitaxis. In addition, we found that TRPA-1, a receptor protein associated with cold and mechanosensation in both mammals and invertebrates, is critical for this behavior. Finally, we show that PVD neurons, which utilize TRPA-1 to detect harsh cold, are also essential for gravity sensation. Conversely, TRNs are not required for this behavior. These findings implicate a complex, interconnected mechanism for gravity sensation involving an ion channel that is also present in the mammalian vestibular system.

Introduction

Gravitropism is a common behavior among Eukaryotes. Members of plants, animals, fungi, and even protists depend on gravity sensation for survival. Small changes in position or orientation result in a mechanical force that is transduced by graviperceptive organs. This force is often conveyed using dense organelles or mineral-rich structures whose displacement triggers a signaling pathway that ultimately results in a behavior[1]. Another common characteristic of gravity transduction pathways is the use of ciliated neurons, in which the deflection of hair-like "stereocilia" opens mechanically gated ion channels[2], [3]. Although this general mechanism has been well characterized, less is known about how mechanoreceptors and mechanosensory cells transduce such minute forces – calculated to be as small as 0.57-1.13pN in *Euglena*, for example –into a robust signal[4].

Given the availability of detailed connectome of the entire nervous system and powerful genetic and optogenetic tools, *C. elegans* has been a powerful model organism for elucidation of the neural circuitry and molecular mechanism governing sensory perception, learning, memory, and behavior[5]–[12]. However, because they are traditionally studied in a two-dimensional environment (on petri dishes containing agar), few studies have examined complex behavior exhibited by *C. elegans* in three-dimensions. In the wild, *C. elegans* nematodes are typically found in compost in wet areas (such as under shrubs or along riverbanks) that can change drastically from season to season[13], [14]. *C. elegans* has evolved an adaptive alternative dauer phase that prioritizes dispersal over reproduction during adverse environmental conditions. Dauers exhibit characteristic nictation behavior in which they "stand" on their tails and wave their heads. This is believed to facilitate dispersal, possibly by allowing worms to "hitch a ride" on nearby isopods[15]. Therefore, we believe that gravity may be a critical input that *C. elegans* use in combination with other cues to navigate to the surface before traveling longer distances (Fig. 1A).

Surprisingly, previous studies have shown that *C. elegans* dauers do not demonstrate a gravitactic preference, unlike *C. japonica*, which negatively gravitax[16] and that adult worms orient downwards

24 when swimming in liquid[17]. However, as in *Drosophila* and *Ciona*, gravitaxis in worms is likely context-
25 dependent[18], [19]. *C. elegans* has a strong aversive response to light[6] and responds to electromagnetic
26 fields[20]–[22]. Therefore, integration with other sensory modalities may influence or mask gravitactic
27 behavior. No gravitactic experiments have been performed in the absence of either stimulus. In this study
28 we show that in the absence of light, *C. elegans* dauers exhibit preferential negative gravitaxis which is
29 enhanced when shielded from electromagnetic fields.

30 While there is less known about behavioral responses to gravity, the effects of hyper- and microgravity
31 on *C. elegans* physiology is well documented[23]–[27]. In response to hypergravitational forces, signaling
32 by the mechanosensory receptor sodium channel proteins MEC-4/MEC-10 DEG/ENaC lead to nuclear
33 localization of DAF-16 FoxO transcription factor[28]. These 'mec' genes were found to be expressed in the
34 six touch receptive neurons (TRNs) in *C. elegans* that specifically mediate gentle touch sensation[29].
35 Additionally, MEC-10 is expressed in the proprioceptive/multimodal neurons PVDL/R and FLPL/R, while
36 MEC-4 is found in the FLP pair only (Fig. 1B). Mechanosensation is also mediated by Trp cation channels
37 which are common in vertebrates and invertebrates[30]–[33]. The TRPA-1 receptor is a polymodal sensor,
38 capable of conveying either high or low temperatures as well as light and the presence of noxious
39 stimuli[34]. In worms, TRPA-1 confers sensitivity to noxious cold in PVDs as well as mechanosensation in
40 OLQ and IL1 neurons. The *trpa-1* homologs *pyx* and *pain* are necessary for gravitaxis in
41 *Drosophila*[35].Therefore, it seems likely that *C. elegans* would have a system for detecting normal
42 gravitational force that may utilize components of known mechanosensory pathways and that some of
43 these neurons might be involved in graviperception.

44 We discovered that *C. elegans* worms prefer to migrate vertically against the force of gravity – negative
45 gravitaxis – and that this behavior is most pronounced in dauer larvae. Further, gravitaxis is influenced by
46 environmental cues such as light and background electromagnetic fields. We also show that the TRPA-1
47 channel and MEC-7/12 microtubules, but not the associated channel proteins DEG/ENaC MEC-4/10, are

48 required for this behavior. Finally, we identified the polymodal PVD neurons, which spans the length of
49 the worm, as essential for this directional bias. Based on these findings, we propose a model of gravity
50 transduction involving parallel mechanosensory pathways and PVD neurons.
51
52 **Results**
53 ***C. elegans* exhibits negative gravitactic behavior**
54 Under stressful conditions, including overcrowding, lack of resources, and extreme temperatures, *C.*
55 *elegans* larvae develop into dauers. Through drastic physical, metabolic, and behavioral changes, dauers
56 become efficient vectors for dispersal that can survive for months without food[36]. Under normal
57 laboratory growth conditions where *C. elegans* are cultivated on flat agar surface in petri dishes, *C. elegans*
58 dauers do not nictate unless contaminated by fungi. Nictation behavior is also observed when grown on
59 three dimensional habitable scaffolds[37]; however, 3D scaffolds are not very convenient to study
60 migratory behavior.
61 To address this issue, we adapted a setup used to study neuromuscular integrity in *C. elegans*[38]
62 to investigate if *C. elegans* dauers exhibit directional bias in response to gravity. We injected dauers into
63 gravitaxis chambers comprised of two serological pipettes that, when stacked end to end, allowed for ~25
64 cm of movement in either direction away from the injection site (Fig.1C). Pipette chambers loaded with
65 worms were oriented either vertically or horizontally and were left overnight and scored the following
66 day. In the absence of any cues, worms would not exhibit any migratory bias in either direction when the
67 chambers are oriented horizontally. For all gravitaxis assays, we included a horizontal comparison to
68 control for any directional preference attributable to the construction and design of the chamber itself. If
69 worms perceive gravity they would exhibit a directional bias in the vertically oriented chamber – a
70 migratory bias to the bottom if exhibiting positive gravitaxis and a migratory bias to the top in case of
71 negative gravitaxis.

72 Contrary to prior assays conducted on petri plates that showed *C. elegans* dauers do not favor
73 any direction in response to gravity[16], N2 dauers exhibited a small but significant directional bias in
74 migration to the top of the vertical chamber in stark contrast to lack of any directional preference in
75 horizontal controls ((n = 3,816 worms over 14 trials; $p < 0.001$) (Fig. 2A, B)). A Gravitaxis Index (GI)
76 analogous to the chemotaxis Index (CI)[39], that represents the fraction of worms that migrate to the top
77 or bottom of the gravitaxis chamber did not represent the migratory behavior of the worms (GI vertical =
78 0.43, SEM = 0.17; GI horizontal = 0.20, SEM = 0.16; $p > 0.05$, two-tailed t test). Instead, we analyzed the
79 distribution of worms across each assay and compared vertical and horizontal distributions using Kruskal-
80 Wallis. We performed Dunn's test for multiple comparisons with Bonferroni's correction when comparing
81 across multiple conditions.
82
83 **Gravity perception competes with light and electromagnetic signals in governing migration**
84 *C. elegans* exhibits strong negative phototactic behavior. It is possible that the vertical negative gravitactic
85 migration might be adversely influenced by the strong response to light (Fig. 1A). We hypothesized that
86 N2 dauer larvae might exhibit a stronger negative gravitaxis effect in the dark. We repeated the assay in
87 the dark using a light-blocking blackout cloth. We found that N2 dauers demonstrated a strong preference
88 for migration towards the top of the chambers under these conditions in stark contrast to lack of any
89 directional preference in horizontal controls (n = 37,869 worms over 108 trials; $p < 0.001$) (Fig. 2A, B).
90 Significantly, this preference increased relative to worms tested under normal laboratory lighting
91 conditions ($p < 0.01$) (Fig. 2C).
92 In addition to photosensitivity, *C. elegans* adults have been shown to respond to electromagnetic
93 fields and their direction of migration is influenced by their satiety state – well fed worms migrate up
94 while starved worms migrated down a vertical column[20]. To test if electromagnetic fields (EMF)
95 influence the gravitaxis behavior of dauers, we decided to shield our assay from background EM fields

