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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.

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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.
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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
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32 Our study demonstrates that in addition to gravity's effects on muscle development, gravity can
33 also impact motor neuron development.

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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

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

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

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

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

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

Quantification of defects: Defects were scored by researcher microscopic observations. Animals were scored as defective if one or more of the 19 DD/VD neurons showed any axonal defect (branch, turn and extend, stop, join and reach). Occasionally, an abnormally high axonal defect rate (50% or more) was observed even in normal 1G conditions. We censured the all the data from these days, and maintained the genetic background of the *juls76* strain by backcrossing this strain with the N2 wild-type strain. Dorsal-ventral locations of defects were approximated by eye and scored as: Ventral – 0% to 15% dorsal-ventral distance, Ventral sublateral – 15% to 50% dorsal-ventral distance, Dorsal sublateral - 50% to 85% dorsal-ventral distance, Dorsal – 85% to 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

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

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

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

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

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

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

188

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

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

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 probability 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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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REFERENCES

- Blanchette CR, Perrat PN, Thackeray A, and Benard CY. 2015. Glypican Is a Modulator of Netrin-Mediated Axon Guidance. *PLoS Biol* 13:e1002183. 10.1371/journal.pbio.1002183
- Brenner S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77:71-94.
- Caceres Ide C, Valmas N, Hilliard MA, and Lu H. 2012. Laterally orienting *C. elegans* using geometry at microscale for high-throughput visual screens in neurodegeneration and neuronal development studies. *PLoS One* 7:e35037. 10.1371/journal.pone.0035037

- 351 Colavita A, Krishna S, Zheng H, Padgett RW, and Culotti JG. 1998. Pioneer axon guidance by
352 UNC-129, a *C. elegans* TGF-beta. *Science* 281:706-709.
- 353 DeFelipe J, Arellano JI, Merchan-Perez A, Gonzalez-Albo MC, Walton K, and Llinas R. 2002.
354 Spaceflight induces changes in the synaptic circuitry of the postnatal developing
355 neocortex. *Cereb Cortex* 12:883-891.
- 356 di Prampero PE, and Narici MV. 2003. Muscles in microgravity: from fibres to human motion. *J*
357 *Biomech* 36:403-412.
- 358 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, and Mello CC. 1998. Potent and specific
359 genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806-
360 811. 10.1038/35888
- 361 Fitts RH, Riley DR, and Widrick JJ. 2001. Functional and structural adaptations of skeletal
362 muscle to microgravity. *J Exp Biol* 204:3201-3208.
- 363 Genchi GG, Ciofani G, Polini A, Liakos I, Iandolo D, Athanassiou A, Pisignano D, Mattoli V,
364 and Menciassi A. 2015. PC12 neuron-like cell response to electrospun poly(3-
365 hydroxybutyrate) substrates. *J Tissue Eng Regen Med* 9:151-161. 10.1002/term.1623
- 366 Gysi S, Rhiner C, Flibotte S, Moerman DG, and Hengartner MO. 2013. A network of HSPG core
367 proteins and HS modifying enzymes regulates netrin-dependent guidance of D-type
368 motor neurons in *Caenorhabditis elegans*. *PLoS One* 8:e74908.
369 10.1371/journal.pone.0074908
- 370 Hammarlund M, and Jin Y. 2014. Axon regeneration in *C. elegans*. *Curr Opin Neurobiol*
371 27:199-207. 10.1016/j.conb.2014.04.001

