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Hypergravity hinders axonal development of motor neurons in *Caenorhabditis elegans*

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As space flight become more accessible in the future, humans will be exposed to gravity conditions other than our 1G environment on Earth. Changes in physiology and anatomy in altered gravity conditions have long been observed, especially the loss of muscle mass during long-term space habitation, the reason for which is not fully understood. Although much effort has gone into studying the effects of gravity in muscle physiology, its effect on the development of neurons has not been thoroughly assessed. Using the nematode model organism *Caenorhabditis elegans*, we examined changes in response to hypergravity in the development of the 19 GABAergic DD/VD motor neurons that innervate body muscle. We found that a high gravity force above 10G significantly increases the number of animals with defects in the development of axonal projections from the DD/VD neurons. We showed that a critical period of hypergravity exposure during the embryonic/early larval stage was sufficient to induce defects. While characterizing the nature of the axonal defects, we found that in normal 1G gravity conditions, DD/VD axonal defects occasionally occurred, with the majority of defects occurring on the dorsal side of the animal and in the mid-body region, and a significantly higher rate of error in the 13 VD axons than the 6 DD axons. Hypergravity exposure increased the rate of DD/VD axonal defects, but did not change the distribution or the characteristics of the defects. Our study demonstrates that in addition to gravity's effects on muscle development, gravity can also impact motor neuron development.

1

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14

15 **ABSTRACT**

16 As space flight become more accessible in the future, humans will be exposed to gravity
17 conditions other than our 1G environment on Earth. Changes in physiology and anatomy in
18 altered gravity conditions have long been observed, especially the loss of muscle mass during
19 long-term space habitation, the reason for which is not fully understood. Although much effort
20 has gone into studying the effects of gravity in muscle physiology, its effect on the development
21 of neurons has not been thoroughly assessed. Using the nematode model organism
22 *Caenorhabditis elegans*, we examined changes in response to hypergravity in the development of
23 the 19 GABAergic DD/VD motor neurons that innervate body muscle. We found that a high
24 gravity force above 10G significantly increases the number of animals with defects in the
25 development of axonal projections from the DD/VD neurons. We showed that a critical period of
26 hypergravity exposure during the embryonic/early larval stage was sufficient to induce defects.
27 While characterizing the nature of the axonal defects, we found that in normal 1G gravity
28 conditions, DD/VD axonal defects occasionally occurred, with the majority of defects occurring
29 on the dorsal side of the animal and in the mid-body region, and a significantly higher rate of
30 error in the 13 VD axons than the 6 DD axons. Hypergravity exposure increased the rate of
31 DD/VD axonal defects, but did not change the distribution or the characteristics of the defects.
32 Our study demonstrates that in addition to gravity's effects on muscle development, gravity can
33 also impact motor neuron development.

34

35

36 **INTRODUCTION**

37 The possibility of human long-term space travel and habitation may be thrust into reality
38 over the next 10 to 20 years. However the effects of low gravity on the human body are costly
39 for human health. Particularly, muscle atrophy in microgravity conditions poses a large problem
40 for long-term space travel and habitation (Vandenburgh et al. 1999). The bulk of the
41 microgravity-induced muscle atrophy has been focused on the muscle (Fitts et al. 2001)for
42 obvious reasons, and has led to suggestions from the scientific community for astronaut fitness
43 during spaceflight (di Prampero & Narici 2003).

44 The effects of gravity on motor neurons that control muscle activity has been less studied
45 than muscle. Previous work showed that microgravity induced a decrease in synaptic density in
46 the hindlimb area of the motor cortex in the brains of rats that were aboard a space mission for
47 16 days (DeFelipe et al. 2002). Alterations in gravity could also affect the development of
48 neuronal projections. In hypergravity conditions, 150G force induced neuron-like cell
49 differentiation and development of longer neurites in cultured PC12 cells (Genchi et al. 2015).
50 Moreover, low gravity during spaceflight can induce alterations in dendritic arbor development
51 in medial spinal motor neurons in rats (Inglis et al. 2000). However, the mechanisms of gravity's
52 effect on motor neuron development is unknown.

53 The nematode *C. elegans* is one of the premier metazoan genetic model organisms, and
54 studies with the worm have led to seminal discoveries including RNA interference, microRNAs,
55 and genes controlling programmed cell death (Fire et al. 1998; Hengartner & Horvitz 1994; Lee
56 et al. 1993). Moreover, because worms are easy to handle, studies using *C. elegans* have been
57 conducted in space. Worms survival and grow well in space and many functions of the worms
58 are normal ((Higashitani et al. 2005; Szewczyk et al. 2008; Zhao et al. 2006). Muscle gene

59 expression is slightly altered in space (Higashibata et al. 2006), as well as markers for aging
60 (Honda et al. 2012). Hypergravity experiments on the ground show that behaviors and muscle
61 structure of the worms are normal at a 100G force, although the FOXO transcription factor DAF-
62 16 translocates to the nucleus (Kim et al. 2007). However, no studies on the effects of altering
63 gravity to the motor neurons have been conducted.

