The role of *crm-1* in ionizing radiation-induced nervous system dysfunction in *Caenorhabditis elegans*

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Graphical Abstract

Effect of ionizing radiation on the function of nervous system
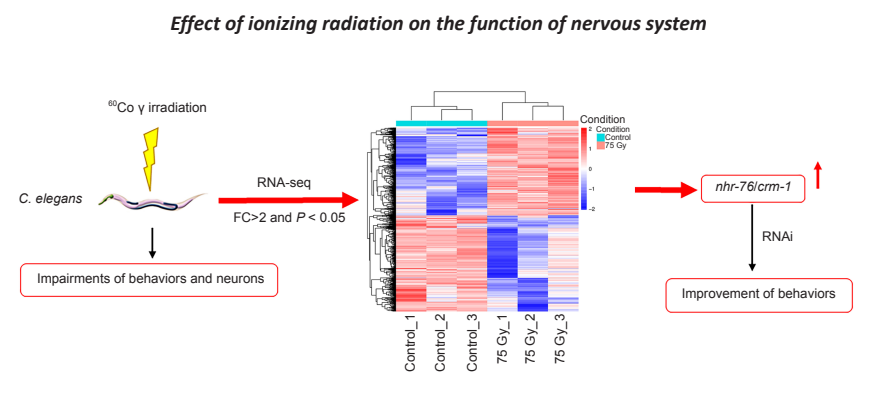
Abstract

Ionizing radiation can cause changes in nervous system function. However, the underlying mechanism remains unclear. In this study, *Caenorhabditis elegans* (*C. elegans*) was irradiated with 75 Gy of ^{60}Co whole-body γ radiation. Behavioral indicators (head thrashes, touch avoidance, and foraging), and the development of dopaminergic neurons related to behavioral function, were evaluated to assess the effects of ionizing radiation on nervous system function in *C. elegans*. Various behaviors were impaired after whole-body irradiation and degeneration of dopamine neurons was observed. This suggests that 75 Gy of γ radiation is sufficient to induce nervous system dysfunction. The genes *nhr-76* and *crm-1*, which are reported to be related to nervous system function in human and mouse, were screened by transcriptome sequencing and bioinformatics analysis after irradiation or sham irradiation. The expression levels of these two genes were increased after radiation. Next, RNAi technology was used to inhibit the expression of *crm-1*, a gene whose homologs are associated with motor neuron development in other species. Downregulation of *crm-1* expression effectively alleviated the deleterious effects of ionizing radiation on head thrashes and touch avoidance. It was also found that the expression level of *crm-1* was regulated by the nuclear receptor gene *nhr-76*. The results of this study suggest that knocking down the expression level of *nhr-76* can reduce the expression level of *crm-1*, while down-regulating the expression level of *crm-1* can alleviate behavioral disorders induced by ionizing radiation. Therefore, inhibition of *crm-1* may be of interest as a potential therapeutic target for ionizing radiation-induced neurological dysfunction.

Key Words: behavior; *Caenorhabditis elegans*; degeneration; disorder; dysfunction; nerve injury; nervous system; neurodevelopment; neuron; radiation

Introduction

The practical applications of ionizing radiation are increasingly extensive. More and more people are becoming involved with the development of both nuclear and radiological technologies. Ionizing radiation sources can be natural or artificial in nature. Artificial radiation sources include the production and testing of nuclear weapons, the application of nuclear technology, nuclear accidents, and medical exposure. Ionizing radiation can affect human health in numerous ways owing to its deleterious effects on biological systems. These include reproductive system damage, hematopoietic system damage, and nervous system dysfunction (Ye, 2011; Han et al., 2019; Lalkovicova, 2022; Wu et al., 2022). Therefore, understanding the pathophysiological processes by which ionizing radiation produces these effects and exploring their mechanisms is of great value in the safe and effective use of ionizing radiation.