- 372 Hedgecock EM, Culotti JG, and Hall DH. 1990. The unc-5, unc-6, and unc-40 genes guide
373 circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C.*
374 *elegans*. *Neuron* 4:61-85.
- 375 Hengartner MO, and Horvitz HR. 1994. Programmed cell death in *Caenorhabditis elegans*. *Curr*
376 *Opin Genet Dev* 4:581-586.
- 377 Higashibata A, Szewczyk NJ, Conley CA, Imamizo-Sato M, Higashitani A, and Ishioka N. 2006.
378 Decreased expression of myogenic transcription factors and myosin heavy chains in
379 *Caenorhabditis elegans* muscles developed during spaceflight. *J Exp Biol* 209:3209-3218.
380 10.1242/jeb.02365
- 381 Higashitani A, Higashibata A, Sasagawa Y, Sugimoto T, Miyazawa Y, Szewczyk NJ, Viso M,
382 Gasset G, Eche B, Fukui K, Shimazu T, Fujimoto N, Kuriyama K, and Ishioka N. 2005.
383 Checkpoint and physiological apoptosis in germ cells proceeds normally in spaceflown
384 *Caenorhabditis elegans*. *Apoptosis* 10:949-954. 10.1007/s10495-005-1323-3
- 385 Honda Y, Higashibata A, Matsunaga Y, Yonezawa Y, Kawano T, Higashitani A, Kuriyama K,
386 Shimazu T, Tanaka M, Szewczyk NJ, Ishioka N, and Honda S. 2012. Genes down-
387 regulated in spaceflight are involved in the control of longevity in *Caenorhabditis*
388 *elegans*. *Sci Rep* 2:487. 10.1038/srep00487
- 389 Inglis FM, Zuckerman KE, and Kalb RG. 2000. Experience-dependent development of spinal
390 motor neurons. *Neuron* 26:299-305.
- 391 Jin Y, Jorgensen E, Hartwig E, and Horvitz HR. 1999. The *Caenorhabditis elegans* gene unc-25
392 encodes glutamic acid decarboxylase and is required for synaptic transmission but not
393 synaptic development. *J Neurosci* 19:539-548.

- 394 Kim N, Dempsey CM, Kuan CJ, Zoval JV, O'Rourke E, Ruvkun G, Madou MJ, and Sze JY.
395 2007. Gravity force transduced by the MEC-4/MEC-10 DEG/ENaC channel modulates
396 DAF-16/FoxO activity in *Caenorhabditis elegans*. *Genetics* 177:835-845.
397 10.1534/genetics.107.076901
- 398 Kim, N; Dempsey, C.M.; Zoval, J.M.; Sze, J.; Madou, M.J. 2007. Automated microfluidic
399 compact disc (CD) cultivation system of *Caenorhabditis elegans*. *Sensors and Actuators*
400 *B: Chemical* 122:511-518. 10.1016/j.snb.2006.06.026
- 401 Lee J, Bandyopadhyay J, Lee JI, Cho I, Park D, and Cho JH. 2015. A role for peroxidase PXN-1
402 in aspects of *C. elegans* development. *Mol Cells* 38:51-57. 10.14348/molcells.2015.2202
- 403 Lee RC, Feinbaum RL, and Ambros V. 1993. The *C. elegans* heterochronic gene *lin-4* encodes
404 small RNAs with antisense complementarity to *lin-14*. *Cell* 75:843-854.
- 405 Lee TY, Yoon KH, and Lee JI. 2016. NGT-3D: a simple nematode cultivation system to study
406 *Caenorhabditis elegans* biology in 3D. *Biol Open* 5:529-534. 10.1242/bio.015743
- 407 Lewis JA, Fleming, J.T. 1995. Basic Culture Methods. In: Epstein HFS, D.C., ed.
408 *Caenorhabditis elegans: Modern Biological Analysis of an Organism*. 1995: Academic
409 Press, Inc., 4-27.
- 410 MacNeil LT, Hardy WR, Pawson T, Wrana JL, and Culotti JG. 2009. UNC-129 regulates the
411 balance between UNC-40 dependent and independent UNC-5 signaling pathways. *Nat*
412 *Neurosci* 12:150-155. 10.1038/nn.2256
- 413 McIntire SL, Jorgensen E, Kaplan J, and Horvitz HR. 1993. The GABAergic nervous system of
414 *Caenorhabditis elegans*. *Nature* 364:337-341. 10.1038/364337a0
- 415 McIntire SL, Reimer RJ, Schuske K, Edwards RH, and Jorgensen EM. 1997. Identification and
416 characterization of the vesicular GABA transporter. *Nature* 389:870-876. 10.1038/39908