64 Motor neurons innervate 4 blocks of body wall muscle that line the ventral, dorsal, and
65 lateral sides of the worm body to control forward and backward movement (Sulston & Horvitz
66 1977). Particularly, D-type GABAergic motor neurons that are found on the ventral side of the
67 animal extend circumferential axonal processes called commissures to the dorsal side where they
68 join with the dorsal nerve cord to control movement (White et al. 1976). Six D-type motor
69 neurons called the DD neurons are born embryonically and extend commisssures dorsally before
70 the worm hatches from the egg shell. Another 13 D-type neurons called the VDs are born at the
71 first larval stage and then extend their commissures dorsally (White et al. 1976). Axon growth
72 cones from the developing DD/VD motor neurons sense secreted attractive or repulsive cues
73 along the body wall to find their targets in the dorsal side of the worm (Colavita et al. 1998;
74 Hedgecock et al. 1990; MacNeil et al. 2009).

75 The proper development of all 19 DD/VD motor neurons requires many genes expressed
76 from multiple cell types, and can be easily altered by genetic manipulation (Wadsworth 2002).
77 We wondered what the effect of altering gravity would have on the development of the DD/VD
78 neurons. In this study we increased the gravity force on developing *C. elegans* worms by
79 centrifugation, resulting in a hypergravity environment. We found high gravity significantly
80 increases the number of animals that had disrupted DD/VD axon projections, and we
81 characterized the defects we found in detail here.

82 **MATERIALS AND METHODS**

83 *Nematode culture and strains.* Animals were grown and maintained at 20°C on Nematode
84 Growth Medium (NGM) plates seeded with *E.coli* OP50 as described previously (Brenner 1974).
85 Strains used for this study: N2, LG II: *juIs76* [(p)*unc-25*::GFP], LG IV: *evIs82a* [(p)*unc-*
86 *129*::GFP)] and LG X: *zdIs5* [(p)*mec-4*::GFP). The genetic background of the *juIs76* strain was
87 maintained by backcrossing this strain with the N2 wild-type strain, which was effective in
88 maintaining a consistent axonal defect rate.

89 *Nematode hypergravity cultivation tube*: 1L NGM media was prepared similar to previous
90 protocols (Lewis 1995), except Difco granulated agar was substituted for Bactoagar (Lee et al.
91 2016). NGM was placed into 1.5 ml centrifuge tubes (150 ml tubes) and transferred to a heat
92 block set at 42°C to prevent immediate solidification of agar. After distribution into tubes, the
93 tubes were spun at 100G for 2 min to solidify the agar and create a surface in which the force of
94 gravity is perpendicular to the flat agar surface in the tube.

95 To create a small lawn of *E. coli* bacteria, a single colony of OP50 strain bacteria was inoculated
96 in LB broth and incubated in a shaker at 37°C overnight, then concentrated by spinning down
97 and removing the supernatant. The pellet was resuspended and 2 µl of bacteria was added to the
98 surface of each tube and allowed to dry at room temperature for at least 24 hours before usage.

99 *Preparation of eggs and hypergravity exposure*: Eggs were harvested by bleaching gravid worms
100 according to a standard protocol (Steiernagle 1999), washed and collected in M9 buffer. 1 µl of
101 this solution was counted to obtain an approximate egg population density. For hypergravity
102 experiments, 100-150 eggs were placed into the cultivation tube, and centrifuged in a
103 temperature-controlled micro centrifuge (Thermo Scientific, Sorvall Legend Micro17R

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104 Centrifuge) at the particular G-force (rpm values for 6G, 10G, 100G and 500G are 300 rpm, 400
105 rpm, 1200 rpm and 2800 rpm, respectively) at 20°C for various exposure times. A control tube
106 (1G) maintained in a 20°C incubator was performed with every experiment. Worms were
107 assessed by microscopy at the L4 larval/young adult stage which is 60 hours after egg harvesting.
108 If the hypergravity exposure time was shorter than 60 hours, the tubes were placed in a 20°C
109 incubator after the hypergravity exposure until 60 hours was reached.

110 *Microscopic analysis*: After 60 hours, animals were washed with M9 buffer and collected into
111 1.5 ml tubes and allowed to settle on the bottom of the tube. After removing the supernatant,
112 animals were mounted onto glass slides with a dry 2% agarose pad. To immobilize the worms, 2
113 µl of 1M sodium azide was added onto the agarose pad. An epi-fluorescent microscope
114 (Olympus BX50) was used to visualize the GFP-labeled neurons. Imaging software (Nikon
115 Elements) was used for extended depth of field (EDF) images, as well as all others images.

116 *Quantification of defects*: Defects were scored by researcher microscopic observations. Animals
117 were scored as defective if one or more of the 19 DD/VD neurons showed any axonal defect
118 (branch, turn and extend, stop, join and reach). Occasionally, an abnormally high axonal defect
119 rate (50% or more) was observed even in normal 1G conditions. We censured the all the data
120 from these days, and maintained the genetic background of the *juIs76* strain by backcrossing this
121 strain with the N2 wild-type strain. Dorsal-ventral locations of defects were approximated by eye
122 and scored as: Ventral – 0% to 15% dorsal-ventral distance, Ventral sublateral – 15% to 50%
123 dorsal-ventral distance, Dorsal sublateral - 50% to 85% dorsal-ventral distance, Dorsal – 85% to
124 100% dorsal-ventral distance.

