

1 **Benzimidazoles cause lethality by inhibiting the function of *Caenorhabditis elegans***

2 **neuronal beta-tubulin**

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30 **Highlights**

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32 • Expressing wild-type *ben-1* only in neurons restores susceptibility to benzimidazoles

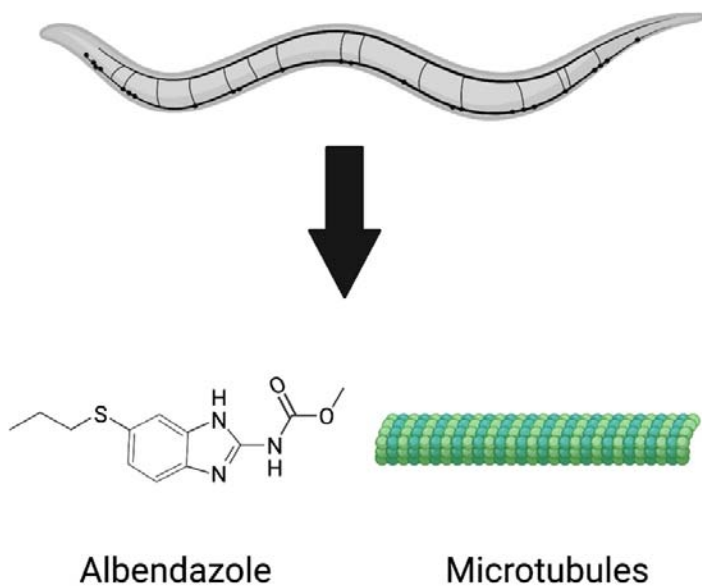
33 • Expression of *ben-1* in cholinergic neurons restores susceptibility to benzimidazoles

34 • GABAergic neurons might also play a role in benzimidazole sensitivity

35 • Broad implications for molecular mechanisms of benzimidazole mode of action

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37 **Graphical Abstract**



38

39 **Abstract**

40

41 Parasitic nematode infections cause an enormous global burden to both human and  
42 livestock populations. Resistance to the limited arsenal of anthelmintic drugs used to combat these  
43 infections is widespread, including resistance to benzimidazole (BZ) compounds commonly found  
44 in livestock parasites. Previous studies using the free-living nematode *Caenorhabditis elegans* to  
45 model parasitic nematode resistance have shown that loss-of-function mutations in the beta-tubulin  
46 gene *ben-1* confer resistance to BZ drugs. However, the mechanism of resistance and the tissue-  
47 specific susceptibility are not well known in any nematode species. To identify in which tissue(s)  
48 *ben-1* function underlies BZ susceptibility, transgenic strains that express *ben-1* in different tissues,  
49 including hypodermis, muscles, neurons, intestine, and ubiquitous expression were generated.  
50 High-throughput fitness assays were performed to measure and compare the quantitative  
51 responses to BZ compounds among different transgenic lines. Significant BZ susceptibility was  
52 observed in animals expressing *ben-1* in neurons, comparable to expression using the *ben-1*  
53 promoter. This result suggests that *ben-1* function in neurons underlies susceptibility to BZ.  
54 Subsetting neuronal expression of *ben-1* based on neurotransmitter system further restricted *ben-1*  
55 function in cholinergic neurons to cause BZ susceptibility. These results better inform our current  
56 understanding of the cellular mode of action of BZ and also suggest additional treatments that  
57 might potentiate the effects of BZs.

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59 **Keywords:** Benzimidazole resistance, *C.elegans*, high-throughput assay

## 60 1. Introduction

61

62 Anthelmintic drugs are crucial to combat parasitic nematode infections, which affect billions  
63 of people and livestock populations each year (Hotez et al., 2014; Kaplan & Vidyashankar, 2012).  
64 However, only a limited arsenal of drugs are approved, comprising four major classes:  
65 benzimidazoles (BZs), nicotinic acetylcholine receptor agonists (nAChRs), macrocyclic lactones  
66 (MLs), and amino-acetonitrile derivatives (AADs). BZs have been used extensively for over 50  
67 years (Abongwa et al., 2017; Roos et al., 1995). Because of the intensity of administration of the  
68 few anthelmintics available, resistance was documented in *Haemonchus contortus* within a few  
69 years of its introduction (Theodorides et al., 1970). Resistance to commonly used BZs continues to  
70 be widespread today (Kaplan & Vidyashankar, 2012).

71 Following the establishment of resistance to BZs, mutations in beta-tubulin genes were first  
72 correlated with resistance in the fungus *Aspergillus nidulans*, followed by the free-living nematode  
73 *Caenorhabditis elegans* (Driscoll et al., 1989; Hastie & Georgopoulos, 1971; Sheir-Neiss et al.,  
74 1978). Loss-of-function mutations in the beta-tubulin gene *ben-1* were identified in strains resistant  
75 to BZs (Driscoll et al., 1989). Redundancy among the six beta-tubulin genes in *C. elegans* allows  
76 strains with non-functional *ben-1* to still develop normally (Driscoll et al., 1989). Resistance alleles  
77 corresponding to point mutations in *ben-1* homologs in parasitic nematode populations continue to  
78 be identified (Avramenko et al., 2019; Dilks et al., 2021; Hahnel et al., 2018; Mohammedsalih et al.,  
79 2020), and many have been validated to cause resistance using genome editing and highly  
80 sensitive *C. elegans* drug response assays (Dilks et al., 2020, 2021; Hahnel et al., 2018). These  
81 techniques are difficult in the parasites, and *C. elegans* has been proven to be a suitable model to  
82 study resistance complementary to parasitic nematodes (Wit, Dilks, et al., 2021).

83 New resistance alleles continue to be identified in parasite populations, which emphasizes  
84 the need to develop compounds that can be used in conjunction with BZs to potentiate their  
85 effects. However, the mechanism of action beyond beta-tubulin binding is still unknown. In  
86 susceptible animals, BZs binding to beta-tubulin inhibits tubulin polymerization necessary to form  
87 microtubules (Ireland et al., 1979; Lacey, 1990; Lacey & Prichard, 1986; Lacleite et al., 1980) but is  
88 not well understood in what tissues and specific cells microtubule formation is inhibited. Preliminary

89 studies of BZ susceptibility in *H. contortus* taken from sheep treated with fenbendazole were found  
90 to have gross disintegration of the interior intestine, suggesting that BZs target beta-tubulin in the  
91 intestine of these animals (Jasmer et al., 2000). We can use genetic tools such as transgenesis  
92 and high-throughput assays developed for *C. elegans* to further investigate the mode of action of  
93 BZs.