- 417 Steiernagle T. 1999. Maintenance of *C. elegans*. In: Hope I, ed. *C elegans: A Practical*
418 *Approach*: Oxford University Press, 51-67.
- 419 Sulston JE. 1976. Post-embryonic development in the ventral cord of *Caenorhabditis elegans*.
420 *Philos Trans R Soc Lond B Biol Sci* 275:287-297.
- 421 Sulston JE, and Horvitz HR. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis*
422 *elegans*. *Dev Biol* 56:110-156.
- 423 Szewczyk NJ, Tillman J, Conley CA, Granger L, Segalat L, Higashitani A, Honda S, Honda Y,
424 Kagawa H, Adachi R, Higashibata A, Fujimoto N, Kuriyama K, Ishioka N, Fukui K,
425 Baillie D, Rose A, Gasset G, Eche B, Chaput D, and Viso M. 2008. Description of
426 International *Caenorhabditis elegans* Experiment first flight (ICE-FIRST). *Adv Space Res*
427 42:1072-1079. 10.1016/j.asr.2008.03.017
- 428 Vandenberg H, Chromiak J, Shansky J, Del Tatto M, and Lemaire J. 1999. Space travel directly
429 induces skeletal muscle atrophy. *FASEB J* 13:1031-1038.
- 430 Wadsworth WG. 2002. Moving around in a worm: netrin UNC-6 and circumferential axon
431 guidance in *C. elegans*. *Trends Neurosci* 25:423-429.
- 432 White JG, Southgate E, Thomson JN, and Brenner S. 1976. The structure of the ventral nerve
433 cord of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci* 275:327-348.
- 434 Zhao Y, Lai K, Cheung I, Youds J, Tarailo M, Tarailo S, and Rose A. 2006. A mutational
435 analysis of *Caenorhabditis elegans* in space. *Mutat Res* 601:19-29.
436 10.1016/j.mrfmmm.2006.05.001

437

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 μ m (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 μ m. (E) Axonal defects in 100G exposed animals. Bar=50 μ m. (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 μ m. (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.