96 present in the lab using a simple Faraday cage made of aluminum foil. To our surprise, removing
97 background EMF also had a drastic effect on gravitactic behavior. Dauers in the -light -EMF condition
98 negatively gravitaxed in comparison with horizontal controls ($p < 0.001$; n = 37,869 worms over 108 trials)
99 (Fig. 2A, B) and were significantly more negatively gravitactic than either the -light +EMF or +light +EMF
100 conditions ($p < 0.001$ in both cases) (Fig. 2C). Over the course of several months, we noticed that gravitaxis
101 in our Faraday cage gradually decreased. After reinforcing the cage once more, we saw a recovery of
102 strong negative gravitatic behavior, adding to our observation that worms rely on both EMF and gravity
103 inputs in determining vertical movement (Fig. S1). Thus, *C. elegans* exhibits negative gravitaxis behavior
104 which becomes pronounced when sensory stimulus by light is blocked and is further enhanced when
105 shielded from electromagnetic fields suggesting that *C. elegans* gravitaxis behavior is complex and a
106 consequence of integration of multiple sensory inputs. We continued to use a Faraday cage for the
107 remainder of the experiments described in this study.

108 In addition to external cues, we were curious if development has any effect on gravitaxis behavior.
109 According to our hypothesis, after worms find suitable conditions and reach adulthood, there is less
110 motivational drive to migrate long distances in any direction. Instead, adult worms are cannibalized by
111 their offspring when resources are scarce allowing the offspring to enter the alternate
112 developmental/dispersal dauer stage. We hypothesized that adult worms would either lose motivation to
113 migrate against the force of gravity or would lose the ability to sense gravity altogether. In both cases, this
114 would result in diminished gravitaxis in our assay.

115 As expected, N2 adults were less gravitactic than dauers in the absence of light and EMF ($p <$
116 0.001) (Fig. 2C). However, adults negatively gravitaxed significantly more than horizontal controls ($p <$
117 0.001; n = 1,297 worms over 11 trials) (Fig. 2A, B). Moreover, reduced gravitaxis in adults may be an
118 artifact of our experimental setup that was designed for dauers. Because of their length, adults can cover
119 a greater distance in a short amount of time with less exertion. It is possible that between the start of the

experiment and when the worms were scored, adults were able to reach the end of the chamber and turn back. Scaling up the assay or monitoring the adults as they move would provide more insight into this question.

Our results that *C. elegans* dauers and adults exhibit negative gravitaxis on solid surface in contradiction to prior study are likely attributable to assay design. Previous experiments have mostly been performed using Petri dishes, which limit the distance worms can travel. We were able to find robust differences between horizontal and vertically oriented chambers in part owing to the long distances they traversed. In addition, the environment of a worm crawling in a thin space between the agar and tube wall may be somewhat more analogous to crawling through a column of soil than the environment worms experience on the side of an open plate. Likewise, earlier experiments on gravitaxis in *C. elegans* make no mention of the lighting conditions used, leading us to believe that signal integration may have complicated these initial findings. A correlation was found between the number of worms in the chamber and the range of distances travelled, which points to a social aspect of this behavior that may also have been unaccounted for (Fig. S2).

Neuron-specific microtubules are required for gravitaxis

Gravitational forces perceived by invertebrates can be very small and consequently invertebrates have evolved extremely sensitive mechanisms for perceiving these tiny forces. In some invertebrates, stretch receptors are imbedded in the cuticle which results in an ion influx upon deformation in a manner analogous to stretch and pressure sensors in mammals[40]. Gentle touch in *C. elegans* is perceived by the mechanosensory DEG/ENaC channels MEC-4 and MEC-10[29] which form a pressure-sensitive channel in Touch Receptor Neurons (TRNs) that respond to cuticle deflections through extracellular matrix proteins MEC-1, MEC-5, and MEC-9[41] (Fig. 3). MEC-12 and MEC-7, which encode alpha and beta tubulin respectively, form unique 15 protofilament microtubules only in the six TRNs. These microtubule bundles

144 are thicker than the typical 11 protofilament microtubules found in most cells, including other
145 neurons[42]. Although the exact role of these unique microtubule structures in touch sensation is not well
146 understood, the 15-protofilament structures function in part by anchoring the MEC-4/10 receptor
147 complex within the cell[43]

148 Dauers defective in some components of the gentle touch perception pathway – *mec-2(e75)*, *mec-*
149 *18(u228)*, *mec-4(e1339))*., *mec-10(tm1552)*, *mec-12(e1605),* and *mec-7(ok2152)* – exhibited impaired
150 negative gravitaxis when compared with N2 dauers (Fig. 4A-C). While the mechanoreceptor subunit
151 mutants *mec-4* and *mec-10* showed a modest, non-significant decrease in their ability to gravitax
152 compared with wildtype controls (Fig. 4C), vertically oriented worms exhibited a significant directional
153 bias from their horizontal controls (n = 1,750 worms over 12 trials; $p < 0.001$ and n = 4,422 worms over
154 25 trials; $p < 0.001$ for *mec-4* and *mec-10*, respectively) (Fig. 4A, B) suggesting that the ability to perceive
155 gravity is not seriously impaired in mutants lacking MEC-4/MEC-10 mechanosensory channel subunits. As
156 a caveat, the *mec-4* allele used here (*e1339*) is a missense mutation that exhibits weaker phenotypes
157 generally than the null, *tu253*. When tested in our assay, *mec-4(tu253)* worms do not gravitax (Fig. S3);
158 however, these worms are also sluggish, which has led to *e1339* being used in place of *tu253* in at least
159 one other study[44]. In contrast to our *mec-4*/*mec-10* results, both microtubule subunit mutants (*mec-7*
160 and *mec-12*) lose directional preference entirely and behave like the horizontal controls (n = 1,441 over 8
161 trials; $p > 0.5$ and n = 10,094 worms over 21 trials; $p > 0.5$, respectively) (Fig. 4A-C).

162 We examined the known *mec-7/12* phenotypes to understand how these genes alone could alter
163 gravitaxis behavior. Because MEC-7 and MEC-12 are tubulin proteins, mutations in either gene alter
164 axonal outgrowth to varying degrees[45], [46]. However, multiple studies have shown that the single
165 missense mutation H192Y in MEC-12, which we used in our experiments, eliminates gentle touch
166 sensation even though TRN development is not significantly affected[43], [47], [48]. MEC-12 is unique in
167 part because it is the only *C. elegans* microtubule subunit that is acetylated at K40[45], [49] and mutations

168 that modify K40 or MEC-17, a transacetylase necessary for microtubule acetylation and stability in TRNs,
169 also prohibit mechanosensation[50]–[52]. We tested whether acetylation of MEC-12 by MEC-17 is
170 necessary for gravitaxis in *mec-17(u265)* worms that lack functional MEC-17. This mutation did not affect
171 gravitaxis, as vertically oriented *mec-17* worms differed significantly from horizontal controls (n = 1,101
172 worms over 7 trials; $p < 0.001$) and were not statistically different from N2 dauers ($p > 0.05$) (Fig. 4A-C).
173 In addition to anchoring MEC-4/MEC-10 ion channels in TRNs, MEC-7/MEC-12 microtubules are
174 involved in regulating gene expression through MAPK signaling, ultimately targeting the transcription
175 factor CEBP-1. MEC-7/MEC-12 could be regulating gravitaxis through CEBP-1. However, the deletion
176 mutant *cebp-1(tm2807)* showed no defect in our gravitaxis assay; *cebp-1* dauers migrated significantly
177 higher in vertically oriented chambers than to the equivalent distance from the origin horizontally and
178 were statistically indistinguishable from N2 dauer controls (n = 1,140 worms over 9 trials; $p < 0.001$ and p
179 > 0.5, respectively) (Fig. 4A-C). Thus, CEBP-1 activation by MEC-7/MEC-12 is not essential for transducing
180 gravitational force.
181 Other candidate genes within the DEG/ENaC pathway were tested to determine the amount of
182 crossover between the canonical system and a potentially new gravitactic mechanism. *mec-2* encodes an
183 accessory protein that is part of the MEC-4/MEC-10 channel complex that acts as a linker between
184 receptors and 15 protofilament microtubules. Like the *mec-4* and *mec-10* mutants, *mec-2(e75)* dauers
185 were not significantly impaired in their ability to gravitax (n = 4,147 worms over 20 trials; $p < 0.001$) (Fig.
186 4A, B). *mec-18(u228)* mutants also exhibited similar results (n = 5,820 worms over 9 trials; $p < 0.001$),
187 although the exact function of MEC-18 and its role in gentle touch sensation is not well characterized[53],
188 [54]. Finally, an allele resulting in defective MEC-3 – a transcription factor required for differentiation of
189 several sensory neurons – did not significantly affect gravitaxis (n = 2,882 worms over 12 trials; $p < 0.001$).
190 Taken together, these results support the conclusion that MEC-7/MEC-12 microtubules might be involved
191 in a distinct role in gravity perception aside from their structural role in axon and dendrite length,