125

126 **RESULTS**

127 **Hypergravity induces DD/VD motor neuron axonal defects**

128 To study the effect of hypergravity on *C. elegans* biology, we used a tabletop refrigerated
129 centrifuge to create a gravity force and designed a small worm cultivation tube from a 1.5 cm
130 centrifuge tube filled with NGM agar and seeded with OP50 *E. coli* bacteria on top (Fig 1A). To
131 visualize the DD/VD motor neurons, we used a *C. elegans* transgenic strain that expresses GFP
132 under the control of the *unc-25* gene promoter. The *unc-25* promoter directs expression of
133 glutamic acid decarboxylase, an enzyme necessary for the production of GABA
134 neurotransmitter, in the DD/VD motor neurons as well as several other neurons (Jin et al. 1999;
135 McIntire et al. 1993). Using the (p)*unc-25*::GFP strain, a total of 19 commissural DD/VD axons
136 can be observed running circumferentially from the ventral to the dorsal side (Fig 1B). Adult
137 hermaphrodite *C. elegans* were bleached to remove the eggs containing developing embryos
138 inside the mothers' bodies, and the eggs were immediately placed in the cultivation tube. The
139 tubes were either spun in the centrifuge to induce a high gravity force or placed in a 20°C
140 incubator as a 1G gravity control. There were no obvious differences observed between 1G and
141 100G: the pace of development in 1G and 100G was identical, and hermaphrodite worms at
142 100G developed normally to gravid adult mothers with no noticeable defects in movement (data
143 not shown). This is consistent with a previous study that cultivated *C. elegans* in a microfluidic
144 compact-disc cultivation system at 100G and showed that worms had normal growth and adult
145 movement and behaviors (Kim et al. 2007; Kim 2007)

146 After 60 hours, the worms had reached mid-late L4 larval stage. At 1G, most of the
147 animals showed normal DD/VD commissural projections that reached the dorsal nerve cord (1B
148 and C), although occasional defects could be observed (1I and J). However, in animals exposed

149 to 100G hypergravity for 60 hours, defective axonal projections could be seen more frequently.
150 When we quantified the defects, we found that 21% of worms grown in 1G displayed axonal
151 defects (Fig 1F). However, worms grown in 100G had at least one axonal defect in over 60% of
152 the worms, which is about a 3-fold higher frequency than in 1G conditions (Fig 1I). Among the
153 total axons we looked at, axonal defects occurred at a frequency of 1.5% (Fig 1J). In 100G,
154 defects occurred at a rate of 5.1%, an over 3-fold increase compared to 1G (Fig 1G).

155 Next, we varied the hypergravity force to observe their effects on DD/VD axon
156 development. Forces of 10G to 500G increased the percent of worms with axonal defects by
157 approximately 30% over 1G. Interestingly, the number of defects did not significantly differ
158 from 10G to 500G. On the other hand, a force of 6G, the minimum speed for our table top
159 centrifuge, could not induce axonal defects (Fig 2A). Therefore it seems that a certain threshold
160 in gravitational force exists between 6G and 10G that induces axonal defects. For our
161 experiments, we decided to use a hypergravity force of 100G, which is the force used in a
162 previous study (Kim et al. 2007).

163

164 **Hypergravity-induced axonal defects requires exposure during a specific period of**
165 **development**

166 We wondered if there was a critical period during development that hypergravity can
167 induce motor neuron axonal defects. Hence, we exposed embryos to hypergravity for different
168 time periods from the embryo stage and assessed DD/VD commissural axon defects in these
169 animals at the 60 hour point or 72 hour point. Exposure of developing *C. elegans* to 100G
170 hypergravity for 18, 60, and 72 hours all resulted in an approximately 2.5 to 3 fold increase in

171 axonal defects (Fig 2B and C). A shorter exposure of 10 hours resulted only in a small increase
172 in worms with axonal defects, and a 3 or 6 hour exposure did not increase axonal defects (Fig 2B
173 and C). We also exposed L4-stage *C. elegans* to 100G hypergravity for 3 hours, but there were
174 no increases in axonal defects. It is important to note that embryos recovered by bleaching the
175 mothers are not at a synchronous stage of development. Instead, these embryos are within an
176 approximately 6 hour range of development. This may explain why we observe an intermediate
177 phenotype at 10 hours hypergravity exposure (Fig 2C). Taking this into consideration, with our
178 methods a minimum of 18 hours in hypergravity is required for an increase in axonal defects.

179 What may be occurring in the DD/VD motor neurons during this time in development?
180 DD neurons are born during the embryonic stage on the ventral side of the animal and send
181 projections to the dorsal side well before hatching (Sulston 1976). The VD neurons, however are
182 not born until the late L1/early L2 stage, more than 20 hours after these eggs are harvested from
183 their mothers (Figure 2C). Thus, during this 18 hour critical exposure period, the VD neurons
184 likely have not yet been born. More surprising was when we restricted exposure to 100G
185 hypergravity from 4 to 18 hours, we saw a decrease in induced axonal defects compared to the
186 full 18 hours to a point (Fig 2B, 2C), further illustrating the importance of early exposure to
187 hypergravity in the observed increase in axonal defects.

188

189 **Characterization of hypergravity-induced axonal defects**

190 We then characterized the DD/VD motor neuron axonal defects more carefully by
191 counting the defects in each commissure. We first checked the structure of the DD neurons in the
192 L1 stage immediately after a 15 hour hypergravity exposure and found that 100% of DD

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193 neuronal commissures are normal at this time point (Table 1). This was puzzling, since this
194 meant that the defects we see in 1G at 60 hours likely arise after the mid-L1 stage, either by
195 some alteration of the DD axons, or some predisposed developmental change to the yet-born VD
196 neurons.