94 Here, we re-introduced the wild-type *ben-1* gene into a *ben-1* knockout strain background  
95 using transgenesis (Rieckher & Tavernarakis, 2017), where multi-copy arrays express *ben-1* in  
96 specific tissues. Plasmids containing the coding sequence of *ben-1* fused to tissue-specific  
97 promoters, including neurons, hypodermis, muscles, and intestine, as well as endogenous and  
98 ubiquitous expression, formed extrachromosomal arrays in transgenic animals. Loss of *ben-1*  
99 causes BZ resistance (Hahnel et al., 2018), so transgenic addition of wild-type *ben-1* can restore  
100 BZ sensitivity. We then performed high-throughput fitness assays to quantitatively assess the  
101 response to albendazole (ABZ). We found that when *ben-1* is expressed in neurons, the wild-type  
102 susceptibility phenotype is restored. We then generated transgenic strains that expressed *ben-1* in  
103 cholinergic, dopaminergic, GABAergic, or glutamatergic neurons to narrow down the neurons  
104 where BZs cause lethality. We found that *ben-1* expression in cholinergic neurons was sufficient to  
105 restore wild-type BZ susceptibility. These results offer insights into the mode of action of BZs and  
106 suggest that BZs might have a similar cellular target as other classes of anthelmintics.

## 107 2. Materials and methods

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### 109 2.1 Strains

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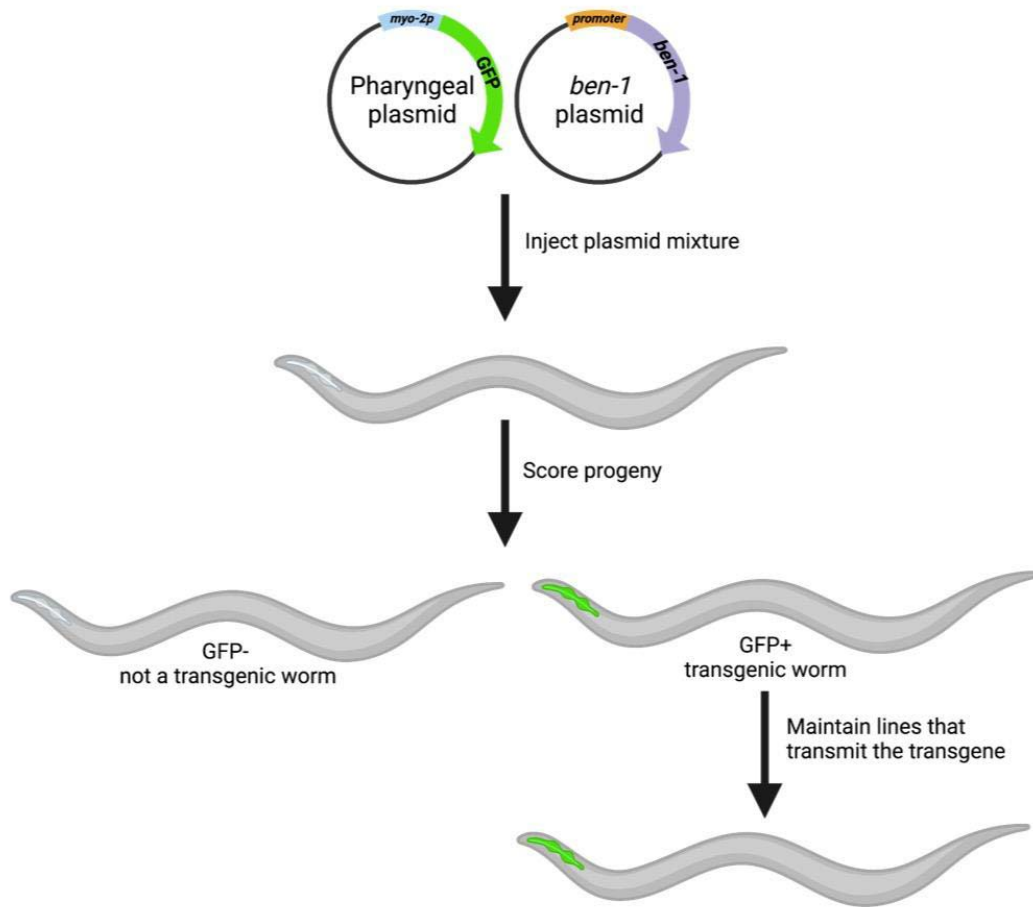
111 Strains were maintained at 20°C on modified nematode growth media plates (NGMA) with  
112 1% agar, 0.7% agarose, and *Escherichia coli* OP50 bacteria for food (Andersen et al., 2014). To  
113 alleviate starvation effects, strains were grown for three generations before each assay (Andersen  
114 et al., 2015). Most strains were generated using the ECA882 strain, *ben-1(ean64)*, which has the  
115 laboratory-derived reference strain background (N2) and a deletion of *ben-1* exons 2 through 4.  
116 This strain has been previously shown to be resistant to albendazole (Hahnel et al., 2018). The N2  
117 strain was also used as a background for some control transgenic strains.

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### 119 2.2 Plasmid construction

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121 All *ben-1* plasmids with alternative promoters were constructed by VectorBuilder (Table  
122 S1). Tissue-specific promoters included *myo-3* (muscles), *unc-119* (pan-neuronal), *col-19*  
123 (hypodermis), and *ges-1* (intestines). A plasmid with the *eft-3* promoter for ubiquitous expression  
124 was designed as well. Neurotransmitter-specific promoters included *unc-17* (cholinergic), *eat-4*  
125 (glutamatergic), *dat-1* (dopaminergic), and *unc-25* (GABAergic). Promoter sequences for  
126 neurotransmitter system-specific expression plasmids have been published previously (Flames &  
127 Hobert, 2009; Serrano-Saiz et al., 2020). The plasmid with the *ben-1* endogenous promoter was  
128 constructed using the *ben-1* cDNA and an amplicon of the *ben-1* promoter and assembled using  
129 Gibson cloning (Gibson et al., 2009). The co-injection marker plasmid, pBCN27 (*myo-*  
130 *2p::GFP::unc-54\_3'UTR*) was a gift from Ben Lehner (Addgene, plasmid #26347) (Semple et al.,  
131 2010).



132

133 **Figure 1.** The generation of transgenic strains with specified expression of *ben-1* using  
134 microinjection. A mixture of *ben-1* plasmid fused to a specific promoter and a GFP co-injection  
135 marker that expressed GFP in the pharynx were injected into the gonads of adult worms. After 48-  
136 72 hours, offspring were scored for presence of GFP in the pharynx, which was indicative of  
137 successful transgenesis. Strains with approximately 70% transmission were selected for high-  
138 throughput fitness assays.