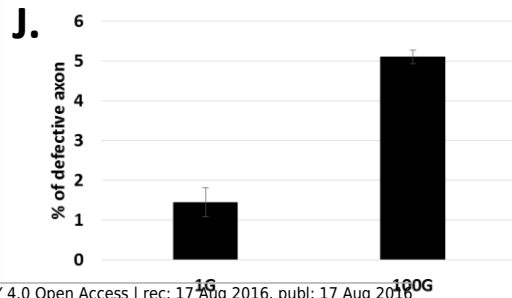
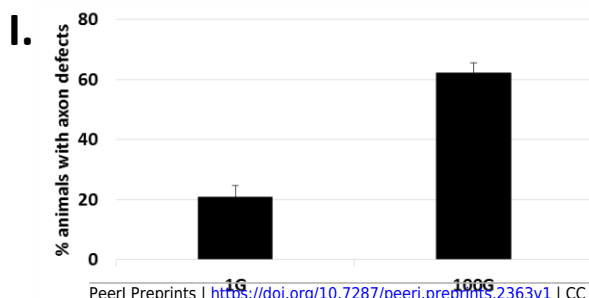
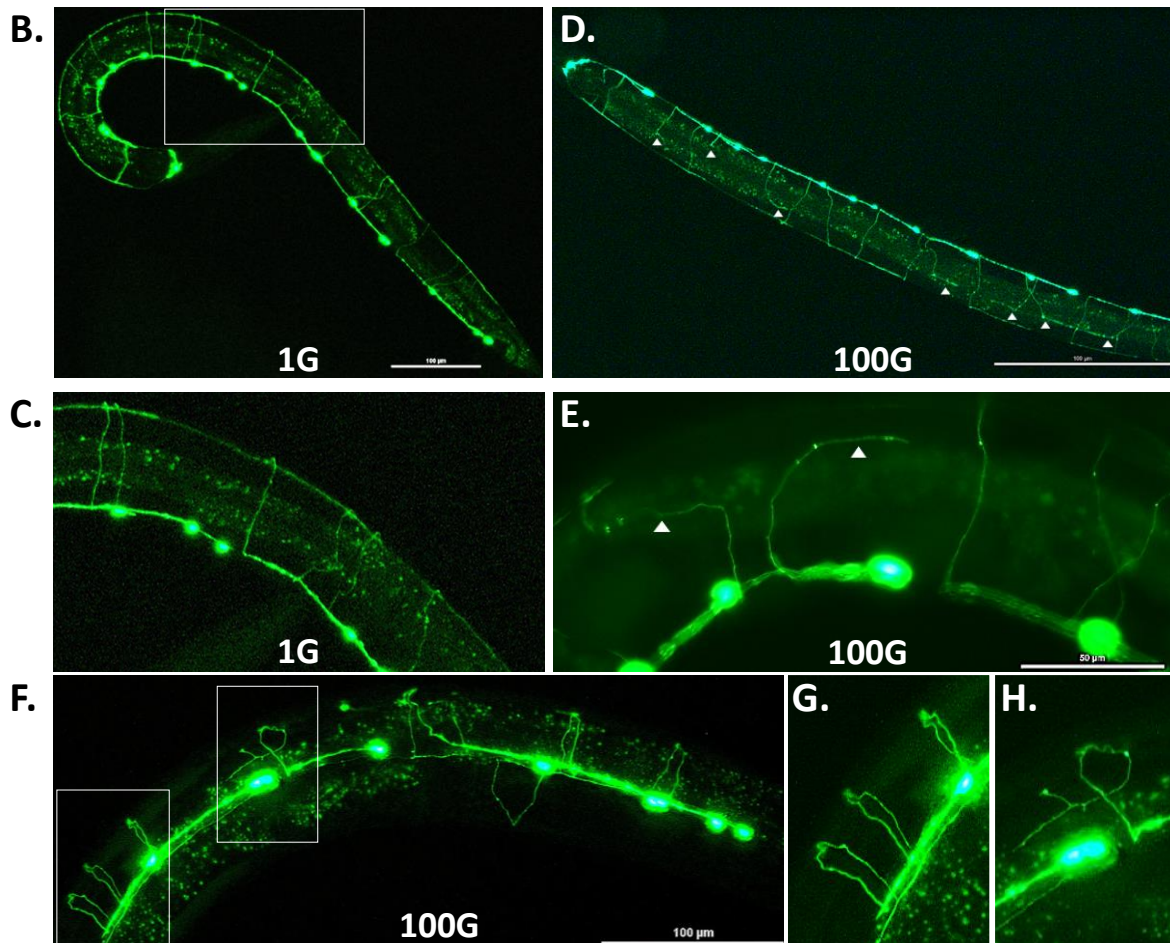
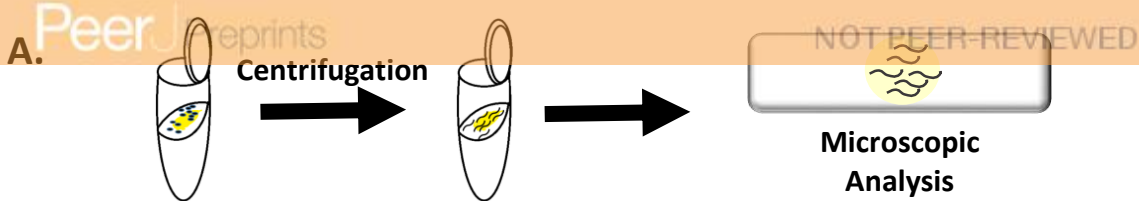
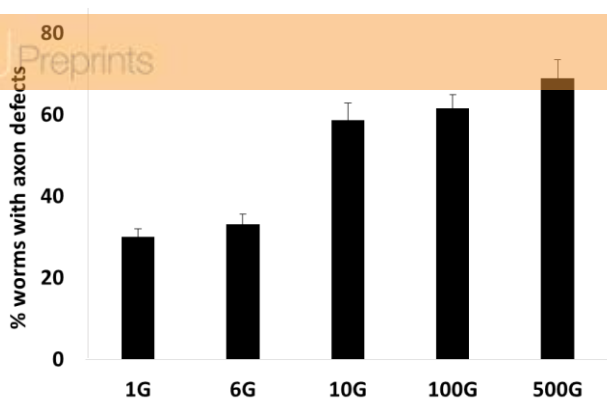