192 anchoring the DEG/ENaC channel subunits, or regulation of CEBP-1 in transducing mechanical force. The
193 lack of a strong effect in *mec-4* and *mec-10* mutants also indicates that other types of mechanosensory
194 channels may be involved in gravity perception.
195
196 **TRPA-1 is critical for gravity sensation**
197 Ion channel protein TRPA-1 is one member of a superfamily of TRP proteins involved in many sensory
198 modalities, including sensitivity to touch, hot and cold temperatures, noxious chemicals, and light[31],
199 [34]. Orthologs have been found across the animal kingdom, including in mammals, fish, and other
200 invertebrates such as *D. melanogaster*. Mouse TRPA-1 is also present in the vestibular system, the primary
201 organ where gravity sensation occurs in mammals[55]. In *C. elegans*, *trpa-1* is expressed in the
202 proprioceptive/nociceptive pair of neurons PVDL/R, among others. Although TRPA-1 is known to play a
203 role in noxious cold sensation in these neurons, *Drosophila* homologs of TRPA channels, *pain* and *pyx,*
204 are required for gravity sensation[35]. Additionally, *trpa-1* knockout mice are shown to have impairments
205 in mechanosensation as well as perceiving noxious cold[33]. Likewise, *C. elegans* TRPA-1 is likely to have
206 mechanosensory functions in addition to its known role in cold sensation[33].
207 The *trpa-1(ok999)* mutation is a large deletion that removes about 1.4 kbp from the N-terminus of the
208 TRPA-1 protein[33]. When *trpa-1* mutants were tested in our assay, they showed no directional
209 preference when compared with horizontal controls ($n = 2,692$ worms over 17 trials; $p > 0.05$) (Fig. 5A, B).
210 Predictably, vertically oriented *trpa-1* mutants differed significantly from vertical N2 controls ($p < 0.001$)
211 (Fig 5A, B). *trpa-1(ok999)* mutant adult worms exhibit several movement defects including decreased
212 forward locomotion as well as several variations in the sinusoidal movement typical to wildtype
213 worms[56] and as such could also affect vertical migration leading to misinterpretation of the role of
214 TRPA-1 in graviperception. However, in our assay, *trpa-1(ok999)* dauers were distributed similarly across

215 the chambers in both the horizontal and vertical conditions, demonstrating that they can travel long
216 distances regardless of orientation.
217
218 **PVD neurons, and not TRNs, are required for gravitaxis**
219 The role of *trpa-1, mec-7,* and *mec-12* and not *mec-4/10* in affecting this behavior presented an interesting
220 challenge. Although MEC-7 and MEC-12 are present in many neurons, their function is not well
221 understood outside of TRNs. There is no reported connection between microtubules containing MEC-7/12
222 and TRPA-1 function. Of the six TRNs, only one expresses *trpa-1*[57]. However, we noticed that PVDs,
223 which detect muscle movement in addition to a host of other stimuli, express all three candidate genes.
224 We hypothesized, therefore, that PVD neurons could be integration centers for two proprioceptive inputs:
225 muscle movement and relative position to Earth's gravity.
226 To test this hypothesis, we genetically ablated the TRNs and PVDs by expression of constitutively active
227 MEC-4 channels and DEG-3, respectively. The *mec-4(d)* gain of function (GOF) mutation results in
228 constitutive MEC-4/10 activation that degrades TRNs specifically, which we confirmed through the
229 absence of *mec-3p::*RFP marked TRNs (Fig. 6A-F). PVD neurons were ablated (PVD-) by the expression of
230 a constitutively active version of the nicotinic acetylcholine receptor (nAChR) channel subunit, *deg-*
231 *3(u662)/*DEG-3, under the *ser2prom3* promoter[58]. By adulthood, the highly branched PVD neurons have
232 developed 4° processes (Fig. 6A). 1°-3° processes can be observed in dauers (Fig. 6E). This ablation was
233 confirmed using *ser2prom3::*GFP (Fig. 6D).
234 As expected, ablation of the TRN neurons did not alter the ability of the worms to perceive gravity.
235 TRN- worms did not perform differently from N2 in our gravitaxis assay ($p > 0.05$) (Fig. 7B) and showed a
236 directional bias to the top compared with horizontal controls ($n = 2,680$ worms over 19 trials; $p < 0.001$)
237 (Fig. 7A, B). Meanwhile, PVD- worms did not gravitax, although their distribution differed significantly
238 from the horizontal condition ($n = 4,855$ worms over 15 trials; $p < 0.001$) (Fig. 7A, B). This difference is

239 attributable to a higher percentage of worms clustered at the center of the vertical assay, whereas worms
240 tested in the horizontal condition showed a slight (but likely random) preference for one side of the
241 chamber. As before, we suspect that this difference is due to choice and not ability, as PVD- worms appear
242 to have normal movement and are capable of reaching the ends of the chamber in any orientation.
243

244 **Discussion**

245 In this study, we found that *C. elegans* demonstrate a strong negative gravitactic preference especially in
246 the dispersal dauer phase, and less prominently in adults. In response to stressors such as overcrowding,
247 lack of food, and poor environmental conditions, *C. elegans* enter an altered developmental dauer
248 pathway that facilitates survival and enables them to seek a better habitat[14], [59]. Therefore, if a habitat
249 underground becomes poor, it is crucial that dauers find their way to the surface and nictate in the
250 absence of other guiding cues. Recent studies have also found that worms are sensitive to the earth's
251 magnetic fields[20]–[22]in addition to photosensitivity[6]. We found that the normal lighting conditions
252 and EM field in our laboratory setting was enough to alter gravitactic behavior. This supports our
253 understanding of signal integration in the nervous system, in which several sensory inputs are constantly
254 compared when making decisions[60].

255 The extent to which light, EM fields, and other inputs override gravity sensation, as well as the circuits
256 required to calculate their respective influence on behavior, are important questions for future studies.
257 Previous assays for gravitaxis by *C. elegans* dauers conducted on plates did not detect any directional
258 preference while *C. japonica* dauers under similar assay conditions were strongly negatively
259 gravitactic[16]. Our altered assay has shown that C. elegans dauers as well as adults also exhibit negative
260 gravitaxis. The reduced ability of N2 dauers to exhibit negative gravitaxis might be an outcome of myriad
261 adaptations to laboratory cultivation as has been observed for several other traits including social feeding,
262 egg laying behavior, oxygen tolerance, as well as nictation[11], [14], [15], [61]–[63]. Interestingly, our

263 results suggest that negative gravitaxis is maintained (albeit weakened) in the LSJ1 sister strain, which has
264 been raised in liquid media over several generations (Fig. S4). It would be interesting to see how these
265 two strains compare gravitactically when swimming rather than crawling on solid media.

266 Finding that *trpa-1* and components of the DEG/ENaC system are both involved in gravitaxis reveals a
267 complex mechanism for transducing gravitational force. Unlike the DEG/ENaC channels, TRPA-1 receptors
268 do not require internal or external machinery to function[33]. Additionally, *trpa-1* is only expressed in one
269 of six TRNs, which are the only neurons containing the 15 protofilament structures formed by MEC-
270 7/MEC-12 tubulins. As described previously, MEC-7/MEC-12 microtubules may have a function in
271 mechanosensation beyond their structure; whether this function can take place when they are not
272 assembled into 15 protofilament structures has not been explored. These findings point to at least two
273 redundant pathways involved in gravity sensation that need to be explored in more detail as well as their
274 possible role in nictation.