Different tissues in the human body have different degrees of sensitivity to ionizing radiation. This is because of differences in the rate of cell division and the degree of cell differentiation within these tissues. The central nervous system is a mildly sensitive tissue. Many radiobiologists consider the brain's tolerant dose to be between 55 and 65 Gy, while the fractional tolerant dose is approximately 2 Gy (Marazziti et al., 2016). However, in recent years it has been reported that the sensitivity of central nervous system to ionizing radiation may have been underestimated. Despite the relatively high resistance of nerve cells to the effects of ionizing radiation, the functional activity of the nervous system can undergo significant changes, even under the influence of doses that do not cause deterministic effects (Marazziti et al., 2016; Kosiakova et al., 2020). These include changes in electrophysiological activity, cognitive decline, mental disorders, and decreased motor function. It has been reported that the electroencephalograms of adolescents with tinea capitis displayed abnormal beta waves after receiving 1.21–1.39 Gy of X-ray radiation treatment to the scalp (Yaar et al., 1982; Aguiar et al., 2015). Similarly, previous research, in which mice were subject to transcriptome analysis before and after irradiation with 0.1 Gy of ^{137}Cs γ-ray whole-body radiation, showed that the expression levels of cognition-related genes were reduced in irradiated mice. Specifically, there was a down-regulation of genes related to synaptic plasticity, such as glutamate receptor, ionotropic, NMDA1 (*zeta 1*) (*Grin1*), and glutamate receptor, ionotropic, *AMPA3* (*alpha 3*) (*Gria3*) (Lowe et al., 2009). Memory loss and language disorders have occurred in staff who have worked in the interventional radiology department for an extended period of time (Marazziti et al., 2015), while clean-up workers in the Chornobyl exclusion zone had significantly higher rates of schizophrenia compared with the general Ukrainian population (Loganovsky and

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Loganovskaja, 2000). In a study of female workers from 12 nuclear weapon plants in the United States, an increased mortality from mental disorders was found (Sibley et al., 2003; Marazziti et al., 2016). York et al. (2012) used [137]Cs γ-rays to irradiate mice with 0.5 and 2 Gy of whole-body radiation and found that the locomotor ability of mice was reduced after radiation. Taking these findings together, it can be seen that even a low dose of ionizing radiation can cause changes in nervous system function that are significant enough to result in diverse clinical symptoms: cognitive impairment, mental disorders, and decreased locomotor capacity. That is to say, low doses of ionizing radiation have a negative effect on the health of the recipient. Therefore, examining the nervous system dysfunction induced by ionizing radiation, exploring its pathophysiology, and finding protective biological targets play an important role in the application of nuclear and radiation technology, especially in the realm of occupational exposure.

To date, there have been relatively few reports in which *Caenorhabditis elegans* (*C. elegans*) has been used as a model to study the impairments of ionizing radiation on the nervous system. However, in other fields, *C. elegans* is a mature model animal for studying neurobiology (Appleby, 2012; Liu et al., 2020; Randi and Leifer, 2020). *C. elegans* has a relatively simple nervous system, but has motor neurons and sensory neurons, as well as many neurotransmitters such as dopamine. These elements make it a useful model in the study of neuroscience (White et al., 1986). Some studies have found that noxious external environmental stimuli (such as arsenic) can lead to nervous system dysfunction in *C. elegans*. These manifest as disorders of behaviors including head thrash and body bend behaviors, as well as degenerative changes in dopaminergic and serotonergic neurons. The degeneration of dopaminergic neurons is implicated as a major cause of behavioral disorders in *C. elegans* (Qu and Wang, 2020; Zhang et al., 2020; Chen et al., 2021). Therefore, we investigated the effects of ionizing radiation on nervous system function in *C. elegans*. To accomplish this, we examined behavioral motor function, sensory function, and dopaminergic neuron development.

Cysteine rich motor neuron protein homolog (*crm-1*) encodes a putative transmembrane protein with multiple cysteine-rich domains that is known to have bone morphogenetic protein (BMPs) binding activity. This protein has been shown to control body size in *C. elegans* (Fung et al., 2007). There are many studies on *crm-1* in mammals. This gene is involved in the regulation of lens development, cell adhesion and migration in human or mouse, and motor neuron development (Zeng et al., 2015; Zhang et al., 2016; Tam et al., 2018; Brązert et al., 2020). *crm-1* has been poorly studied in *C. elegans*. This study mainly explores whether crm-1 is involved in the regulation of nervous system function in *C. elegans*.