Figure 2(on next page)

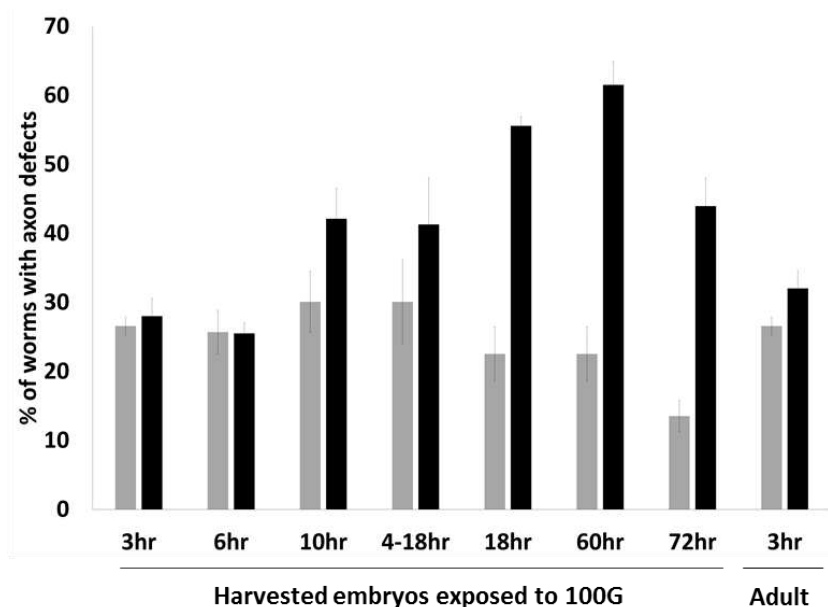
Hypergravity force and exposure time affect DD/VD neuron axon development.

(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.

A.



B.



C.

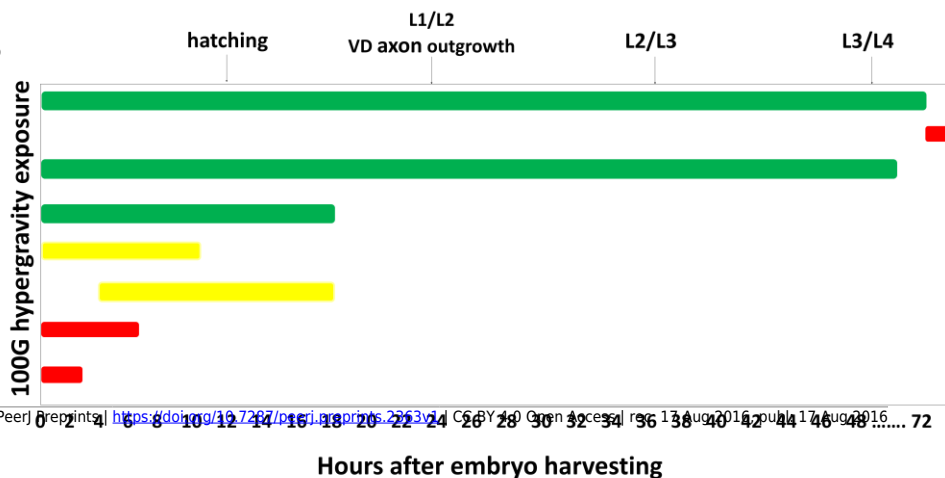
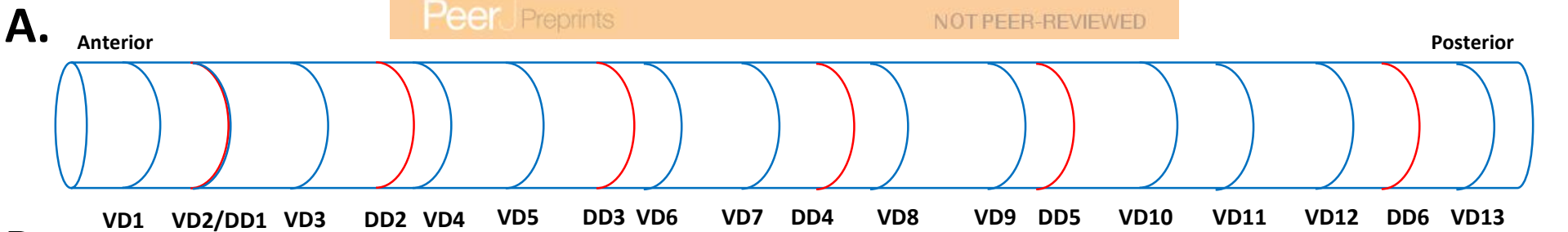