275 Finally, we found evidence pointing to a pair of neurons, PVDL/R, as primary gravity-sensors in worms.
276 Unlike TRN- worms, which behave normally in our gravitaxis assay when compared with controls, PVD-
277 worms have a distinct lack of preference in response to gravity. These results, in conjunction with our
278 *trpa-1* data, provides a sketch of how gravity may be transduced at a molecular and cellular level. We
279 propose that PVD neurons utilize TRPA-1 to provide information about the direction of gravity. However,
280 we are still missing key details about this mechanism – we cannot conclude, for example, that TRPA-1 is
281 the primary gravity receptor in worms. As with all TRPs, TRPA-1 is known to have primary and secondary
282 roles in a variety of sensory pathways[30]. Likewise, we do not know how MEC-7 and MEC-12 microtubule
283 subunits may be acting in gravity transduction. They may be performing a vital function within PVD
284 neurons that is required to transmit a signal; it is also possible that they play a role in other important
285 supportive or downstream neurons. As stated previously, ciliated neurons are frequently used in gravity
286 sensation[2], [3]. C. elegans have 60 ciliated neurons[58] – of these, IL2 neurons may also be involved in

287 detecting gravity because of their known role in nictation[16]. Other ciliated neurons express MEC-12,
288 TRPA-1, and the likely gravity sensitive mechanoreceptor in mammals, TMC-1[60]–[66]. Thus, PVDs may
289 be secondary neurons that act within a circuit of cells that make decisions about movements with respect
290 to gravity.

291 Understanding how *C. elegans* perceives gravity has implications for both worm and mammalian
292 biology. For years, *C. elegans* has been used as a model for understanding the effects of microgravity on
293 human physiology in space[23], [24], [26], [67]–[71]. Despite this, only a few studies have investigated
294 whether worms are capable of sensing gravity in the nervous system. Additionally, gravity perception is
295 linked evolutionarily and developmentally with hearing[72], [73]. The vestibular and auditory systems in
296 mammals share similar sensory structures, transduction machinery, and gene expression[74].
297 Mammalian TRPA1 has already been identified for its putative role in sensory transduction within inner
298 ear hair cells in mice[75]. The similarities between these systems makes the study of gravity sensation
299 important not only for understanding mammalian vestibular sensation, but auditory sensation as well. In
300 addition to its potential function in the inner ear, *trpa-1* homologs may also be involved in pain and
301 inflammation, as well as childhood asthma in humans[76], [77]. Remarkably a recent study shows that C.
302 elegans is able to sense sound through mechanical perturbations of the skin and involves the multimodal
303 PVD neurons and the FLP mechanosensory neurons[78]. Uncovering the role of TRPA-1 as a
304 mechanoreceptor opens the door to using *C. elegans* as a model for studying audition and auditory
305 disorders and has further implications for translational research.

306

307

Materials and Methods

Worm rearing

C. elegans worms were grown and maintained on OP50 *E.coli* at room temperature (23°C) using standard methods[85]. Dauer formation was induced by overcrowding, which occurred 8-12 days after chunking onto seeded NGM plates. Adults used for gravitaxis assays and imaging were taken from synchronized populations 2 days after hatching. Crosses were performed as described previously[86], using fluorescence microscopy and/or PCR to confirm the outcome of each cross. All strains used in this study are included in Table S1.

Dauer isolation

Dauer larvae were isolated from 1-5 starved NGM plates using 1% SDS and a 30% sucrose gradient as described previously[59], [87]. Briefly, worms were rinsed and collected in M9, then pelleted and resuspended in 7mL 1% SDS in a 15 mL falcon tube. Worms were left rotating for 30 minutes in SDS to kill all non-dauer lifestages. After 3-5 rinses with M9, worms were resuspended in 10 mL 30% sucrose solution and centrifuged for 5 minutes. Surviving dauers were collected at the interface of the water-sucrose gradient with a large bore glass Pasteur pipette and rinsed 3-5X in M9. Worm were used immediately or after rotating in solution overnight.

Synchronization

Mixed stage worms were rinsed from confluent plates with M9 and collected in 1.5 mL tubes. Worms were then rinsed and pelleted. A 1 mL bleach solution containing 150 µL bleach and 50 µL 5N KOH was added and worms were lysed in this solution for 3 minutes, or until most corpses were dissolved. The embryo pellet was then rinsed 3-5X in M9 and plated onto OP50 NGM plates until adulthood (~2 days at 23°C).

Assay preparation

Gravitaxis assay chambers were created using 5 mL serological pipettes and 4% NGM agar. To assemble each chamber, a heated blade was used to cut the tapered end of one pipette and the cotton plug was removed from the end of a second pipette. The two pipettes were then joined end-to-end by melting the two ends slightly over a Bunsen burner and fusing them with moderate pressure, ensuring that no gaps remained. The NGM media was then autoclaved and then taken up by each double pipette using a standard serological pipettor while the agar was still molten. Best results were achieved by orienting each pipette parallel with the benchtop as much as possible during the procedure and as the agar solidified. Pipettes were used within 24 hours of construction or stored at 4°C to be used within 48 hrs.

Once solidified, a heated 3mm Allen key was used to punch a hole through one wall of the pipette about 5mm below the fusion point (for reference, the cotton-plug end is the “top”). Each end of the pipette was removed using a heated blade and sealed with parafilm.

Gravitaxis assay

Worms were pelleted in M9 and were either extracted directly from the pellet or pipetted onto a square of parafilm. After cutting the tip to increase the bore, a pipette was used to aspirate 0.5-2 uL of the concentrated worm pellet, letting in a small volume of air at the end to facilitate injection. Worms were

injected into the agar at the opening created by the Allen key. This opening was then sealed with parafilm, and the chamber was immediately oriented vertically or horizontally. Worms were allowed to gravitax overnight and were scored the following day (typically 12-24 hrs after injection).

Pipettes were examined under a dissecting microscope and a marker was used to mark the location of each worm. Pipettes were scored immediately after removal from the assay location. Worms were only scored if they appeared alive, healthy, and were not swimming (chambers were only scored if a majority of worms were alive and crawling). Worms were not scored within 2.5 cm to either side of the injection point.

To tally the location of each worm, pipettes were divided into 7 sections on each side of the 5 cm injection site. Each section was 3.5 cm long (the same length as a 1 mL volume indicated on the pipettes). The number of marker points in each section was then recorded.

Imaging

Fluorescent micrographs were taken using a Leica SP8 Resonant Scanning Confocal microscope at 10-40x. Brightness and contrast were adjusted and overlays added using ImageJ.

Quantification and Statistical Analysis

Data were collected in Excel and analyzed using RStudio. Statistical tests, p values, sample sizes, and replicates are detailed in the results and figure legends.

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Figure 1. Behavioral model, wiring diagram, and gravitaxis assay design