Methods

C. elegans culture
C. elegans strains were cultured on bacterial lawns of either Escherichia coli OP50 (OP50) or Escherichia coli HT115(DE3) (HT115(DE3)) (Shanghai Weidi Biotechnology Co., Ltd., Shanghai, China) at 20°C in the dark and ~50% humidity, according to standard methods (Brenner, 1974). *C. elegans* was grown on standard nematode growth medium (3 g NaCl, 20 g agar, 2.5 g peptone, 975 mL H_2O, 1 mL 1 M $CaCl_2$, 1 mL 5 mg/mL cholesterol, 1 mL 1 M $MgSO_4$ and 25 mL 1 M K_3PO_4). It fed on OP50 or HT115(DE3) and took approximately 48 hours to develop from the L1 stage to the young adult stage (Byerly et al., 1976). The *C. elegans* strains used included wild-type strain N2, transgenic strain BZ555(dat-1p::GFP), and transgenic strain PHX5872 *crm-1* (syb5872) (SunyBiotech). In BZ555, the sodium-dependent dopamine transporter (dat-1) is co-expressed with GFP. In PHX5872 *crm-1* (syb5872), *crm-1* and GFP are co-expressed. Wild-type strains N2 and OP50 were obtained from Soochow University, and transgenic strain BZ555 was obtained from Wenzhou Medical University. When examining behavioral indicators, wild-type strains were divided into two groups: a control and a radiation group. To examine behavioral indicators after irradiation and/or inhibition of *crm-1*, wild-type strains were divided into four groups: a control group, a crm-1 RNAi group, a radiation group, and a *crm-1* RNAi and radiation group. When examining dopaminergic neurons, transgenic strains BZ555 were divided into two groups: a control group and a radiation group. Worms of the transgenic strain PHX5872 *crm-1* (syb5872) were divided into two groups: a control group and a radiation group after irradiation or a control group and a *nhr-76* RNAi group after inhibition of *nhr-76*. The *C. elegans*-related experiments were reviewed by the Experimental Animal Ethics Committee of Soochow University on March 16, 2020.

Irradiation
The [60]Co γ irradiator in the State Key Laboratory of Radiation Medicine and Protection, School of Radiation Medicine and Protection, Soochow University, was used in this study. In preliminary experiments carried out prior to this study, it was found that 75 Gy could induce abnormal behaviors in *C. elegans*. Therefore, *C. elegans* in the L2 stage was exposed to 75 Gy γ-ray radiation. The dose rate was 25 Gy/min and the irradiation time was 3 minutes. Worms were then cultured to the young adult stage for follow-up experiments (**Figure 1**).
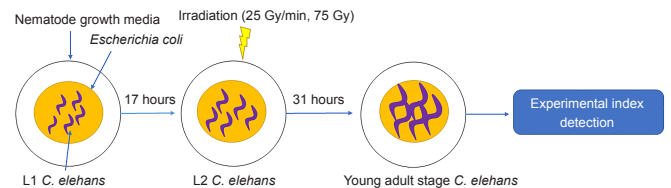

Figure 1 │ **Flow chart showing radiation in *Caenorhabditis elegans* (*C. elegans*).**

Measurement of behavioral motor and sensory function
In *C. elegans*, changes of behavioral motor and sensory function are reflected in changes in head thrash, touch avoidance, and foraging behaviors. Measurement of head thrashes was carried out as described previously (Zeng et al., 2017). The young adult stage *C. elegans* was transferred to nematode growth media medium without food. M9 buffer (60 μL; 5 g NaCl, 3 g K_2HPO_4, 15 g $Na_2HPO_4·12H_2O$, 1 mL 1 M $MgSO_4$, H_2O to 1000 mL; Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) was added to a single *C. elegans* worm, and the number of head thrashes occurring within 60 seconds was recorded. The movement of the head of *C. elegans* to one side and then back was recorded as a head thrash. Thirty *C. elegans* per group were analyzed.

Touch avoidance was measured as described in previous studies (Kaplan and Horvitz, 1993; Hobert et al., 1999; Hart, 2006). An eyebrow hair was used to gently touch the area between the posterior bulb and the vulva of young adult stage *C. elegans* (**Figure 2A**), and each *C. elegans* was touched 10 times. The number of backwards movements after touching was recorded. Thirty *C. elegans* per group were analyzed.

Foraging behavior was studied as described in a previous study (Kohra et al., 2002). Before the test, a petri dish was prepared. One end of the nematode growth medium was set as the starting point. A target circle with a radius of 0.5 cm was coated with OP50. The center of this target circle was 4 cm from the starting point (**Figure 2B**). Twenty young adult stage *C. elegans* were transferred to the starting point. The number of *C. elegans* reaching the target circle was observed after 2, 4, 6, 8, and 24 hours, and six parallel samples were set in each group. The foraging level was considered to be the number of *C. elegans* reaching the target circle divided by the total number of *C. elegans*.