### 139 2.3 *C. elegans* transgenesis

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141 The *C. elegans* microinjection technique has been previously described (Rieckher &  
142 Tavernarakis, 2017). Briefly, a *ben-1* expression plasmid was combined with the *myo-2p::GFP* co-  
143 injection marker (5 ng/uL) and 1 kb DNA ladder (Invitrogen #10787018) at the specified  
144 concentrations (Table S2, Abrahante et al., 1998; Carvelli et al., 2004; Eastman et al., 1999;  
145 Frøkjær-Jensen et al., 2008; Frøkjær-Jensen et al., 2012; Hao et al., 2012; Kaymak et al., 2016;  
146 Marshall & McGhee, 2001; Muñoz-Jiménez et al., 2017; Serrano-Saiz et al., 2013; Thomas et al.,  
147 2019). This mixture was injected into the gonads of adult hermaphrodites harboring the *ben-1*  
148 deletion (Figure 1). Post-injection, a single adult was placed onto a 6 cm plate. F1 progeny  
149 expressing GFP in the pharynx were identified 48-72 hours following injection and subsequently  
150 singled (Figure 1). Two independent lines per rescue construct were selected based on the  
151 presence of the transgene in the F2 generation (Figure 1). Wild-type GFP control strains were  
152 made by injecting the co-injection marker into the N2 strain. Deletion GFP control strains were  
153 constructed using the co-injection marker injected into ECA882.

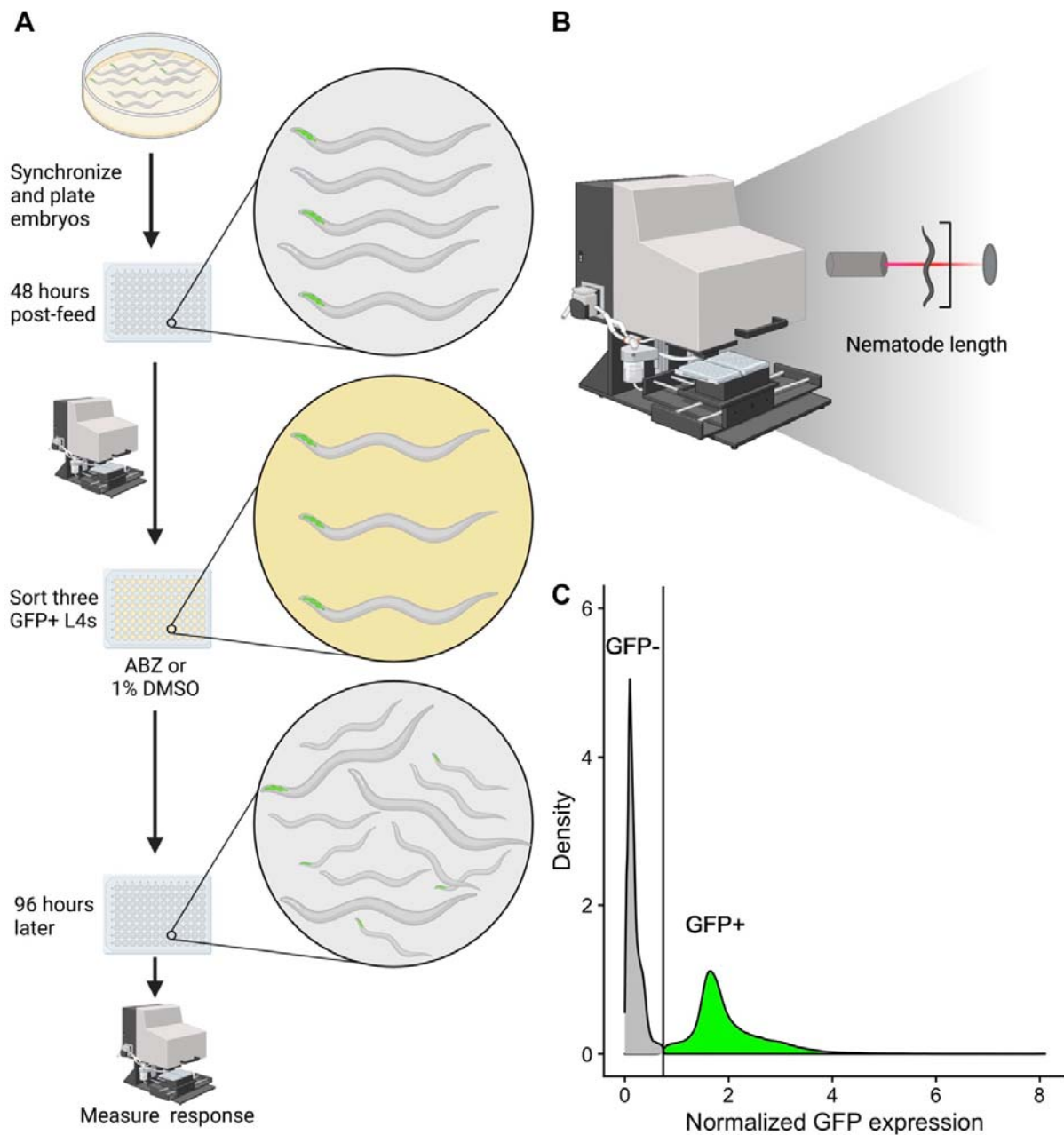
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### 155 2.4 High-throughput fitness assays

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157 The COPAS BIOSORT high-throughput phenotyping assay has been previously described  
158 (Andersen et al., 2015; Brady et al., 2019; Dilks et al., 2020, 2021; Evans et al., 2018; Evans &  
159 Andersen, 2020; Hahnel et al., 2018; Zdraljevic et al., 2017). Additional measures were taken at  
160 each step of the propagation protocol to select for animals with the transgene. Briefly, a small  
161 chunk from a starved 6 cm NGMA plate was placed onto a fresh plate. After 48 hours, GFP-  
162 positive, gravid hermaphrodites were transferred to a plate with a bleach solution (40 mL NaOCl  
163 (Fisher #SS290-1), 10 mL of 10 M NaOH added to 150 mL of distilled water). Approximately 24  
164 hours later, GFP-positive L1 larvae were transferred to a fresh 6 cm NGMA plate. After 48 hours,  
165 five animals at the L4 stage were picked to a new plate. After 72 hours to allow for offspring to  
166 grow to the L4 stage, five GFP-positive L4s were placed onto a fresh 6 cm NGMA plate and  
167 allowed to develop and propagate. After 96 hours, strains were washed off of plates using M9

168 buffer into 15 mL conicals and treated with fresh bleach solution to dissolve gravid adults and  
169 obtain a large number of unhatched embryos. Embryo pools were washed three times using M9  
170 and once using K medium (51 mM NaCl, 32 mM KCl, 3 mM CaCl<sub>2</sub>, and 3 mM MgSO<sub>4</sub> in distilled  
171 water) (Boyd et al., 2012) before being resuspended in K medium. Clean embryos were diluted to  
172 approximately one embryo per  $\mu$ L in K medium and then aliquoted into 96-well plates at  
173 approximately 50 embryos in each well (Figure 2A). After hatching overnight, arrested L1 larvae  
174 were fed lyophilized *E. coli* strain HB101 (Pennsylvania State University Shared Fermentation  
175 Facility, State College, PA) at a concentration of 5 mg/mL (García-González et al., 2017). After 48  
176 hours, L4 larvae were sorted using the COPAS BIOSORT (Union Biometrica, Holliston MA). The  
177 COPAS BIOSORT is able to measure the time-of-flight (TOF) and green fluorescence of each  
178 object as it flows through the device (Figure 2B, 2C) (Andersen et al., 2015; Brady et al., 2019;  
179 Dilks et al., 2020, 2021; Evans et al., 2018; Evans & Andersen, 2020; Hahnel et al., 2018;  
180 Zdraljovic et al., 2017). Three GFP-positive L4 larvae were sorted from each well to wells in a new  
181 96-well plate containing HB101 lysate at 10 mg/mL and 12.5  $\mu$ M albendazole in 1% DMSO or 1%  
182 DMSO alone (Figure 2A). This concentration of albendazole has been previously used with this  
183 protocol (Hahnel et al., 2018). Four days after exposure to albendazole, wells were treated with 50  
184 mM sodium azide and scored using the COPAS BIOSORT (Figure 2A).