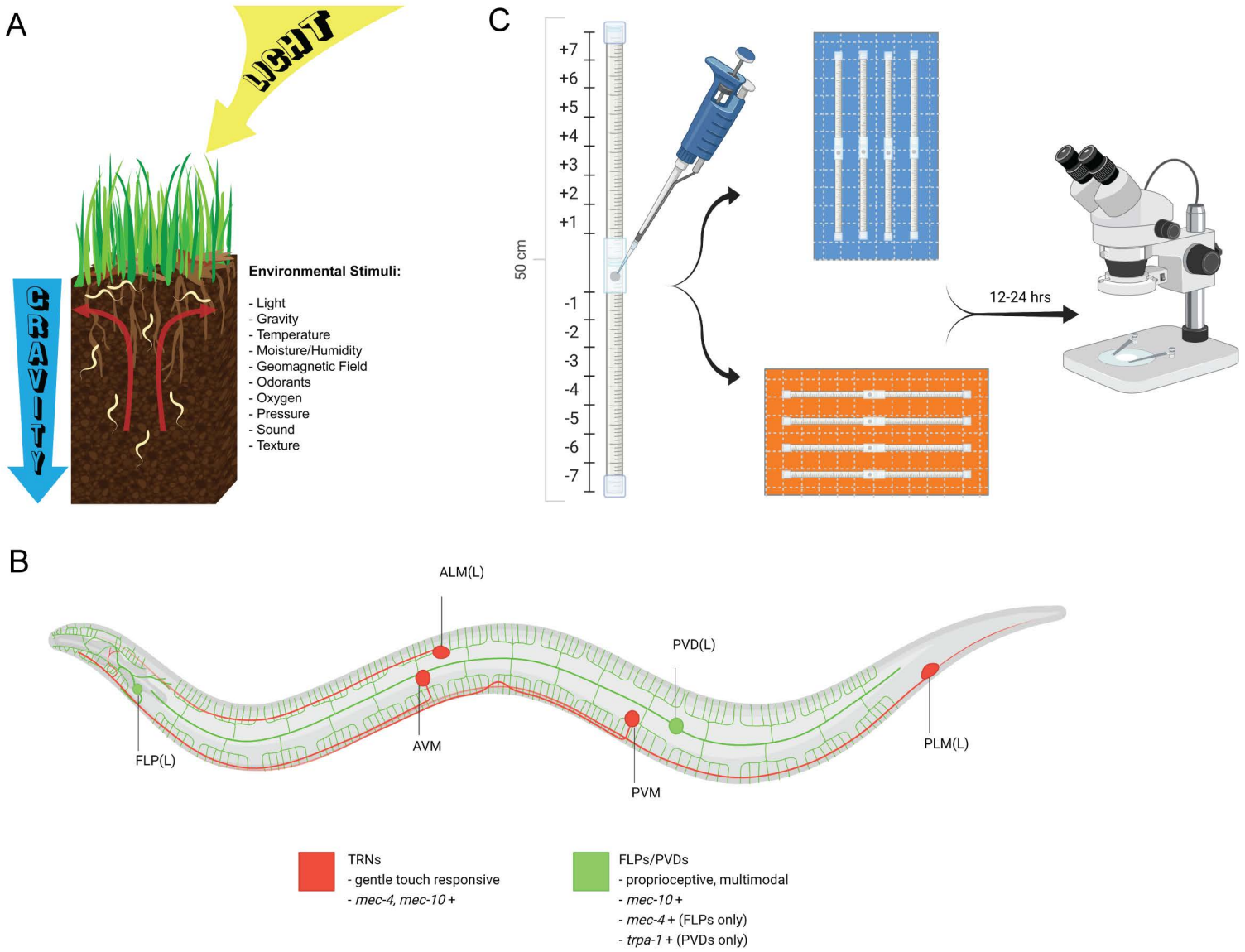


Figure 2. *C. elegans* negatively gravitax under different environmental conditions and developmental stages