Dopamine neurons analysis
Morphological changes in dopamine neurons of the transgenic strain BZ555 were observed (Nass et al., 2002; Zhang et al., 2020). The young adult stage *C. elegans* were transferred to a 3% agarose pads, anesthetized by dropwise addition of 10 μL of 4 mM levamisole hydrochloride (Sangon Biotech (Shanghai) Co., Ltd.), and covered with a coverslip. Dopamine neurons of whole *C. elegans* were counted under a fluorescent microscope (Guangzhou Micro-shot Technology Co., Ltd., Guangzhou, China). The number of discontinuous dendrites and the comparative fluorescent intensity of cell bodies were considered to reflect the extent to which the neuronal development of dopamine neurons was negatively affected. Fifty *C. elegans* per group were analyzed.

RNA sequencing analysis
Gene expression of irradiated and control nematodes was analyzed using RNA sequencing (RNA-seq). After the total RNA of whole *C. elegans* was extracted, mRNA was enriched and cut into short fragments before being synthesized into complementary DNA (cDNA) and purified. End repair was performed, a poly(A) tail was added, and it was connected with the sequencing adapter. Appropriate fragment size was selected for polymerase chain reaction (PCR) amplification and Illumina HiSeq X Ten was used for sequencing. Protein-coding gene expression was calculated using the fragments per kb per million reads method to compare different samples (Roberts et al., 2011). Differentially expressed genes with a significance level of $P < 0.05$ and a fold change greater than 2 were screened for using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis tools (Viau et al., 2020). Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

String online protein-protein interaction networks analysis and literature survey
The string online protein-protein interaction networks (PPI) analysis system (https://string-db.org/) was used to screen out genes that were highly related to cysteine rich motor neuron protein homolog (*crm-1*) interaction, and *C. elegans* was selected as the species. Results were analyzed using the cytoHubba plug-in in cytoscape3.8.2 software (https://github.com/cytoscape/cytoscape/releases/3.8.2/). This assigns values to each gene using the topological network algorithm. Key genes (hub genes) related to *crm-1* were subsequently sorted and mined. The following parameters were used: a degree threshold of 2, a node score threshold of 0.2, a k score of 2, and a maximum depth of 100.

Differentially expressed genes were screened out using to the KEGG pathway

analysis, GO function analysis and string online PPI analysis systems. Literature searches for each of these genes using PubMed (https://pubmed.ncbi.nlm.nih.gov/) and wormbase (https://wormbase.org///#012-34-5) were carried out to understand the relevant function of these genes.

RNA interference (RNAi)

An 807 bp fragment of *crm-1* was amplified by PCR using cDNA as a template and inserted into the *Hind* III site of vector L4440. The recombinant plasmid was transformed into HT115(DE3) to construct a crm-1 RNAi clone. A 1918 bp fragment of nuclear hormone receptor family (*nhr-76*) was amplified by PCR using genomic DNA as a template and inserted into the *Hind* III site of vector L4440, and then transformed into HT115 (DE3) to construct an nhr-76 RNAi clone. The primers used were as follows: for the *crm-1* RNAi clone, the forward primer 5′-gtc gac ggt atc gat aag ctt GAA ATG CGT TCC ATC CGT GC-3′ and the reverse primer 5′-cag gaa ttc gat atc aag ctt TCA CGA CGA AGT CCA TTG GC-3′ (lowercase letters are homologous end sequences). For the nhr-76 RNAi clone, forward primer 5′-gtc gac ggt atc gat aag ctt ATT CAG GAA GCC GAG GAG GA-3′ and reverse primer 5′-cag gaa ttc gat atc aag ctt CGA CTC GCG TCC GAT AAG TT-3′. *crm-1* RNAi was performed by feeding synchronized L1 stage with HT115(DE3) carrying the *crm-1* RNAi clone. *nhr-76* RNAi was performed in the same way. HT115(DE3) carrying the empty vector L4440 plasmid was used as a negative control (Fung et al., 2007). The HT115 strain was streaked on Luria-Bertani media (LB) agar (2 g tryptone, 1 g yeast, 1 g NaCl, 3 g agar, H_2O to 200 mL, pH 7.5) with 50 μg/mL ampicillin and 10 μg/mL tetracycline. If the LB agar was covered with monoclonal strains, the transformation was considered successful. The irradiation conditions were the same as above, as were the measurements of head thrashes, touch avoidance and foraging.