197 There are a total of 19 commissures, DD1 to DD6 and VD1 to VD13, with VD1 being
198 the most anterior commissure, and VD13 at the most posterior end (Fig 3A). The DD1 and VD2
199 commissures overlay each other in the same commissural tract and are indistinguishable.
200 Therefore, we considered DD1/VD2 as one commissure, and count a total of 18 DD/VD
201 commissures. At normal 1G gravity conditions, occasional axon defects were observed in most
202 commissures spanning the anterior and posterior ends of the worm (Fig 3B). We found that
203 certain commissures had a higher probably of having axon defects than others. For instance, the
204 5 commissures in the midbody region that include DD3, VD6, VD7, DD4, and VD8 accounted
205 for 54.48% of the total axonal defects whereas the 5 commissures on the anterior or posterior end
206 including VD1, VD2/DD1, VD12, DD6, and VD13 accounted for only 7.41% of the defects. We
207 also found that axonal defects were skewed towards the VD commissures and that DD neurons
208 showed less defects. We observed that 1.9% of all the VD commissures were defective, whereas
209 only 0.8% of the DD commissures showed defects (Table 1). In conclusion, although axonal
210 outgrowth and guidance is a faithful biological process that allows neuronal projections to reach
211 their targets, the development of the DD and particularly the VD motor neuron commissures are
212 slightly error prone.

213 While defects sporadically occur during the development of the DD and VD axons in 1G
214 gravity conditions, 100G hypergravity clearly aggravates this error rate. In 100G, the rate of
215 defective axons jumps from 0.88% to 2.82% for DD commissures and 1.92% to 6.39% for VD

216 commissures (Table 1), a 3.2-fold and 3.3-fold increase, respectively. Although hypergravity
217 induces more axonal defects, it does not significantly change the distribution of those errors. For
218 every DD axonal defect there are 2.2 VD defects at 1G, and this exact ratio is remarkably
219 maintained in 100G. In respect to location along the worm body, the five midbody commissures
220 still account for 48% of the axonal defects in 100G, whereas the five anterior/posterior end
221 neurons again only account for 8% of the errors. Hence, hypergravity only exacerbates the total
222 axon defect rate.

223 Next, we determined the location of each of the axonal defects in the ventral/dorsal
224 direction. We grouped the defects into four categories: Ventral, Ventral sub-lateral, Dorsal sub-
225 lateral, and Dorsal. At 1G, we found that most of the defects (68.7%) were located in the dorsal
226 sub-lateral region which is defined as the area dorsal of the lateral mid-line, but ventral to the
227 area of the dorsal nerve cord (Fig 3C, see Materials and Methods). Interestingly, this dorsal sub-
228 lateral bias in axonal defects was maintained or slightly higher at 100G (86.5%). We observed a
229 decrease in the number of dorsal defects in 100G, however the overall dorsal side bias of errors
230 (96.9% in 1G, 97.5% in 100G) is quite consistent. Once again, we find that hypergravity does
231 not alter the pattern of axonal defects observed in normal gravity conditions.

232 We also characterized the axon commissural defects based on morphology. We defined
233 four categories of defects: branched, turn and extend, stop, and joined and reach (Fig 4A). Axons
234 that abruptly stop, branch, or turn and extend never reach the dorsal nerve cord, whereas axons
235 that join together does reach the dorsal nerve cord. Most of the defect were in the turn and
236 extend category at close to 80%, while others were all less than 10%. Interestingly, the
237 distribution of axonal defects in 1G conditions was strikingly maintained in 100G hypergravity
238 (Fig 4B). Taken together, we find that hypergravity increases the overall rate of DD/VD

239 commissural axon defects compared to normal gravity, but does not alter the distribution of axon
240 defects.

241

242 **Hypergravity-induces axonal defects are specific for the DD/VD motor neurons.**

243 In a previous study, it was reported that 100G hypergravity did not affect the structure of
244 the chemosensory neurons ASI and ADF, and touch sensory neurons AVM, ALM, PVM, and
245 PLM, as well as the function of the AWA olfactory sensory neurons (Kim et al. 2007). We
246 exposed a strain of *C. elegans* that expressed GFP in the touch sensory neurons to 100G and
247 confirmed that the structure of the touch sensory neurons were not affected by 100G
248 hypergravity in our cultivation system (Table 1). To determine whether the structure of other
249 motor neurons could be affected, we exposed a strain of *C. elegans* that expressed GFP in the
250 DA/DB cholinergic motor neurons. The axonal commissures from the DA/DB motor neurons
251 project from the ventral to the dorsal side of the animal similar to the DD/VD motor neurons.
252 However, 100G hypergravity for 60 hours did not induce any defects in the DA/DB motor
253 neuron axons (Table 1). Thus, the hypergravity-induced axonal defects we have observed and
254 characterized may be specific for the DD/VD GABAergic motor neurons.

255

256 **DISCUSSION**

257 The physiology and cellular functions of organisms are adapted to the 1G gravity
258 conditions here on earth. This is clearly demonstrated by the biological effects that altered
259 gravity in spaceflight has on vertebrates, particularly muscle and bone atrophy. However broader
260 effects of altered gravity at the cellular level remain obscure. Here, we evaluated the effect that

261 high gravity has on motor neuron development in the nematode *C. elegans* and characterized
262 these changes. 10G to 500G hypergravity induces DD/VD motorneuron axon defects in 30%
263 more animals than 1G. Nearly all of the axon defects were found in the dorsal sublateral region,
264 and none of the defective axons reached their targets in the dorsal nerve cord. We found that an
265 18 hr exposure from the embryonic stage to the first larval stage was sufficient to cause the
266 axonal defects, whereas an acute 3 hour exposure in the adult animal did not induce defects.
267 Although hypergravity increases the overall DD/VD motor neuron axon defects compared to 1G,
268 it does not alter the distribution or the characteristics of these defects. Finally, these defects seem
269 to be specific for the DD/VD motor neurons.