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**Figure 2.** COPAS BIOSORT high-throughput fitness assay. **A)** Illustration of the high-throughput fitness assay is shown. Synchronized worms were treated with bleach solution and embryos were distributed into 96-well plates. 48 hours post-feeding, three GFP-positive L4 animals were sorted using the COPAS BIOSORT into each well of a 96-well plate containing 12.5  $\mu$ M albendazole in 1% DMSO or 1% DMSO alone. After 96 hours, animals were scored for GFP fluorescence and length using the COPAS BIOSORT. **B)** Illustration of COPAS BIOSORT is shown. Animals were passed through a flow cell and a laser. The time the detector is interrupted is equated to the length of the nematode. Illustration adapted from (Wit, Rodriguez, et al., 2021). **C)** Distribution of GFP fluorescence of animals is shown. The x-axis is the GFP expression of each animal normalized by the length of each animal. The y-axis is the distribution of the population. The vertical line represents the threshold for establishing if animals are GFP-positive (green) or GFP-negative (gray).

## 199 2.4 Data analysis

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201 Raw data from the COPAS BIOSORT were processed using the R package *easysorter*  
202 (Shimko & Andersen, 2014) as previously described (Dilks et al., 2020, 2021; Hahnel et al., 2018).  
203 An average value for each phenotypic trait measured in the control condition (1% DMSO) was  
204 deducted from the albendazole condition data to normalize the data for each strain. The  
205 distribution of green fluorescence values was analyzed to establish a threshold to filter for GFP-  
206 positive animals that had the transgene (Figure 2C). The average mean TOF value was  
207 summarized for each well per strain and the distribution of mean TOF values for each transgenic  
208 strain was compared to the mean TOF values for the *ben-1* deletion strain. Statistical tests and  
209 analyses were performed in R using the *tukeyHSD* function in the *Rstatix* package. The ANOVA  
210 model (*phenotype ~ strain*) was used to compare differences in phenotypic responses to BZs  
211 between the *ben-1* deletion strain and the other strains.

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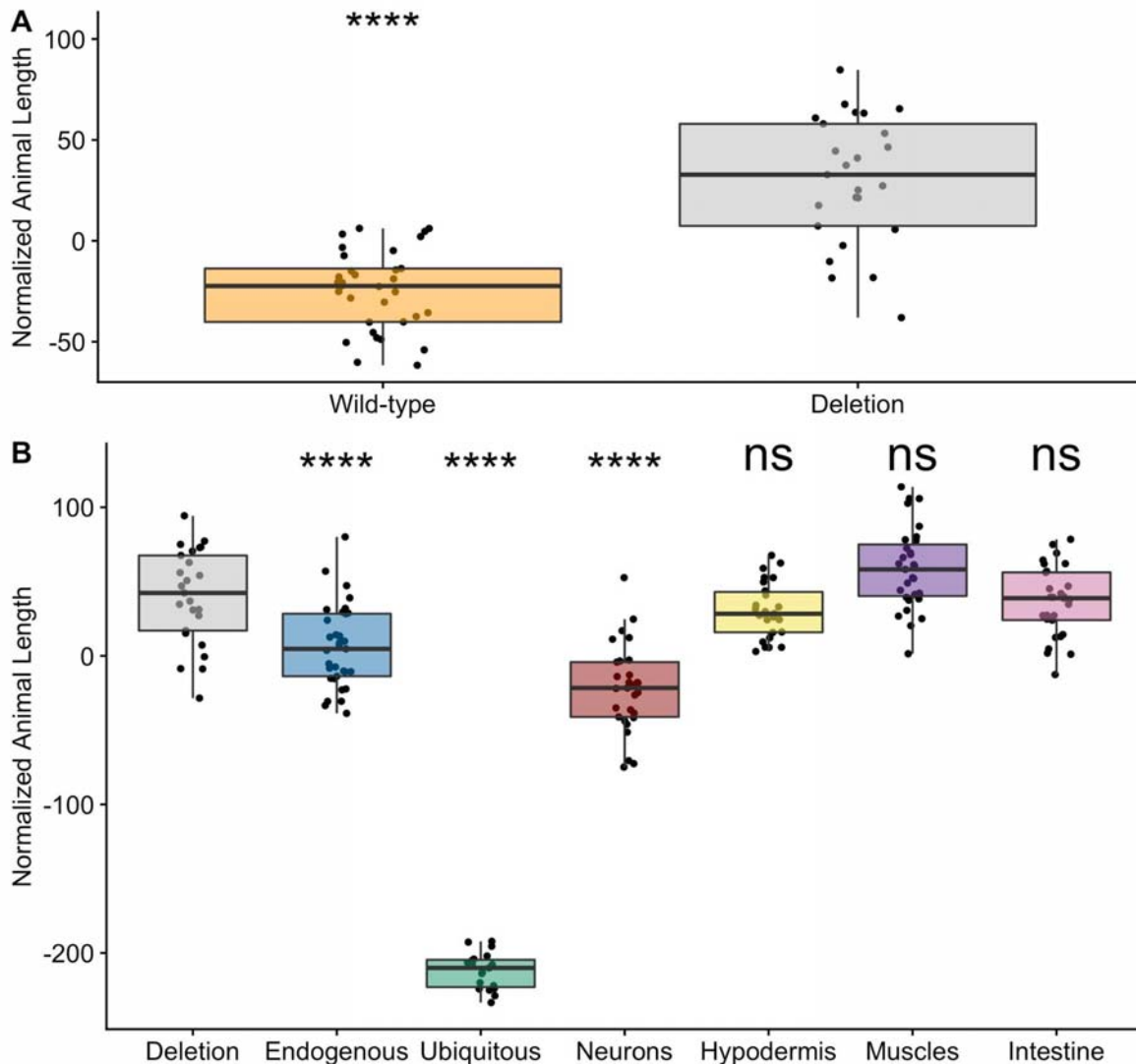
## 214 2.5 Data availability

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216 A list of plasmids used with vendor ID information and a list of *C. elegans* strains and  
217 genotypes used in experiments are included as supplementary information (Tables S1 and S2).  
218 The data and code used to process these data are available at  
219 [https://github.com/AndersenLab/2022\\_ben1sensitivity\\_SBG](https://github.com/AndersenLab/2022_ben1sensitivity_SBG).