Quantitative PCR

Young adult stage *C. elegans* was collected. The total RNA extracted and then reverse transcribed into cDNA (Zhang et al., 2020). Quantitative PCR (qPCR) was performed using Novozymes ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) to verify RNA-seq results and RNAi efficiency. The instrument setup conditions were as follows: initial denaturation for 30 seconds at 95°C, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The relative expression level of target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with the gene *tba-1* being used as the internal control. The primers used for qPCR are listed in **Table 1**.

Table 1 | qPCR primer sequences

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product length (bp)
tba-1	TCA ACA CTG CCA TCG CCG CC	TCC AAG CGA GAC CAG GCT TCA G	193
crm-1	ATG TTC GTT GTG CAA GTG TCG	TCC ACC ATT CTC CAC CAT CTC	173
nhr-76	GTC CGC TGG ATA GAC GAC AC	ATC ATT TCT GCT GGG GAC CG	133

crm-1: Cysteine rich motor neuron protein homolog; *nhr-76*: nuclear hormone receptor family; qPCR: quantitative polymerase chain reaction; *tba-1*: tubulin alpha chain.

Semi-quantitative analysis of PHX5872 crm-1 (syb5872)

The fluorescence intensity value in the head was scored under a fluorescent microscope after irradiation/sham irradiation or *nhr-76* RNAi in the transgenic strain PHX5872 crm-1 (syb5872). Thirty young adult stage *C. elegans* per group were analyzed.

Statistical analysis

All experimental results in this study were statistically analyzed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The measurement data are expressed as mean ± standard deviation (SD). For statistical analysis between two independent samples, an independent sample *t*-test was used. For multiple groups of samples, one-way analysis of variance followed by least significant difference (LSD) *post hoc* test (equal variance) or Dunnett's T3 *post hoc* test (unequal variances) was used. Each experiment was repeated at least three times. Thirty *C. elegans* were selected per group for this study when examining head thrashes and touch avoidance behaviors. When detecting foraging behavior, six parallel samples were set in each group and 20 *C. elegans* were selected per parallel sample. Fifty *C. elegans* were selected per group when detecting dopamine neurons. Thirty *C. elegans* were selected per group when measuring the fluorescence intensity of PHX5872 crm-1 (syb5872).

Results

The effects of ^{60}Co γ-rays on nervous system function of *C. elegans*

To evaluate the effects of ^{60}Co γ-rays on nervous system function in *C. elegans*, changes in the head thrash, touch avoidance and foraging behaviors of wild-type strain were examined before and after irradiation. Changes in dopaminergic neurons in the head the transgenic strain BZ555 after radiation were observed to reflect the functional changes of the nervous system of *C. elegans*.

Behavioral motor and sensory dysfunction

Worms of the wild-type N2 strains were divided into a control group and a radiation group. After being irradiated with ^{75}Gy γ-rays, changes in the behavioral motor and sensory function were detected. The head thrash frequency of *C. elegans* decreased by ~8% from 109.83 times to 101.07 times per minute ($P = 0.005$; **Figure 2C**), the proportion of touch avoidance was significantly reduced by ~23% from 0.78 to 0.60 ($P < 0.001$; **Figure 2D**), and the level of foraging behavior was significantly lessened in the radiation group by ~89%, 83%, 71%, 70%, and 47% at 2, 4, 6, 8, and 24 hours compared with the control group, respectively ($P = 0.003$, $P = 0.001$, $P = 0.001$, $P = 0.001$, $P = 0.003$, respectively; **Figure 2E**). It has been reported that head thrash frequency is dependent on motor neurons such as dorsal A-type and dorsal B-type (White et al., 1976, 1986), while foraging is associated with amphid wing neuron A and amphid wing neuron C olfactory neurons, outer labial quadrant and inner labial 1 sensory neurons, and ring motor neuron G motor neurons (Zeng et al., 2017). Anterior ventral microtubule and anterior lateral microtubule sensory neurons of *C. elegans* transmit information to anterior ventral process D and anterior ventral process A interneurons after gentle touch stimulation. These interneurons then innervate Dorsal A-type motor neurons to give rise to backward avoidance responses (de Bono and Maricq, 2005). Therefore, this study evaluated the effects of γ-rays on the behavioral motor and sensory function of *C. elegans* by examining changes in head thrash, touch avoidance and foraging behaviors. The experimental results show that 75Gy γ radiation can induce nervous system dysfunction in wild-type strains, which manifests as decreased behavioral motor ability and abnormal sensory function.