Figure 3(on next page)

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.



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

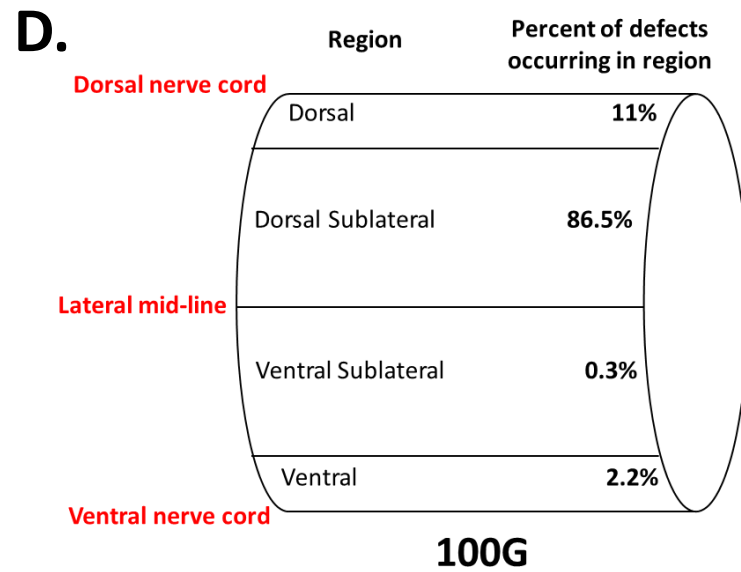
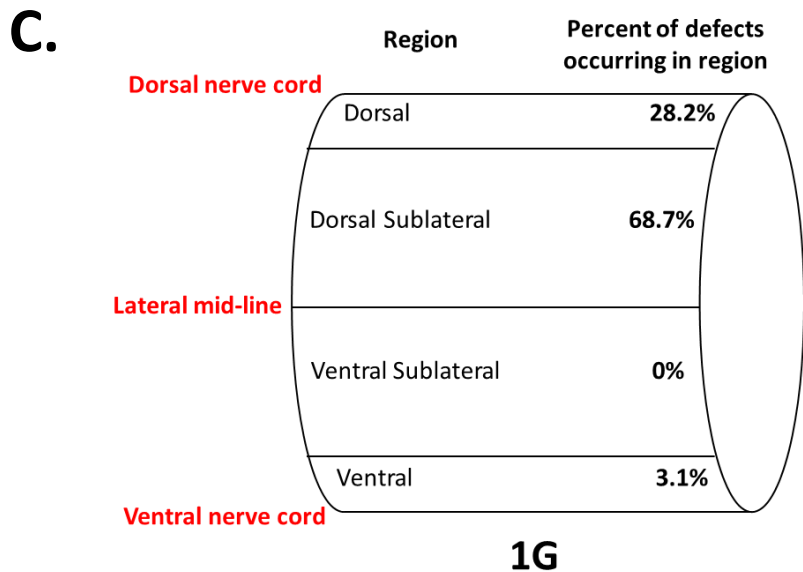
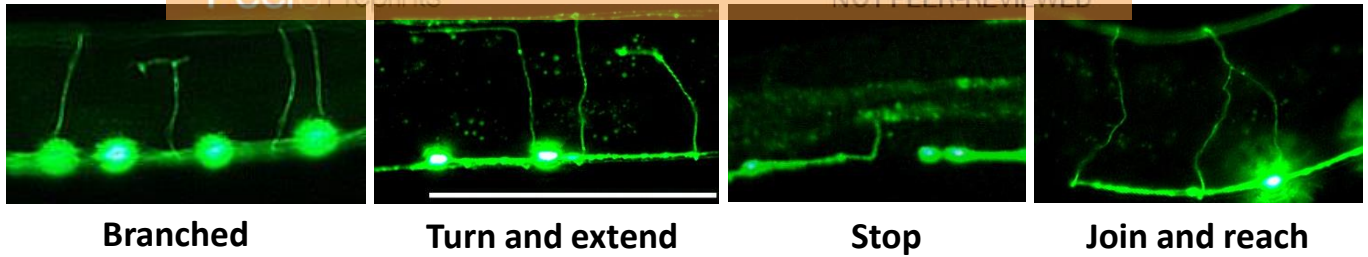