220 **3. Results**

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222

223 **Figure 3.** *ben-1* function in neurons underlies BZ sensitivity. **A)** The x-axis denotes the genetic  
224 background at the *ben-1* locus for each strain. The y-axis is the normalized length of animals after  
225 exposure to 12.5  $\mu$ M of albendazole. Phenotypic data was normalized by subtracting an average  
226 value for each trait from the control data. Each data point is the mean value for the population of  
227 GFP-positive worms in a single well. Tukey box plots have horizontal lines at the third quartile on  
228 the top, the median in the middle and the first quartile at the bottom. The whiskers are extended  
229 within 1.5 range from each quartile. The significant difference between the wild-type genotype and  
230 the deletion genotype is shown above the wild-type results (\*\*\*\* =  $p < 0.0001$ , one-way ANOVA,  
231 Tukey HSD). **B)** The x-axis denotes the specificity of the *ben-1* expression from the transgene. The  
232 y-axis is the normalized length of animals after exposure to 12.5  $\mu$ M of albendazole. Phenotypic  
233 data was normalized by subtracting an average value for each trait from the control data. Each  
234 data point is the mean value for the population of GFP-positive worms in a single well. Tukey  
235 box plots have horizontal lines at the third quartile on the top, the median in the middle and the  
236 first quartile at the bottom. The whiskers are extended within 1.5 range from each quartile. The  
237 significant difference between the transgene and the deletion genotype is shown above the  
238 transgene (\*\*\*\* =  $p < 0.0001$ , one-way ANOVA, Tukey HSD)

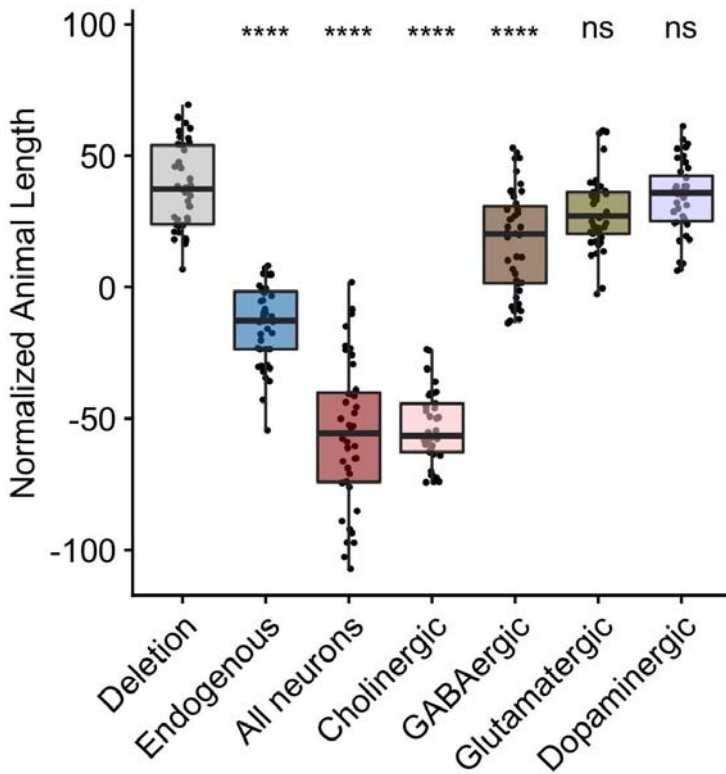
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### 240 3.1 *ben-1* function in neurons rescues BZ sensitive phenotype

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242 We created transgenic strains where *ben-1* was expressed in different tissues, including  
243 muscles, neurons, hypodermis, intestines, as well as ubiquitous expression. These different  
244 expression constructs were made in the ECA882 *ben-1* deletion background. For each candidate  
245 tissue-specific expression strain, two independent strains were generated and assayed to ensure  
246 that the measured effects were caused by the transgenes and not a vagary of the injection  
247 process. Assay results with the second strain are available as supplemental information (Figures  
248 S1 and S2). Animals with a *ben-1* specific transgene were identifiable by a green, fluorescent  
249 pharynx caused by the pharyngeal expression of the co-injection transgenesis marker. We then  
250 performed high-throughput assays to test if any of the tissue-specific expression strains rescued  
251 susceptibility to albendazole. A strain with the resistant *ben-1* knockout background and the  
252 pharyngeal expression marker and a strain with the susceptible wild-type background and the  
253 pharyngeal expression marker were used as controls. We measured growth in control conditions  
254 (DMSO) and albendazole (ABZ) conditions in 44 replicates per strain. The high-throughput assay  
255 was performed as described (See 2.4, figure 2A). Following 48 hours of growth, three GFP-positive  
256 animals for each strain were sorted into each well of a 96-well plate using the COPAS BIOSORT.  
257 These animals gave rise to a population in each well that was then scored 96 hours after exposure  
258 to ABZ using the COPAS BIOSORT. The green fluorescence measurements were used to filter the  
259 data to only include animals that had the transgene (Figure 2C). We used the length of animals as  
260 a measure of developmental rate. Strains with lower average length values, when compared to the  
261 *ben-1* deletion strain, indicate susceptibility to ABZ. As expected, we measured a significant  
262 difference in animal length between the wild-type strain as compared to the *ben-1* deletion in  
263 response to ABZ. (Figure 3A). We found that expression of *ben-1* under its endogenous promoter  
264 or when highly expressed in all tissues caused susceptibility to ABZ (Figures 3B and S1).  
265 Furthermore, the high level of expression using the *eff-3* promoter caused ABZ susceptibility far  
266 beyond wild-type levels. When *ben-1* expression was driven solely in neurons using the *unc-119*  
267 promoter, the animals were equally susceptible to ABZ as the strains that had endogenous  
268 expression of *ben-1*. These results suggest that neuronal *ben-1* might be the endogenous target of

269 BZ compounds. Expression of *ben-1* in hypodermis, muscles, and intestine did not restore the ABZ  
270 susceptibility phenotype (Figure 3B), indicating that neurons might be the sole target of BZs.  
271



272 **Figure 4.** *ben-1* function in cholinergic and GABAergic neurons underlies BZ sensitivity. The x-axis  
273 denotes the specificity of the *ben-1* expression from the transgene. The y-axis is the normalized  
274 lengths of animals after exposure to 12.5  $\mu$ M of albendazole. Phenotypic data was normalized by  
275 subtracting an average value for each trait from the control data. Each data point is the mean value  
276 for the population of GFP-positive worms in a single well. Tukey box plots have horizontal lines at  
277 the third quartile on the top, the median in the middle and the first quartile at the bottom. The  
278 whiskers are extended within 1.5 range from each quartile. The significant difference between the  
279 transgene and the deletion genotype is shown above the transgene (\*\*\*\* =  $p < 0.0001$ , one-way  
280 ANOVA, Tukey HSD)



### 281 3.2 *ben-1* function in cholinergic and GABAergic neurons rescues BZ sensitive phenotype

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283 Although beta-tubulin genes are expressed in every cell of an organism, *ben-1* has been  
284 specifically shown to be broadly expressed in neurons (Hurd, 2018). We used the *C. elegans*  
285 Neuronal Gene Expression Map and Network (CeNGEN) dataset (Taylor et al., 2021) to further  
286 explore neuron-specific expression of *ben-1* and found that it is expressed in 97 of the 128 cell  
287 types distinguished in the dataset and primarily in cholinergic and glutamatergic neurons (Figure  
288 S3). The thorough characterization of each neuron in the *C. elegans* nervous system offered the  
289 opportunity to generate transgenic animals with *ben-1* expression specific to subsets of neurons.  
290 We hoped to narrow down which neurons are specifically targeted by BZs by creating transgenic  
291 strains with *ben-1* expression separated by neurotransmitter system and perform the same high-  
292 throughput analyses.