Degeneration of dopamine neurons

In trangenic strain BZ555, the sodium-dependent dopamine transporter (*dat-1*) is co-expressed with GFP (Bijwadia et al., 2021). The *dat-1* gene encodes a dopamine transporter and so regulates dopamine levels during dopaminergic neurotransmission (Nirenberg et al., 1996). Therefore, fluorescent neurons observed in BZ555 may be considered to be dopaminergic in nature, and the morphology of these neurons can be directly observed using this model. The transgenic strain BZ555 has a total of four pairs of dopaminergic neurons, including one pair of posterior deirid neurons in the tail, two pairs of cephalic sensilla (CEP) neurons and one pair of anterior deirid (ADE) neurons in the head (**Figure 3A–C**). In this study, the effects of ^{60}Co γ-rays on dopamine neurons of *C. elegans* were evaluated by observing the integrity of CEP dendrites and the fluorescence intensity of CEP and ADE cell bodies in transgenic strain BZ555. After 75 Gy γ irradiation, the fluorescence area in the head region of *C. elegans* had shrunk, the fluorescence appeared to be discontinuous and the integrity of CEP dendrites decreased by 3% from 1.00 to 0.97 ($P = 0.014$). After irradiation, the fluorescence area of CEP and ADE cell bodies diminished, and the fluorescence intensity weakened by 10% from 1.00 to 0.90 ($P = 0.024$), indicating that CEP and ADE cell bodies had atrophied (**Figure 3D–I**). The change in fluorescence level after ^{60}Co γ irradiation suggests that ionizing radiation induces degeneration of dopamine neurons in *C. elegans*.

Genome-wide transcriptional analysis and bioinformatics analysis

To search for key genes that play a regulatory role in ionizing radiation-induced nervous system dysfunction, we performed RNA-seq analysis in wild-type strains, comparing the radiation and control groups. We then screened out differentially expressed genes related to nervous system function through bioinformatics analysis.

Genome-wide transcription analysis and string online protein-protein interaction networks analysis of wild-type strains after radiation exposure

The screened differentially expressed genes were analyzed using horizontal clustering (**Figure 4A**) and a volcano plot (**Figure 4B**) to understand their overall distribution. The genes were then subjected to GO enrichment analysis and KEGG pathway enrichment analysis. On the premise of stable RNA quality, a total of 20,174 genes were sequenced. Compared with the control group, 1338 genes were significantly different after radiation. Of these, 638 genes were up-regulated and 700 genes were down-regulated (**Figure 4C**). According to the KEGG analysis, the enriched KEGG pathways included the transforming growth factor-beta signaling pathway, apoptosis, nitrogen metabolism, and tryptophan metabolism. (**Figure 4D**). According to GO analysis, the GO functions enriched in this study included nervous system development, insulin-like growth factor-activated receptor activity, insulin-like growth factor binding, the extracellular region, serine-type endopeptidase inhibitor activity, and regulation of cell growth (**Figure 4E**).

According to GO functional enrichment analysis, KEGG pathway analysis, and a literature survey (Kolle et al., 2000; Fung et al., 2007), the differentially expressed gene *crm-1* is closely related to nervous system development (GO:0007399). The string online PPI analysis system was used to screen out the genes that were highly related to *crm-1* interaction in *C. elegans*. The results were analyzed using the cytoHubba plug-in in cytoscape3.8.2 software, focusing on the hub genes of crm-1. Network modules were mined from the constructed PPI network using the plugin MCODE in cytoscape3.8.2. The parameters were set as follows: a degree threshold of 2, a node score threshold of 0.2, a k score of 2, and a maximum depth of 100. A score greater

than 5 indicates a high probability of an interaction between two genes. Therefore, the genes with a score > 5 were selected for analysis (**Figure 5**). Finally, the differentially expressed gene *nhr-76* (score = 6) was screened. It has been reported that nhr-76 is closely related to nervous system function in human or mouse (Li et al., 2020; Yang et al., 2021; Ben-Zvi and Liebner, 2022), and is closely related to fatty acid metabolism in *C. elegans* (Noble et al., 2013). Therefore, it was speculated that *crm-1* and *nhr-76* could be involved in the regulation of ionizing radiation-induced nervous system dysfunction in *C. elegans*.