Figure 4(on next page)

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.

A.



B.

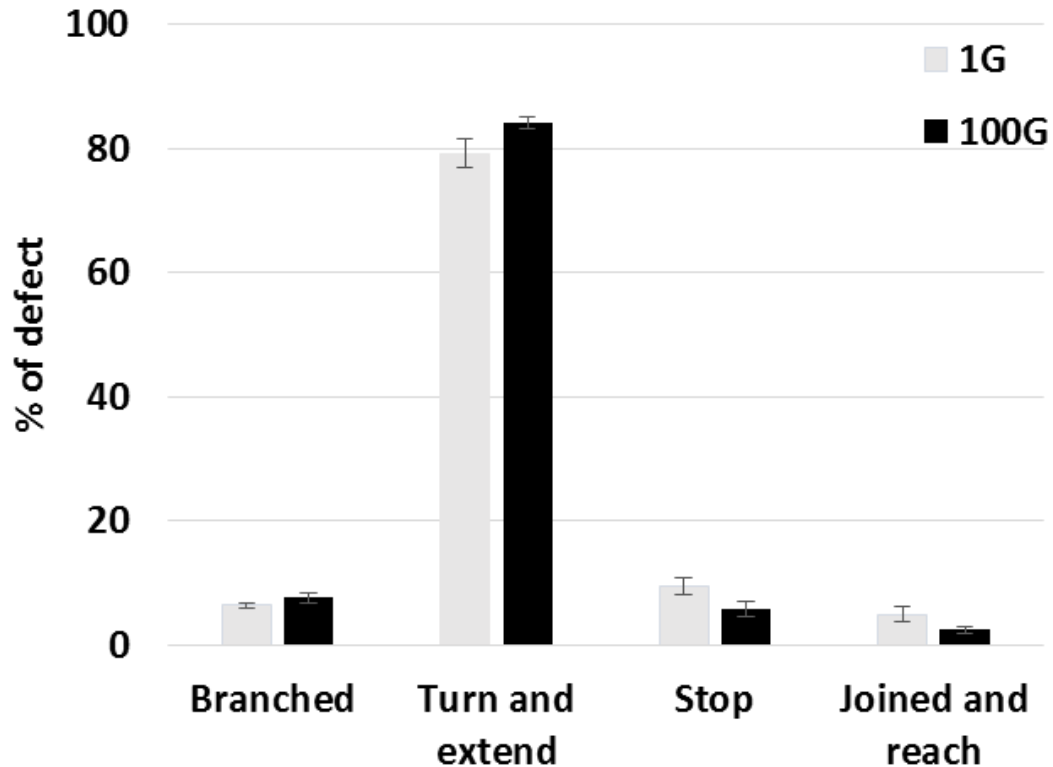


Table 1(on next page)

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.

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

n=number of animals