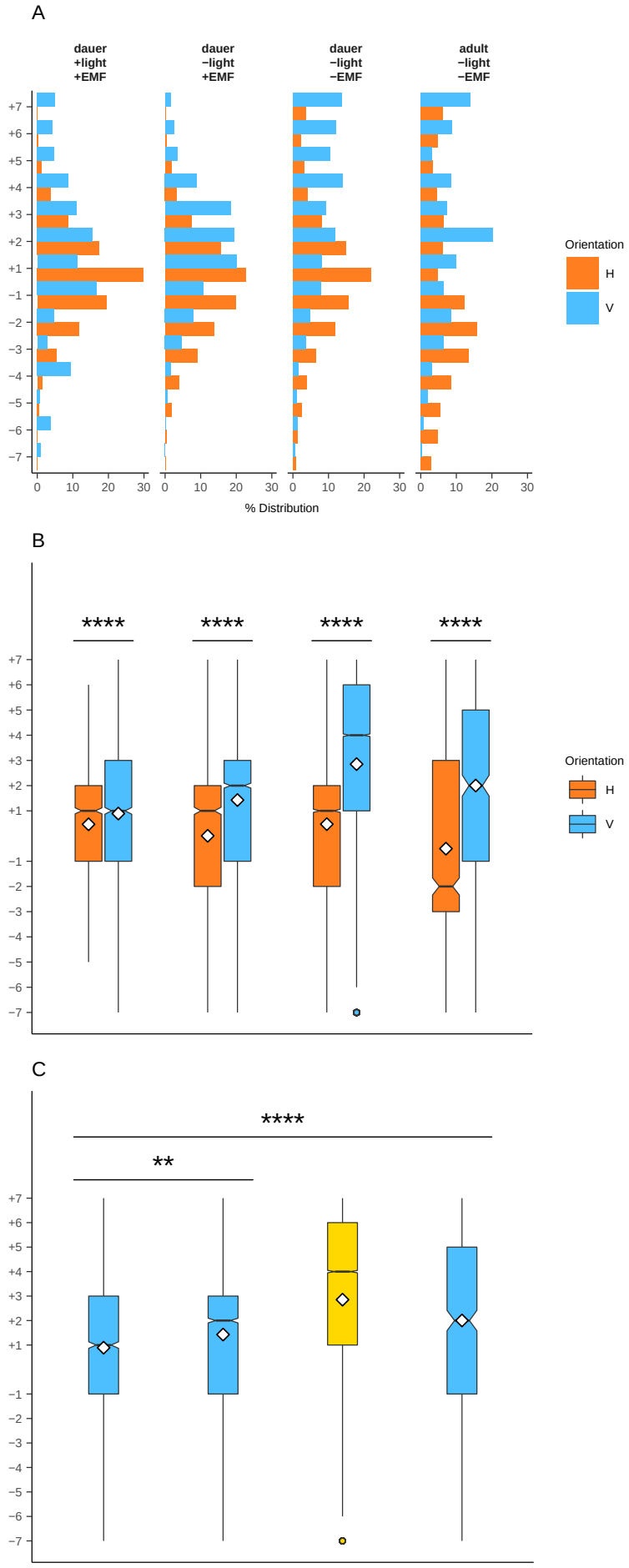


Figure 3. DEG/ENaC components

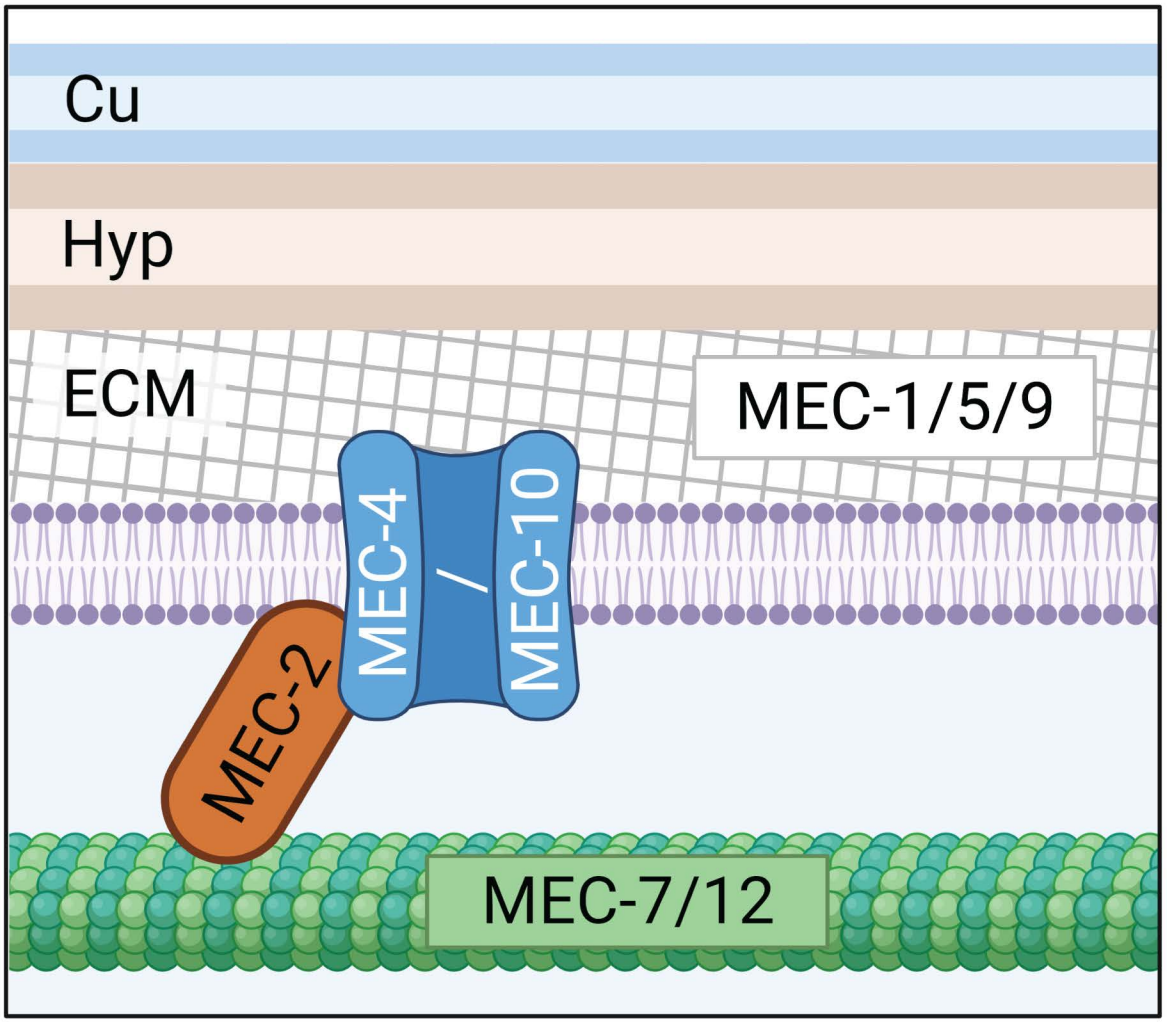
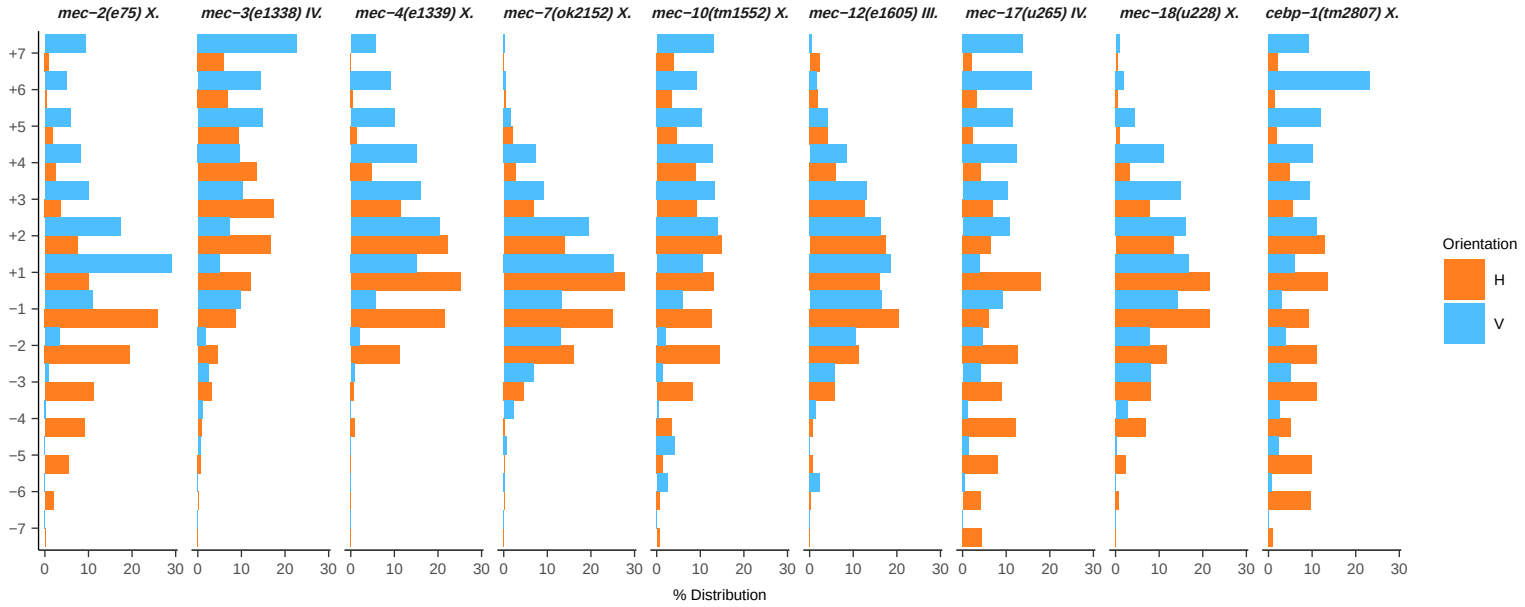
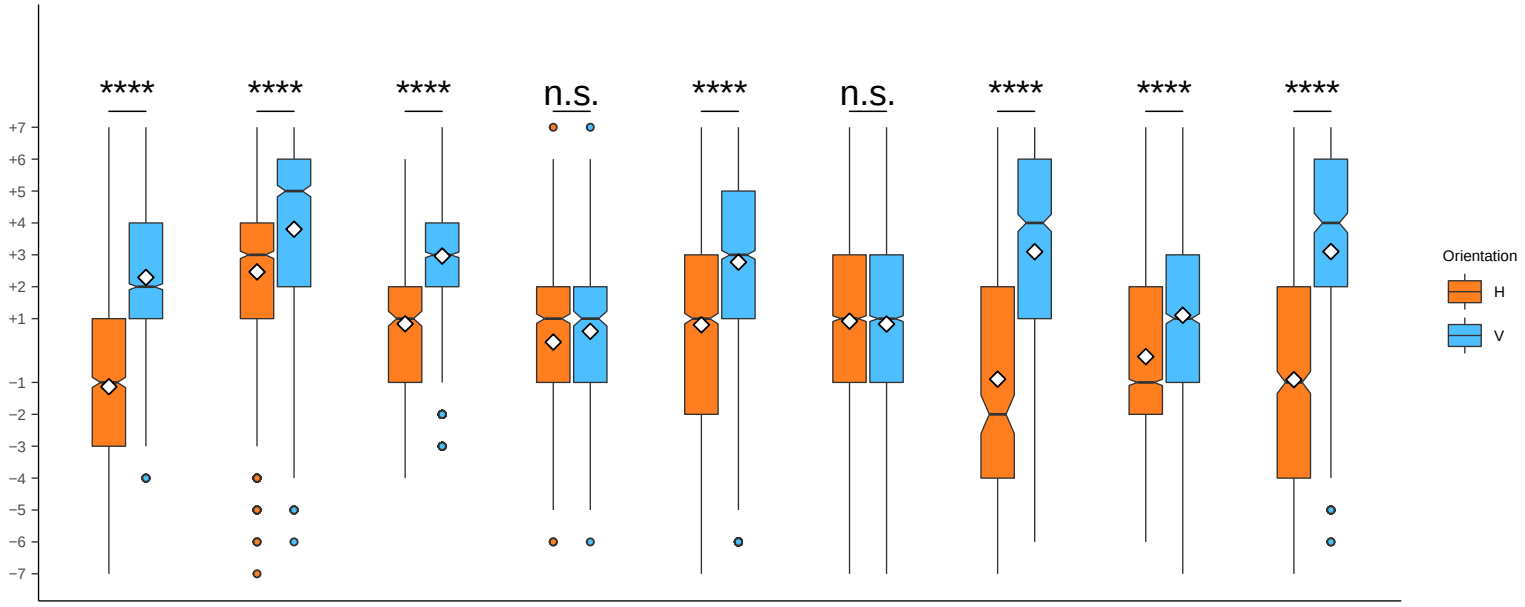


Figure 4. MEC-7/12 microtubule subunits are necessary for gravitaxis in contrast to other DEG/ENaC components