Changes in the expression levels of crm-1 and nhr-76 genes in C. elegans after γ-ray irradiation

Changes in the expression levels of *crm-1* and *nhr-76* after radiation were assessed by qPCR. It was found that the expression levels of *crm-1* ($P = 0.028$) and *nhr-76* ($P = 0.014$) induced by 75 Gy γ-rays were increased by approximately 49% (an increase from 1.02 to 1.52) and approximately 50% (an increase of 1.01 to 1.51), respectively (**Figure 5C**).

The role of nhr-76/crm-1 in ionizing radiation-induced nervous system dysfunction in C. elegans

To confirm how *nhr-76* and *crm-1* interacted and participated in the regulation of ionizing radiation-induced nervous system dysfunction in *C. elegans*, RNAi technology was used to knock down *crm-1*, and expression levels of nhr-76 were measured. Changes in head thrash, touch avoidance and foraging behaviors of *C. elegans* were observed after successful knockdown of *crm-1*.

The interaction between crm-1 and nhr-76

To verify whether *crm-1* interacted with *nhr-76*, RNAi technology was used in this study to knockdown *crm-1* and *nhr-76* in wild-type N2 strains. Their gene expression levels were then measured after successful interference. On knocking down the expression level of the *crm-1* gene ($P < 0.001$), there was no statistical difference in the expression level of the *nhr-76* gene ($P = 0.241$) in *C. elegans*. However, knocking down the expression level of the *nhr-76* gene ($P = 0.015$) produced a change in the expression level of the *crm-1* gene ($P = 0.016$) decreased by approximately 59% (a decrease from 1.01 to 0.41) **Figure 6**). Therefore, *nhr-76* appears to act upstream of *crm-1* at the gene level.

Fluorescence intensity changes of PHX5872 crm-1 (syb5872)

To demonstrate whether the change in *crm-1* expression that occurs after irradiation is regulated by *nhr-76*, and to further investigate whether *crm-1* is regulated by *nhr-76*, the transgenic strain PHX5872 crm-1 (syb5872) was used. The fluorescence intensity of the transgenic strain increased by 35% from 1.00 to 1.35 after irradiation when compared with the control group. This suggests that *crm-1* expression is increased by irradiation. Conversely, the fluorescence intensity weakened by 36% from 1.00 to 0.64 compared with the control group after knocking down the expression level of the *nhr-76* gene ($P < 0.001$, $P < 0.001$; **Figure 7**). This provides further evidence that *crm-1* is regulated by *nhr-76*.

The role of crm-1 in ionizing radiation-induced nervous system dysfunction in C. elegans

To verify whether *crm-1* is involved in the regulation of ionizing radiation-induced nervous system dysfunction in *C. elegans*, we used RNAi technology to knockdown *crm-1*. Changes in head thrash frequency, touch avoidance, and foraging behaviors of *C. elegans* were observed. Four experimental groups were set up: the control group, the *crm-1* RNAi group, the radiation group, and the *crm-1* RNAi and radiation group. Comparing the control and *crm-1* RNAi group, there were no significant changes in head thrash frequency ($P = 0.264$), touch avoidance ($P = 1.000$), or foraging at 2 ($P = 0.078$), 4 ($P = 0.376$), 6 ($P = 0.285$), 8 ($P = 0.315$), or 24 hours ($P = 0.659$). The head thrash frequency and the proportion of touch avoidance were increased by about 12% from 94.17 times to 105.63 times per minute ($P = 0.001$) and 24% from 0.58 to 0.72 ($P = 0.007$), respectively in the *crm-1* RNAi and radiation group when compared with the radiation group. There was no statistical difference in foraging at 2 ($P = 0.903$), 4 ($P = 0.823$), 6 ($P = 0.690$), 8 ($P = 0.986$), or 24 hours ($P = 0.659$) (**Figure 8**). This suggests that inhibition of *crm-1* can effectively alleviate ionizing radiation-induced head thrash and touch avoidance, but cannot alleviate ionizing radiation-induced foraging. It could be seen that knocking down the expression level of the *crm-1* gene effectively improved the behavioral disorders observed in *C. elegans* that are induced by ionizing radiation. From previous experimental results, the expression of *crm-1* gene was regulated by *nhr-76*. Therefore, the *nhr-76/crm-1* pathway may be involved in the regulation of ionizing radiation-induced nervous system dysfunction.