293 We created transgenic strains where *ben-1* expression in neurons was subsetted by the  
294 neurotransmitter system, including cholinergic, glutamatergic, dopaminergic, and GABAergic  
295 neurons. Two independent lines for each type were generated in the ECA882 *ben-1* deletion  
296 background. The same pharyngeal expression marker was used to identify transgenic animals as  
297 well as the same wild-type and resistant control strains. We compared the responses of the four  
298 neurotransmitter system-specific transgenes as well as the endogenous and pan-neuronal  
299 expression strains to the two control strains with 44 replicates per strain. Offspring were scored 96  
300 hours after three GFP-positive parents were sorted into each well of plates containing either ABZ  
301 or the control DMSO condition. The score data were filtered to only compare the lengths of GFP-  
302 positive animals. We found that animals expressing *ben-1* in cholinergic regions were significantly  
303 smaller (e.g., developmentally delayed because of susceptibility to ABZ) than the resistant strain  
304 when exposed to ABZ and were comparable in size to animals expressing *ben-1* in all neurons  
305 (Figure 4). It is worth noting that a large proportion of the neuron classes that express *ben-1* are  
306 cholinergic, including a few of the neurons with the highest levels of expression (Figure S3, Loer &  
307 Rand, 2022, Taylor et al., 2021). We observed no difference in animal length between the resistant  
308 strain and animals expressing *ben-1* in glutamatergic and dopaminergic neurons, suggesting that  
309 BZs do not target these neurons (Figure 4). In animals that expressed *ben-1* in GABAergic

310 neurons, we found a less extreme ABZ response (Figure 4), suggesting that *ben-1* function in  
311 cholinergic and potentially GABAergic neurons was sufficient to restore ABZ susceptibility.

## 312 **4. Discussion**

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### 314 *4.1 ben-1 function in cholinergic and GABAergic neurons is sufficient to rescue organismal BZ* 315 *susceptibility*

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317 Although it is well understood that reduction- or loss-of-function variation in *ben-1* confers  
318 resistance to BZs (Driscoll et al., 1989; Hahnel et al., 2018), little has been done to better  
319 characterize BZ sensitivity at the cellular level. Understanding cell-type specific targets of BZs  
320 could enable development of co-treatments that potentiate BZ effects. Here, we generated  
321 transgenic *C. elegans* where *ben-1* expression was limited to one of four tissue types or ubiquitous  
322 expression and compared to endogenous expression. Each strain's response to the BZ drug  
323 albendazole (ABZ) was compared to ABZ responses in the resistant *ben-1* deletion strain. When  
324 *ben-1* is expressed only in neurons, we see that animal development is delayed in ABZ conditions  
325 (Figure 3), suggesting that *ben-1* expression in neurons is sufficient to rescue sensitivity to BZs.  
326 Although in the replicate experiment we also found a statistically significant difference in animal  
327 length when *ben-1* was expressed in the hypodermis or the intestine (Figure S1), these results  
328 were not reproducible between experiments so could be attributed to the overexpression of genes  
329 by different extrachromosomal arrays. These experiments all rely on the overexpression of *ben-1*  
330 in a tissue-specific manner. This overexpression could cause an imbalance in endogenous levels  
331 of beta-tubulins and could cause a slight deleterious effect on fitness. The normalized length  
332 values for strains expressing *ben-1* in the hypodermis and the intestine are also close to zero or  
333 positive, unlike the negative normalized values for animals expressing *ben-1* in neurons where the  
334 difference between the response of the resistant strain is much more pronounced (Figures 3B, S1).  
335 Single-cell expression studies show that *ben-1* is primarily expressed in neurons (Hurd, 2018), so  
336 neurons might require this specific form of beta-tubulin. Future experiments can confirm if *ben-1*  
337 function in neurons is necessary for the BZ susceptibility phenotype.

338 The detailed understanding of the *C. elegans* nervous system anatomy and function  
339 allowed us to identify specific neurons that are targeted by BZs (Taylor et al., 2021; White et al.,  
340 1986). We subsetted the 118 neuron classes by neurotransmitter system to determine which

341 neurons might require *ben-1* function to be susceptible to BZ compounds. Transgenic strains were  
342 generated with *ben-1* fused to cholinergic, dopaminergic, GABAergic, or glutamatergic-specific  
343 promoters (Flames & Hobert, 2009; Serrano-Saiz et al., 2020) and assayed like the tissue-  
344 specificity experiments. When *ben-1* is expressed in cholinergic neurons, the drug-treated animals  
345 were significantly smaller than the resistant strain, similar to when expression is driven in all  
346 neurons (Figure 4). Although some glutamatergic and dopaminergic neurons also have higher  
347 levels of *ben-1* expression, a significant phenotypic effect was not observed in animals driving *ben-*  
348 *1* expression in these neurons (Figures 4 and S2). However, it is possible that BZs also target  
349 GABAergic neurons as a less-significant deleterious effect was consistently observed across  
350 experiments (Figures 4 and S2). Neuron expression data of *ben-1* supports that cholinergic  
351 neurons are more likely to be the target of BZs because they express *ben-1* at higher levels than  
352 the few GABAergic neurons with *ben-1* expression (Loer & Rand, 2022, Taylor et al., 2021).

353

#### 354 *4.2 Further investigation of ben-1 function in specific tissues is required*

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356 *C. elegans* transgenesis using microinjection of plasmids containing *ben-1* is an effective  
357 model to suggest where *ben-1* function might cause susceptibility to BZs. The re-introduction of  
358 *ben-1* as an extrachromosomal array causes overexpression, which might not reflect endogenous  
359 levels and can cause artifactual conclusions. To definitively show the role of *ben-1* in specific  
360 neurons, we require experiments to test the requirement of *ben-1* in BZ resistance. Conditional  
361 knockout (cKO) experiments where the *ben-1* gene is removed from wild-type animals in specific  
362 tissues and/or cells is achievable using the *Cre/loxP* system developed for *C. elegans* (Kage-  
363 Nakadai et al., 2014). Genes flanked with *loxP* sites are removed from the genome using the *Cre*  
364 recombinase (Austin et al., 1981; Kage-Nakadai et al., 2014). Conditionally expressed *Cre* will only  
365 excise *ben-1* from specific target tissues and/or cells, and the remaining tissues of the organism  
366 will express *ben-1* endogenously. We can create strains with *ben-1* knocked out in specific tissue  
367 types or neurons and perform the same high-throughput assays to assess the relative fitness of  
368 each strain when exposed to BZs. If animals with *ben-1* knocked out only in neurons are resistant

369 to BZs, *ben-1* function in this tissue is necessary for BZ sensitivity. Neuron-specific knockout  
370 experiments will confirm specific targets of BZs.