270 Though we saw the effects of hypergravity at a range of G forces, we used 100G for most
271 of our experiments. A previous study applied a 100G force using a compact disc-type cultivation
272 apparatus (Kim et al. 2007). This study showed that hypergravity induced the nuclear
273 localization of the conserved FOXO transcription factor DAF-16, and this was dependent on the
274 ENaC/degenerin sodium channel that functions in mechanosensation. Many features in the worm
275 were preserved in 100G, including muscle structure, olfactory behavior, feeding behavior, and
276 the structure of several neurons ((Kim et al. 2007); Table 1). However, the effect of
277 hypergravity on the structure of *C. elegans* motor neurons had not been assessed yet. Although
278 our hypergravity cultivation system was different than the compact-disc type apparatus
279 previously used (Kim 2007), we confirmed that growth progressed normally, movement was
280 normal, and the structure of touch sensory neurons was normal ((Kim et al. 2007); Table 1).

281 Although hypergravity can increase axonal defects in the VD motor neurons, we still
282 observe a range of DD/VD axonal defects in a portion of the control animals at normal gravity.
283 This is consistent with observations from other groups that have studied DD/VD commissures

284 using the *juIs76* strain that expresses (p)*unc-25*::GFP (Caceres Ide et al. 2012; Lee et al. 2015).
285 In addition to the *juIs76* strain, we used the *oxIs12* strain that also expresses (p)*unc-47*::GFP in
286 the DD/VD neurons (McIntire et al. 1997). Interestingly, we did not detect any DD/VD axonal
287 defects at either 1G or 100G in this strain (data not shown). However, a previous report
288 cautioned that the oxIs12 transgenic strain affected the expression of X-linked genes involved in
289 axon guidance such as *unc-6*/netrin and *lon-2*/glypican genes, altering axon guidance phenotypes
290 in different genetic backgrounds (Gysi et al. 2013). In addition, previous studies using electron
291 microscopy have shown that the DD motor neurons can develop in slightly different locations in
292 individual animals (White et al. 1976), and the number of axons innervating the dorsal nerve
293 cord can also vary in individual animals (Hedgecock et al. 1990). These studies, along with the
294 other studies using the *juIs76* strain, provide evidence that the development of D-type motor
295 neurons may show slight differences between individuals. Thus, we have confidence that our
296 observations using the (p)*unc-25*::GFP strain are valid, and this lends credence to the fact that
297 DD/VD axon development normally error prone.

298 Two major questions remain unanswered: what causes the errors in DD/VD axonal
299 development, and how is hypergravity increasing these errors? Clues to answer the first question
300 may lie in the distribution of the axonal defects. We find that commissures in the mid-body
301 region tend to show more defects than the commissures at the ends of the animal. One possibility
302 that may cause this to arise is that the length of the commissures are slightly longer in the mid-
303 body area than the ends of the animal. Other differences in the mid-body compared to the ends of
304 the animal are structures such as gonads, vulva, and muscle that could interfere with the
305 guidance or migration of the axons. However, no evidence can account for these explanations.
306 We also find more defects in VD axons compared to DD axons. DD axons develop much earlier

307 than the VD axons, and we show that at the L1 stage, the DD axons are completely normal
308 (Table 1). However, by the L4 stage, defects have arisen in those same DD axons. DD axons
309 migrate to the dorsal cord during the embryonic stage, but the animal continues to grow to an
310 adult. It is not known how the DD axons maintain and grow with the developing body, and we
311 wonder whether this errors can arise in the axon during this process.

312 How does hypergravity affect DD/VD neuron axonal development? The force of gravity
313 itself might be strong enough to break or damage the axons. However, an acute exposure to
314 hypergravity of 3 hours was not sufficient to cause damage to the axons (Fig 2B). In addition,
315 DD/VD motor neuron axons in *C. elegans* have the ability to repair and regenerate after damage
316 (Hammarlund & Jin 2014). In our experiments, we exposed the worms to 100G for 18 and 36
317 hours, and then allowed the worms to recover until 60 hours at 1G. This is enough time to
318 regenerate any axons damaged by 100G force, yet we still observe hypergravity-induced axon
319 defects. Thus, we are not certain whether high gravity induces axon damage.

320 We show an 18 hour exposure early in development and during the 1st larval stage is
321 sufficient to cause the axon defects (Fig 2B, 2C). The VD neurons have yet to be born when the
322 critical period of hypergravity exposure occurs, yet we clearly observe defects in the VD
323 neurons. Thus, we assume that hypergravity may be affecting another cell or tissue rather than
324 the VD neurons themselves. Hypodermis and muscle is a major source for axon guidance cues
325 such as UNC-6/netrin, UNC-129/TGF-beta, and LON-2/glypican (Blanchette et al. 2015;
326 Wadsworth 2002), and we wonder whether hypergravity can somehow be altering these cues We
327 are currently conducting experiments to determine whether problems in axon guidance occur in
328 response to hypergravity.