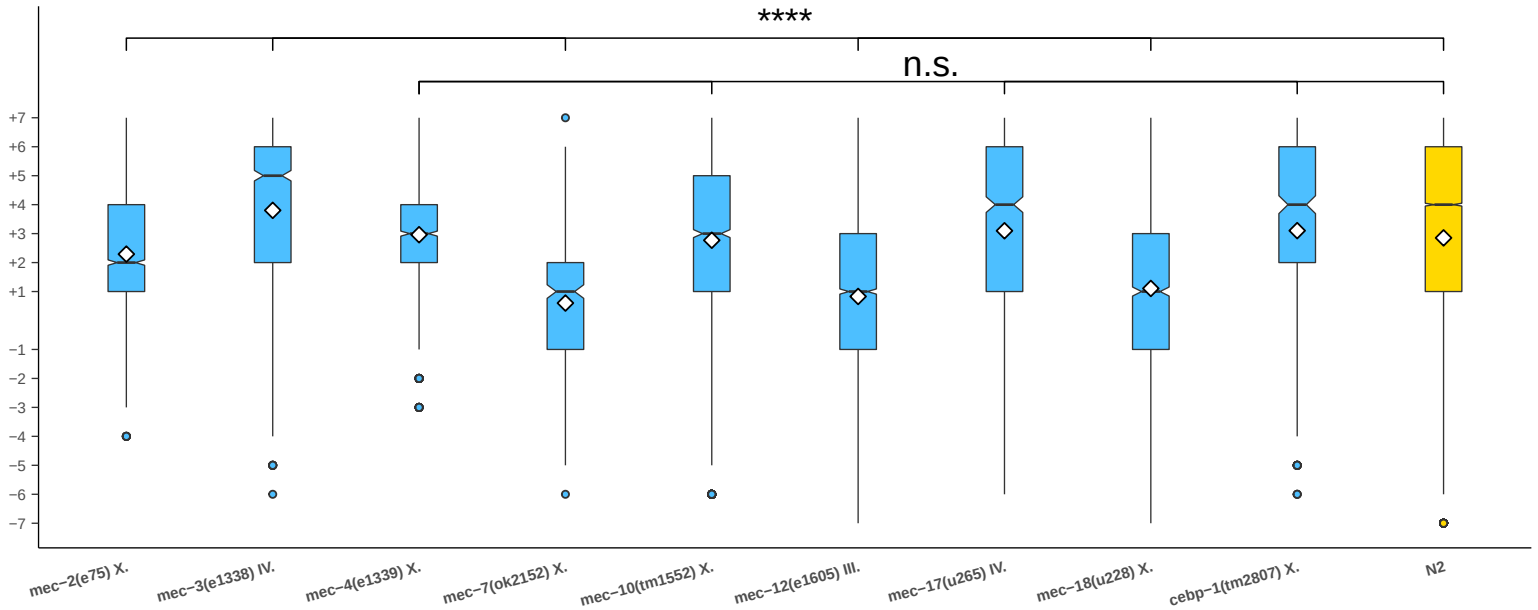
A



B



C



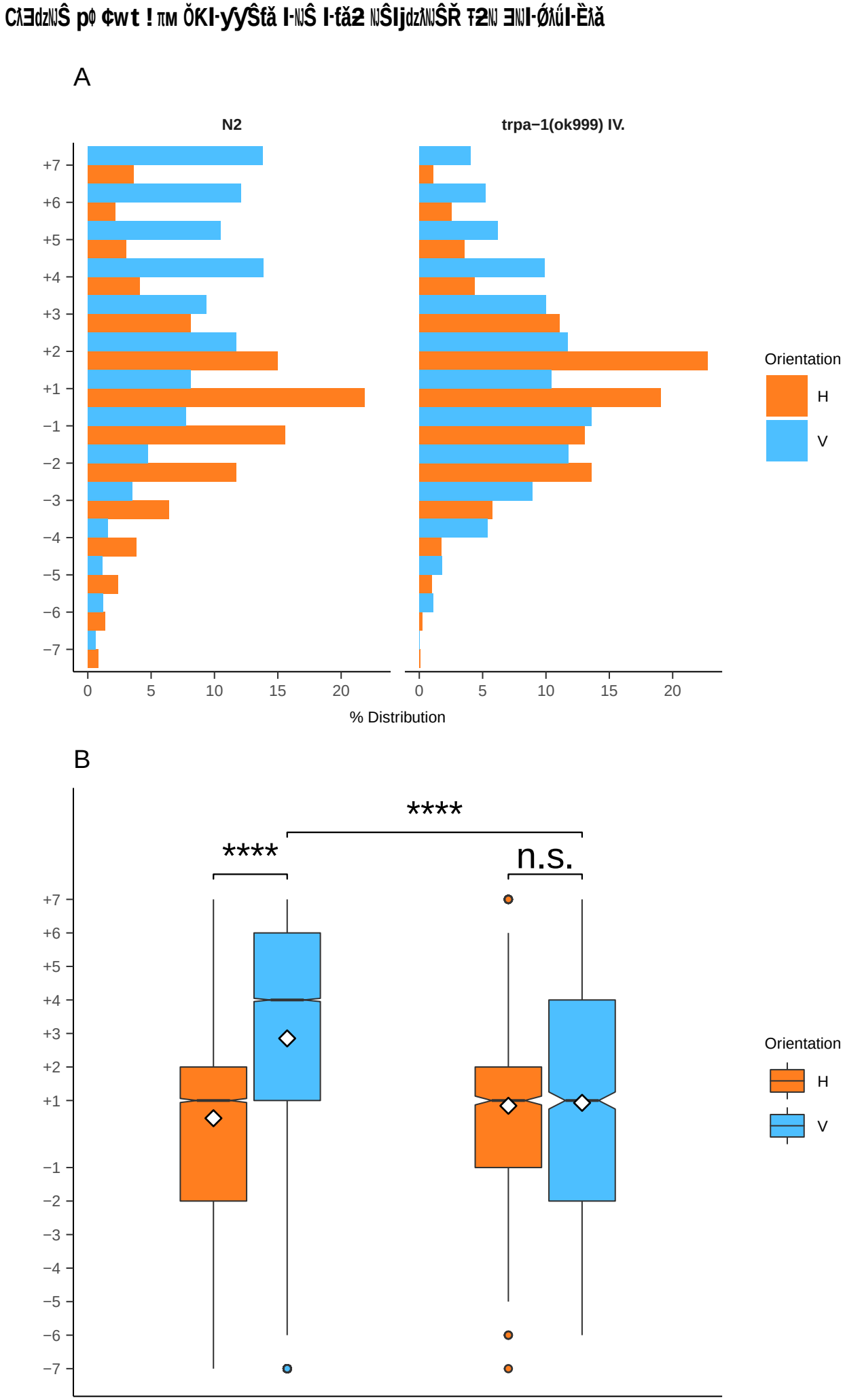

A
N2
trpa−1(ok999) IV.
+7
+6
+5
+4
+3
+2
+1
−1
−2
−3
−4
−5
−6
−7
0
5
10
15
20
% Distribution
Orientation
H
V
B
****
****
n.s.
Orientation
H
V