371

#### 372 4.3 Identify which specific neurons are targeted by BZs

373

374 With the suggestion of *ben-1* function in cholinergic and GABA neurons underlying BZ  
375 sensitivity, we can further narrow down the neurons targeted by BZs. We can continue to generate  
376 transgenic worms using promoters for genes specific to different neuron classes and leverage  
377 other tools unique to *C. elegans* such as the NeuroPAL strain set (Yemini et al., 2021) and the  
378 CRF\_ID annotation framework (Chaudhary et al., 2021). NeuroPAL (Neuronal Polychromic Atlas of  
379 Landmarks) strains are transgenic animals with a differentiated fluorescence pattern for every  
380 neuron in the organism (Yemini et al., 2021). Specialized software was developed for the system  
381 that can be used to identify each neuron from images based on fluorescence (Yemini et al., 2021).  
382 However, the annotation software is semi-automatic and requires some manual input to label  
383 microscopy images (Yemini et al., 2021). An automated annotation framework called CRF\_ID has  
384 been developed to label cells with an algorithm based on the graphical-model based framework  
385 Conditional Random Fields (CRF) (Chaudhary et al., 2021). It has been shown to improve  
386 accuracy in labeling whole-brain images from *C. elegans* and be compatible with labeling color-  
387 based NeuroPAL animal images (Chaudhary et al., 2021). This sophisticated imaging and  
388 annotation system would allow us to simultaneously assay the entire *C. elegans* nervous system to  
389 identify specific targets of BZs. Neurons affected by BZs would be identified by comparing  
390 NeuroPAL animals treated with BZs to ones developing in control conditions. The ability to assay  
391 the entire nervous system will be particularly useful as not all of the 118 neuron classes were  
392 accounted for in the neurotransmitter system-specific assays as a few neurons expressing *ben-1*  
393 use other neurotransmitters or their neurotransmitter system is unknown (Figure S3).

394

#### 395 4.4 Benzimidazole neuron targets might be shared with other anthelmintics

396

397 The finding of BZs targeting beta-tubulin in neurons suggests that the specific neurons  
398 targeted might be in common with other anthelmintic compounds. Resistance to two other widely  
399 used drug classes, macrocyclic lactones (MLs) and nicotinic acetylcholine receptor agonists  
400 (nAChRs), has been associated with genes expressed in neurons. Resistance to MLs has been  
401 linked to genes coding for the subunits of glutamate-gated chloride channels, including *glc-1*, *avr-*  
402 *14*, and *avr-15* (Dent et al., 2000), and genes involved in resistance to nAChRs, including *unc-63*,  
403 *unc-38*, *unc-29*, *lev-1*, and *lev-8* are known (Lewis et al., 1980; Qian et al., 2008). Analyzing  
404 overlaps in expression between ML and nAChR resistance genes and *ben-1* using the CeNGEN  
405 data set (Taylor et al., 2021) could identify potential neurons targeted by multiple anthelmintic  
406 compounds. Comparing responses NeuroPAL animals have to each compound could confirm if  
407 some neurons might be targeted and inhibited/killed by multiple anthelmintic drugs. Identification of  
408 neurons that are targeted by multiple anthelmintics might allow for the development of co-  
409 treatments compatible with more than one anthelmintic compound. Overall, the development of  
410 sophisticated analytical systems for assaying the entire *C. elegans* nervous system has the  
411 potential to improve our understanding of mechanisms of action for multiple anthelmintic classes.

412

413

#### 414 **Declaration of competing interest**

415

416 The authors have no competing financial interests that impacted the research presented in  
417 this paper.

418

419

#### 420 **Acknowledgements**

421

422 We would like to thank Clay Dilks, Katie Evans, and Nicole Banks for their help with  
423 experiments and analysis and Amanda Shaver for her helpful comments on this manuscript. This

424 work was supported by the National Institutes of Health NIAID grant R01AI153088 to ECA. This  
425 study used data made available by the *C. elegans* Neuronal Gene Expression Map and Network  
426 (NIH NINDS R01NS100547). Neurotransmitter system data are from WormAtlas neurotransmitter  
427 tables by Curtis M. Loer and James B. Rand (Loer & Rand, 2022, compiled from Gendrel et al.,  
428 2016; Pereira et al., 2015; Serrano-Saiz et al., 2017). Some diagrams in figures were created using  
429 BioRender.com.