329 In this study we have addressed the role that high gravity conditions have on DD/VD
330 motor neuron development. What effect does low gravity have on motor neuron development?
331 Previous studies in rats showed that development of the dendrites of spiny motor neurons during
332 spaceflight could be altered (Inglis et al. 2000). To determine the effects of microgravity on *C.*
333 *elegans* motor neurons, we will conduct ground-based experiments with microgravity
334 environments. We hope to ultimately answer these questions with space flight or space station-
335 based experiments at the genetic level. *C. elegans* provides a simple *in vivo* platform where
336 neuron development can be easily studied aboard a space station or spacecraft. These studies
337 may clarify whether low gravity effects on motor neuron development can also contribute to the
338 muscle weakness and atrophy observed in astronauts during long-term spaceflight and habitation.

339

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343

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Figure 1 (on next page)

100G hypergravity induces axonal defects in DD/VD motor neurons.

(A) Harvested embryos were exposed to hypergravity by centrifugation and then analyzed by microscopy as adults for neuronal defects. (B) (p)*unc-25::*GFP control animal at 1G gravity shows normal axonal commissures. Bar=100 µm (C) Magnified view of boxed area in (B). (D) (p)*unc-25::*GFP animal exposed to 100G hypergravity shows multiple axon commissural defects (white triangles) Bar=100 µm. (E) Axonal defects in 100G exposed animals. Bar=50 µm. (F) 3D extended depth of field (EDF) image of (p)*unc-25::*GFP exposed to 100 G hypergravity. Ventral in front, dorsal in back. Bar=100 µm. (G) Magnified image of white box in (F) showing normal axonal commissures. Note the circumferential axons traveling along the body wall from the ventral to dorsal side during development. (H) Magnified image of white box in (F) showing defective axon that turned and extended and formed branches before it approached the dorsal side of the animal. (I) Percent animals that display at least one axon defect for 1G and 100G. (J) Percent axons that are defective in 1G and 100 G. Error bars represent SE.

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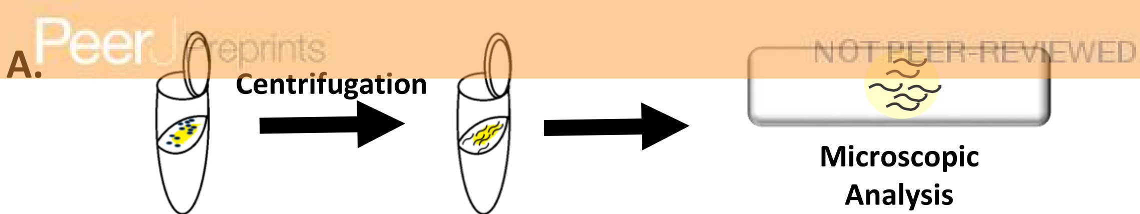


B.
1G
100 μm

D.
100G
100 μm

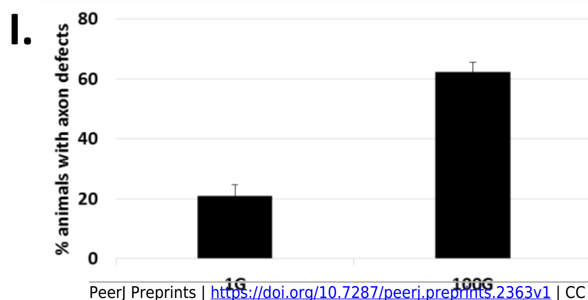
C.
1G

E.
100G
50 μm

F.
100G
100 μm

G.

H.

I.


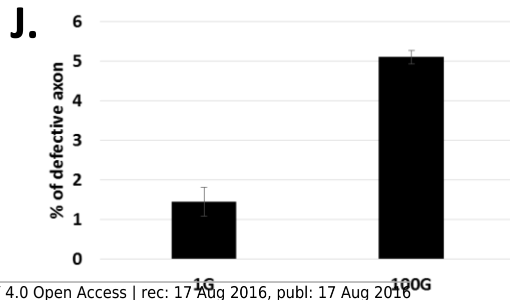
J.


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Figure 2 (on next page)

Hypergravity force and exposure time affect DD/VD neuron axon development.

(A) Animals were exposed to 1 to 500G hypergravity for 60 hours after embryo harvesting. Gravity force of over 10G increased axonal defects. (B) Animals were exposed to 100G hypergravity for various times after embryo harvesting, or to 3 hours during adulthood (far right bars). Grey bars=1G control, black bars=100G. (C) Data in (B) represented by exposure time over the major developmental events of the *C. elegans* (top of graph). Green represents axonal defects induces, red represents axonal defects not induced, yellow represents axonal defects slightly induced. Bars represent SE.

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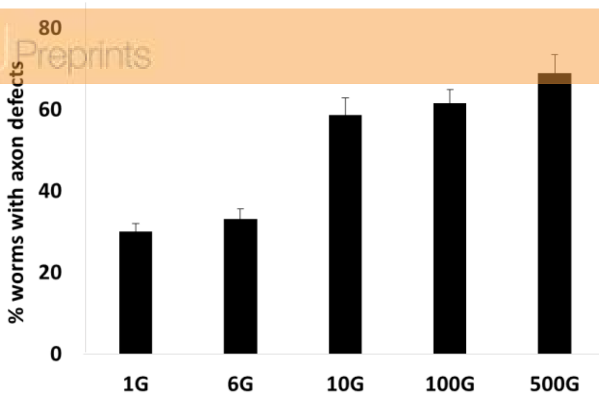