Figure 6. PVD and TRN neurons in adult and dauer worms

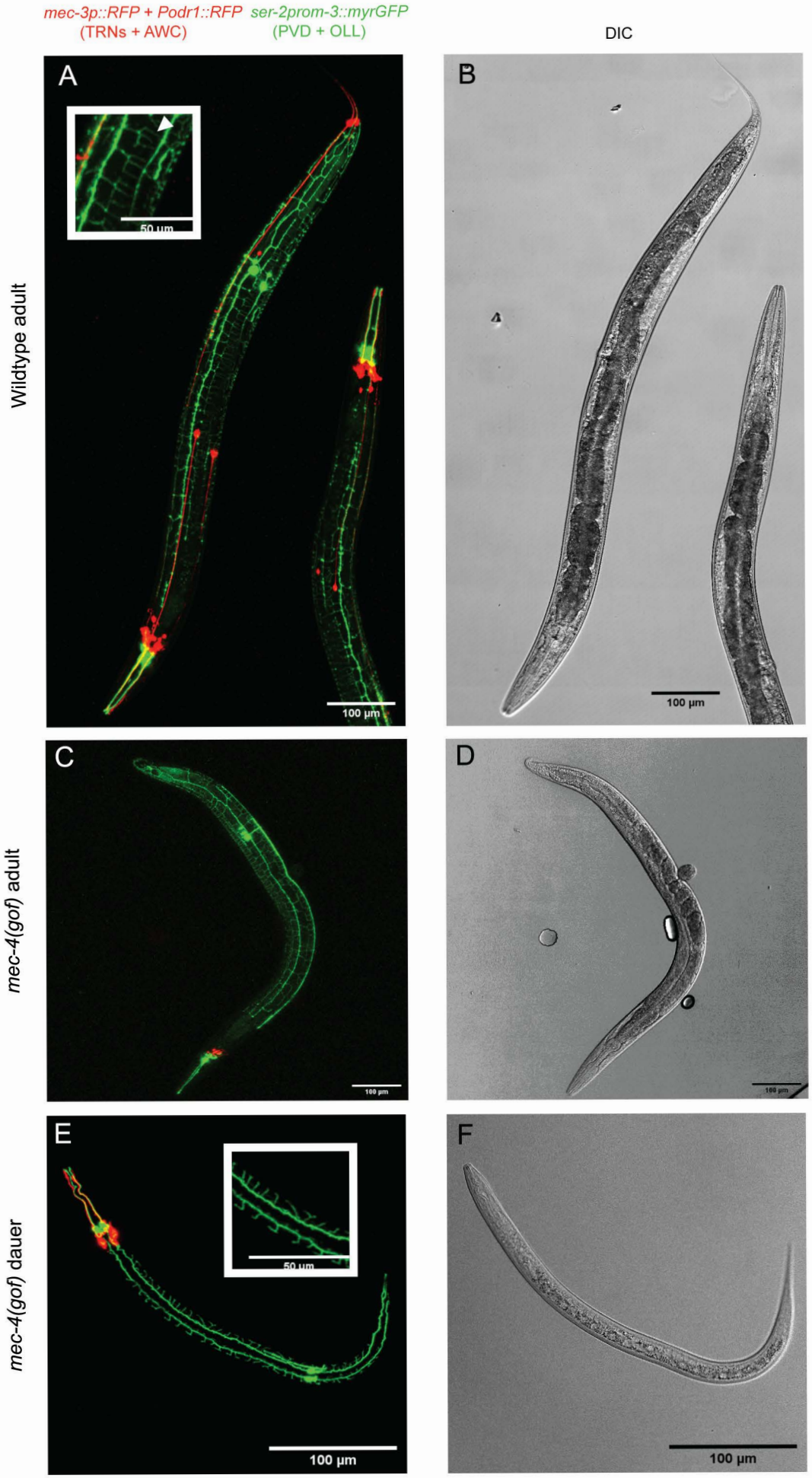


Figure 7. Genetic ablation of PVD but not TRNs affects gravitaxis

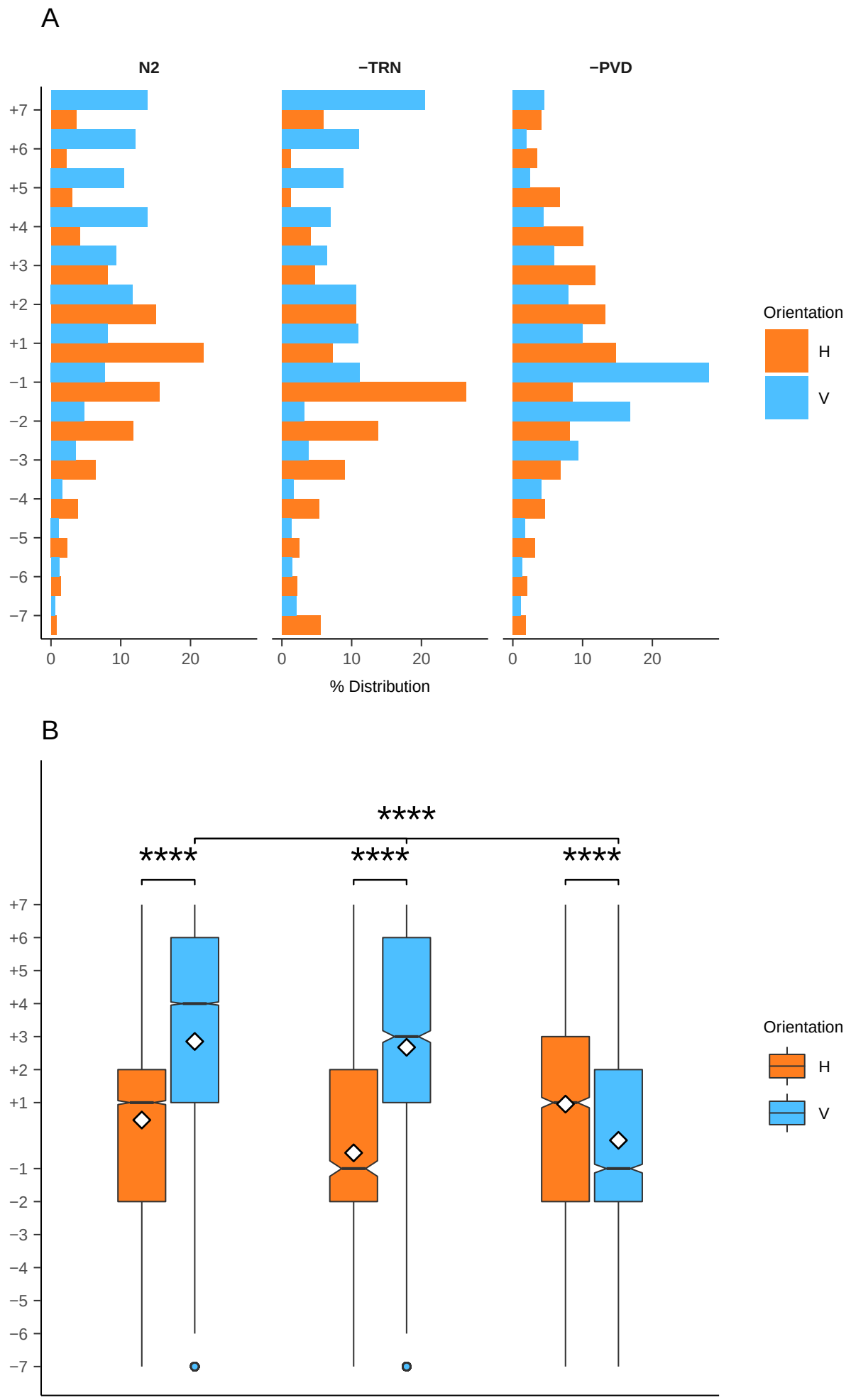


Figure Legends

Figure 1. Behavioral model, wiring diagram, and gravitaxis assay design

(A) Graphic depicting environmental stimuli that inform *C. elegans* behavior in a natural setting. **(B)** Diagram of key neurons mentioned in this study. TRN neurons are labeled in red, while FLP and PVD neurons are green (for neurons with left and right pairs, only the left neuron is displayed). Adapted from individual neuron illustrations available on WormAtlas[79]. **(C)** Gravitaxis chamber and experimental design used in this study. Figures **(B)** and **(C)** created with BioRender.com.

Figure 2. *C. elegans* negatively gravitax under different environmental conditions and developmental stages

(A) Histograms depicting cumulative distribution of *C. elegans* worms over multiple trials in vertical (blue) and horizontal (orange) assays. Presence or absence of normal overhead light and background EMF (no Faraday cage) are denoted with +/-. **(B-C)** Boxplots depicting data shown in A. Vertical conditions only compared in **C**. Dauers tested in a dark Faraday cage (yellow) are used in future comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n.s. is not significant using Kruskal-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

Figure 3. DEG/ENaC components

MEC-4 and MEC-10 subunits form a sodium channel that is required for gentle touch sensation in TRNs. MEC-1, -5, and -9 components of the extracellular matrix (ECM) provide connection points between touch receptors and the cuticle[80]. MEC-7/12 microtubules form 15 protofilament structures in the TRNs only; however, these β and α subunits (respectively) are found in other neuronal 11 protofilament structures[43]. MEC-2 proteins associate with both the Deg/ENaC channels and TRN microtubules and enhance channel activity[81]–[83]. (Adapted from images by *Tavernarakis et. al 1997* [84] and others). Created with BioRender.com.

Figure 4. MEC-7/12 microtubule subunits are necessary for gravitaxis in contrast to other DEG/ENaC components

(A-C) Dauers with mutations in DEG/ENaC genes tested compared with N2 dauers. **(A)** Histograms comparing horizontal (orange) and vertical (blue) distributions of each strain over multiple trials. **(B-C)** Boxplots summarizing data shown in **A**; vertical comparisons with N2 dauers shown in **C** (yellow boxplot)**.** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

Figure 5. TRPA-1 channels are also required for gravitaxis

(A-B) *trpa-1* mutants in vertical (blue) and horizontal (assays) compared with N2 controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

Figure 6. PVD and TRN neurons in adult and dauer worms

Confocal fluorescent **(A, C, and E)** and DIC **(B, D, and F)** micrographs of *mec-3p::RFP* and *ser-2prom-3::GFP* expression labelling the TRN and AWC neurons and PVD and OLL neurons, respectively. **(A-B)** Wildtype adult hermaphrodites; 4° process indicated with white arrowhead in inset. **(C-D)** *mec-4(e1611)* gain of function adult hermaphrodite, in which TRNs are ablated. **(E-F)** *mec-4(e1611)* dauer worm showing development of 2° and 3° processes in inset. Scale bar 100µm, 50µm in insets.

Figure 7. Genetic ablation of PVD but not TRNs affects gravitaxis

(A-C) Gravitaxis behavior of dauers lacking TRNs only (*mec-4* gof), dauers lacking PVD neurons only (*ser2prom-3::deg-3*), and N2 dauer controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

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Declaration of Interests:

The authors declare no competing interests.