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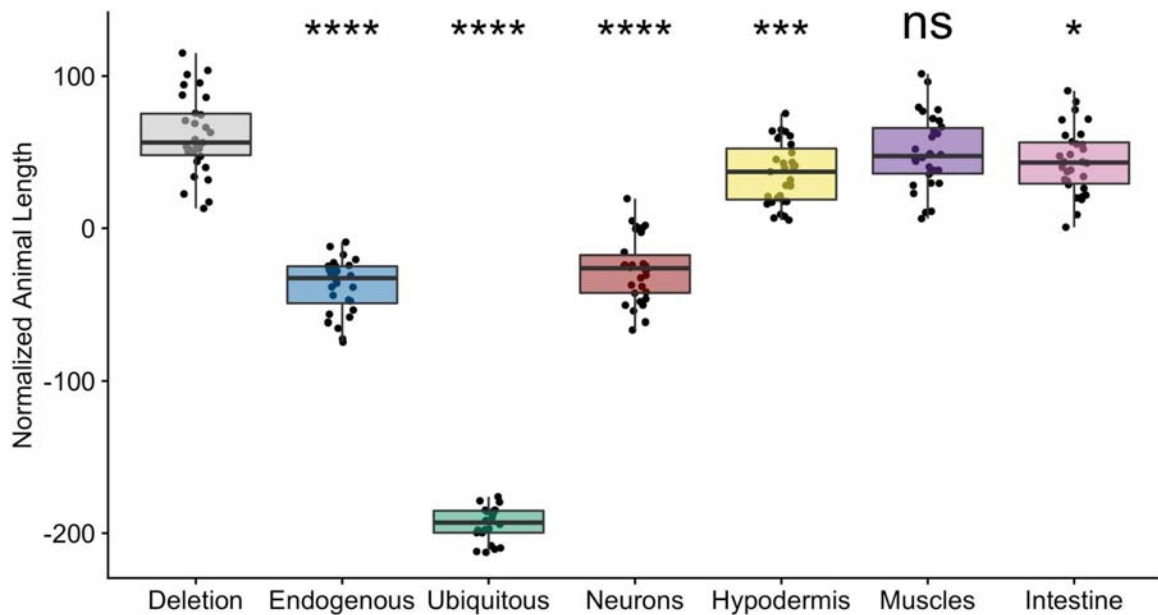
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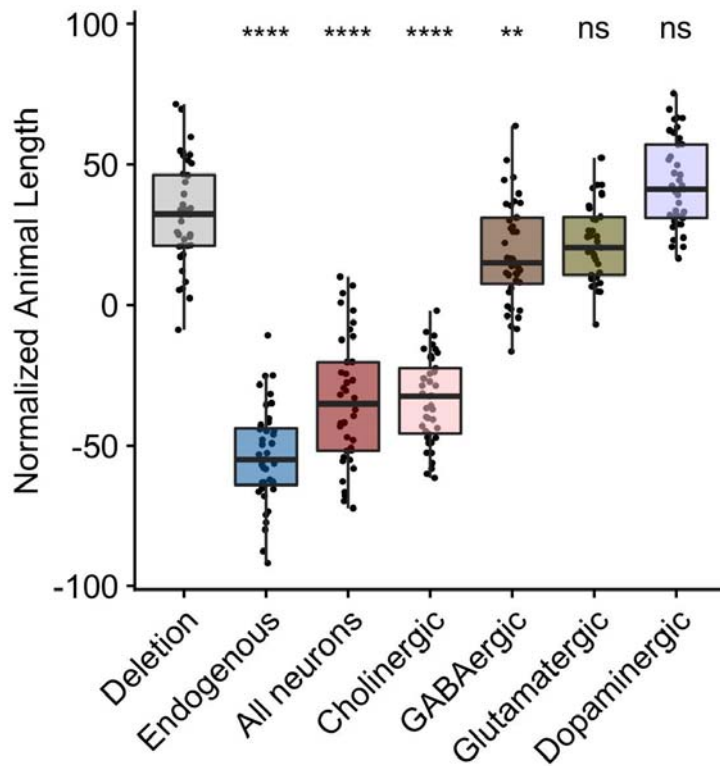
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600 **Supplementary figures and legends**



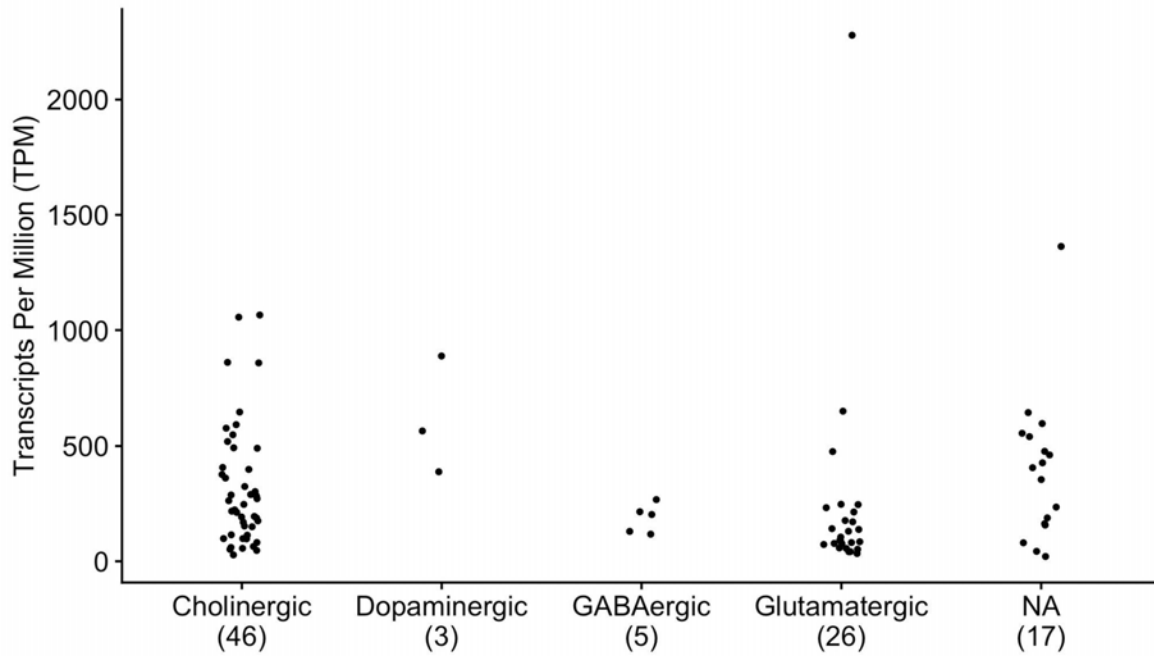
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602 **Figure S1.** Second independent line results. The x-axis denotes the specificity of the *ben-1*  
603 expression from the transgene. The y-axis is the normalized lengths of animals after exposure to  
604 12.5  $\mu$ M of albendazole. Phenotypic data was normalized by subtracting an average value for each  
605 trait from the control data. Each data point is the mean value for the population of GFP-positive  
606 worms in a single well. Tukey box plots have horizontal lines at the third quartile on the top, the  
607 median in the middle, and the first quartile at the bottom. The whiskers are extended within 1.5  
608 range from each quartile. The significant difference between the wild-type genotype and the  
609 deletion genotype is shown above the wild-type results (\* =  $p < 0.05$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p <$   
610  $0.0001$ , one-way ANOVA, Tukey HSD).



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613 **Figure S2.** Second independent line results. The x-axis denotes the specificity of the *ben-1*  
614 expression from the transgene. The y-axis is the normalized lengths of animals after exposure to  
615 12.5  $\mu\text{M}$  of albendazole. Phenotypic data was normalized by subtracting an average value for each  
616 trait from the control data. Each data point is the mean value for the population of GFP-positive  
617 worms in a single well. Tukey box plots have horizontal lines at the third quartile on the top, the  
618 median in the middle, and the first quartile at the bottom. The whiskers are extended within 1.5  
619 range from each quartile. The significant difference between the wild-type genotype and the  
620 deletion genotype is shown above the wild-type results (\*\* =  $p < 0.01$ , < \*\*\*\* =  $p < 0.0001$ , one-way  
621 ANOVA, Tukey HSD).



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**Figure S3.** Distribution of neurotransmitter systems for neuron types expressing *ben-1*. The expression data used for this figure are from the CeNGEN data set (Taylor et al., 2021). Neurotransmitter type data are from WormAtlas neurotransmitter tables by Curtis M. Loer and James B. Rand (Loer & Rand, 2022, compiled from Gendrel et al., 2016; Pereira et al., 2015; Serrano-Saiz et al., 2017). The x-axis denotes the neurotransmitter system for each neuron. The number of neuron classes in each group is included below the neurotransmitter system. NA denotes neurons where the neurotransmitter is unknown or not one of four neurotransmitter systems was included in our experiments. The y-axis is the expression level of *ben-1* for each neurotransmitter type measured as transcripts per million (TPM).