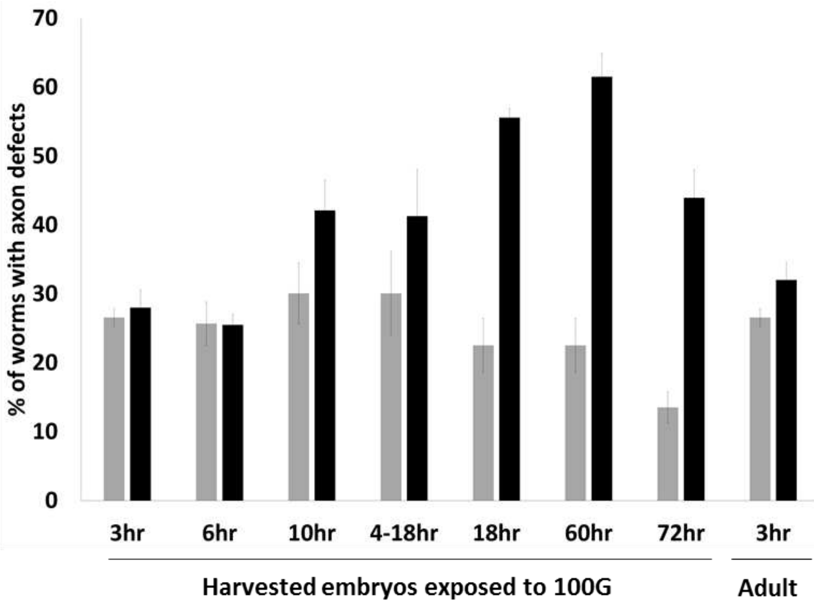

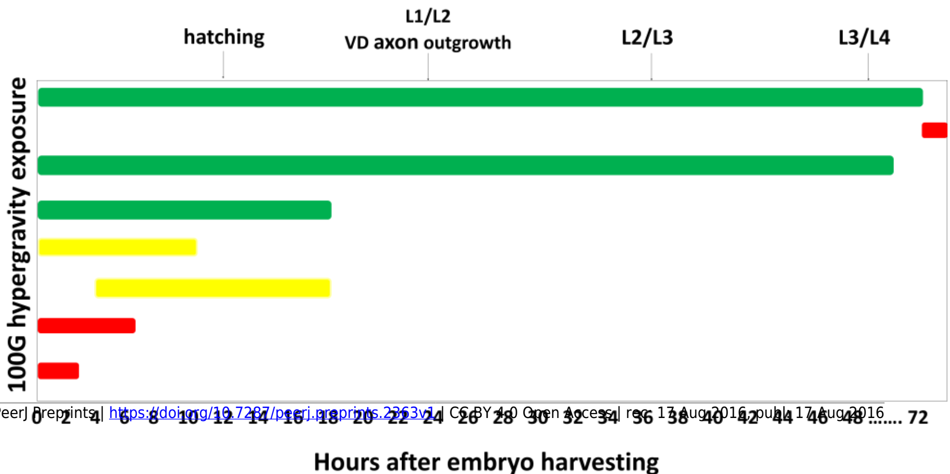

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Figure 3(on next page)

Spatial distribution of axonal defects in 1G and 100G hypergravity exposed animals.

(A) Location of the DD and VD axon commissures along the anterior-posterior axis of the worm. VD2 and DD1 are overlapped commissures and are indistinguishable. (B) Axon defects for each VD/DD commissure. Top table shows the contribution of axon defects for each commissure to the total number of defects for 1G and 100G. The percents for each commissure add up to 100% for 1G and 100G, respectively. Heat maps in purple/lavender shades show the percents indicated in the legend on the right. n=number of worms. Bottom table shows the percent defects for each individual commissure for 1G and 100G. Heat maps in orange/yellow shades show the percents indicated in the legend on the right. (C) Localization of defects in the dorsal-ventral direction for 1G animals. Defects were categorized into the four regions listed and percent of defects in each region is shown. (D) Localization of defects in the dorsal-ventral direction for 100G hypergravity exposed animals.

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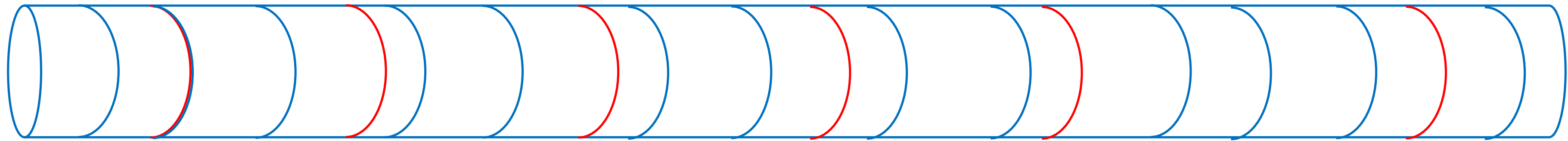
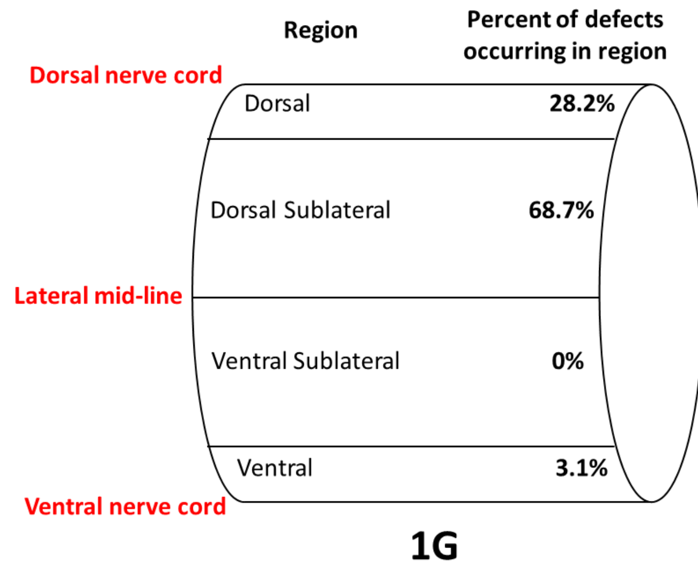
A.



B.

		VD1	VD2/DD1	VD3	DD2	VD4	VD5	DD3	VD6	VD7	DD4	VD8	VD9	DD5	VD10	VD11	VD12	DD6	VD13	n
% of overall axon defects	1G	0.00	0.00	6.35	1.06	11.11	7.94	8.47	10.58	15.34	4.23	15.87	3.17	2.65	4.76	1.06	0.53	1.06	5.82	624
	100G	0.33	0.16	7.82	2.28	12.54	12.38	6.68	14.50	12.38	3.42	10.59	4.56	2.61	1.30	0.81	0.16	1.95	5.54	614
% defects per commissure	1G	0.00	0.00	0.35	0.06	0.62	0.44	0.47	0.59	0.85	0.23	0.88	0.18	0.15	0.26	0.06	0.03	0.06	0.32	624
	100G	0.06	0.03	1.38	0.40	2.22	2.19	1.18	2.57	2.19	0.61	1.88	0.81	0.46	0.23	0.14	0.03	0.35	0.98	614

C.



D.

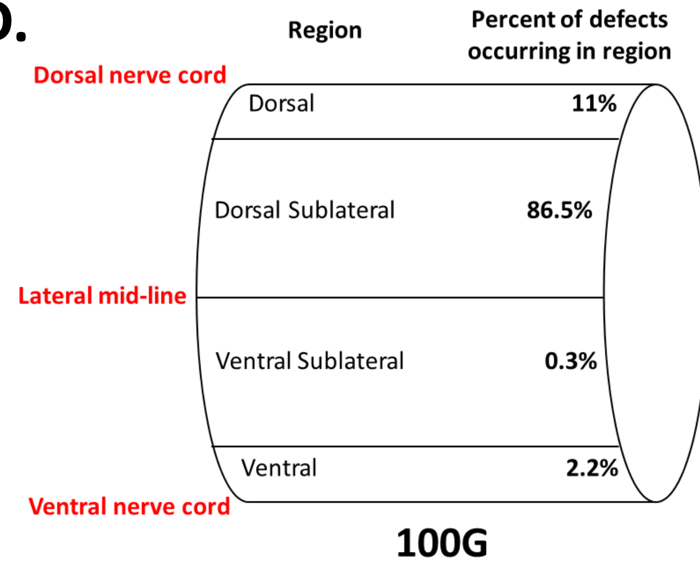


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Figure 4 (on next page)

Qualitative characterization of axonal defects in 1G and 100G hypergravity exposed animals.

(A) Axonal defects were categorized into the four groups shown here. (B) Quantification of axonal defects by category for 1G and 100G exposed animals. Bars represent SE.

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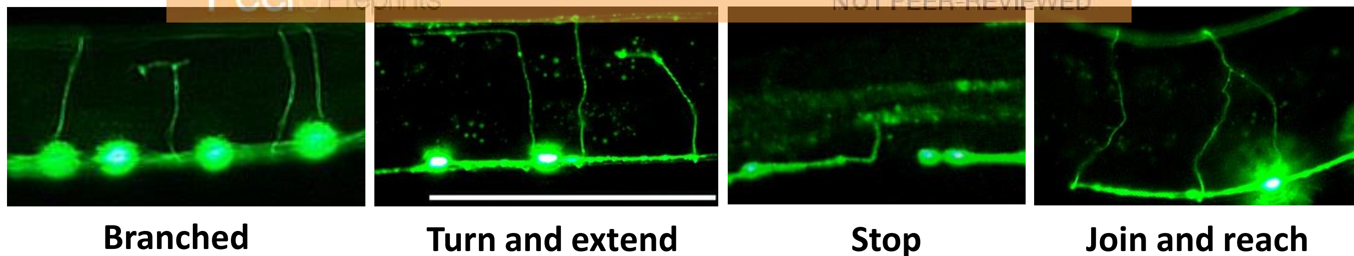

B.

100
80
60
40
20
0
% of defect
1G
100G
Branched
Turn and extend
Stop
Joined and reach

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Table 1(on next page)

Axon defects in different neuron types at 1G and 100G.

n=number of animals

Table 1. Axon defects in different neuron types at 1G and 100G.

		1G		100G	
Neuron type	Reporter strain	% defective axon	n	% defective axon	n
DD motor neurons	(p)*unc-25*::GFP	0.88	624	2.82	614
VD motor neurons	(p)*unc-25*::GFP	1.92	624	6.39	614
DD motor neurons (L1)	(p)*unc-25*::GFP	0	375	0	316
Mechanosensory neurons	(p)*mec-4*::GFP	0	50	0	45
Cholinergic neurons	(p)*unc-129*::GFP	0	102	0	118

n=number of animals

1

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