Discussion

The nervous system of *C. elegans* has 302 neurons, which may be classified into motor neurons, sensory neurons, interneurons, and polymodal neurons. It is relatively simple, transmitting information through about 6400 chemical synapses, 900 gap junctions and 1500 neuromuscular junctions. Similar to higher animals, the neurons of *C. elegans* have many neurotransmitters, such as dopamine, serotonin and glutamate. The behavior of *C. elegans* includes

not only basic behaviors such as locomotion, foraging, feeding and defecation, but also complex behaviors such as the avoidance response and chemotaxis. Many behaviors of *C. elegans* are quantifiable. Researchers can therefore use these to evaluate the functional activities of the nervous system (Hart, 2006). Many researchers have examined behavioral indicators such as head thrash, body bend, foraging and chemotaxis, as well as morphological changes in dopaminergic neurons and glutamatergic neurons, to evaluate the nervous system function of *C. elegans* (Cao et al., 2019; Liu et al., 2020). Therefore, we used *C. elegans* as a model animal to study the impairment of nervous system function induced by ionizing radiation in this study.

C. elegans larvae are still able to develop into adults after being irradiated with 1000 Gy X-rays whole-body radiation (Ishii and Suzuki, 1990). Similarly, the mortality of *C. elegans* is not changed significantly when they are irradiated with 500 Gy γ-rays whole-body radiation (Weidhaas et al., 2006). Therefore, we consider 75 Gy to be a relatively low dose in *C. elegans*. Some studies have shown that ionizing radiation can induce nervous system dysfunction in *C. elegans*, which is reflected in behavioral disorders such as body bend and chemotaxis (Sakashita et al., 2008; Suzuki et al., 2009, 2017, 2020; Ye, 2011). In this study, it was also found that 75 Gy of γ-rays could induce behavioral motor and sensory dysfunction in *C. elegans*, manifesting as disorders of head thrash, touch avoidance and foraging behaviors. This indicates that ionizing radiation inhibits nervous system functions in *C. elegans*, producing dysfunction in both motor and sensory functions. Using the transgenic strain BZ555, we also found that γ-rays could induce neuronal degeneration, indicated by an increase in dendrite defects and cell body atrophy in dopaminergic neurons. Dopamine signaling plays a key role in many aspects of nervous system function, and dopamine is able to affect crucial functions including learning and locomotion (Wise, 2004; Schultz, 2007). Abnormal dopamine function is associated with a variety of diseases, including attention deficit hyperactivity disorder and Parkinson's disease (Rangel-Barajas et al., 2015; Cooper and Van Raamsdonk, 2018; Abrantes Dias et al., 2020; Miyazaki and Asanuma, 2020). It has been reported that a reduction in dopamine signaling can lead to apparent behavioral abnormalities in both mammals and *C. elegans* (Omura et al., 2012; Alghasham and Rasheed, 2014; Kim et al., 2014). Previous studies have also found that dopaminergic neuron damage could be the cause of decline in behavioral motor ability (Qu and Wang, 2020; Zhang et al., 2020; Chen et al., 2021). Therefore, degeneration in dopamine neurons could be responsible for ionizing radiation-induced behavioral disorders in *C. elegans*.

The transcriptomes of *C. elegans* in the control group and the radiation group were sequenced to explore the molecular mechanisms of ionizing radiation-induced nervous system dysfunction in *C. elegans*. Through a literature survey, bioinformatics analysis, and the string online PPI analysis system, we screened out the genes related to nervous system function: *crm-1* and *nhr-76*. The human homolog of crm-1 is cysteine rich transmembrane bone morphogenetic protein (BMP) regulator 1 (*CRIM1*), which is involved in the formation of motor neurons and the regulation of growth and development. A previous study found that cysteine rich transmembrane BMP regulator 1 (chordin like) (*Crim1*) was expressed in the notochord, floor plate, early motor neurons, and interneuron subpopulations within the developing spinal cord. This indicates that *Crim1* is closely related to the development of the central nervous system (Kolle et al., 2000). It was found that *crm-1* could affect the growth and development of *C. elegans* through the BMP pathway and is expressed in both dorsal A-type and dorsal B-type motor neurons of the ventral nerve cord (Fung et al., 2007). The human homolog of nhr-76 is peroxisome proliferator activated receptor delta (*PPARD*), a nuclear receptor that regulates neurogenesis, homeostasis, and fatty acid metabolism in mammals and in *C. elegans* (Noble et al., 2013; Ji et al., 2015; Ben-Zvi and Liebner, 2022). Previous study has shown that murine peroxisome proliferator activator receptor delta (*Ppard*) gene is involved in the proliferation and differentiation of neural stem cells in the mouse hippocampus (Ji et al., 2015). Ppard may also be involved in regulating the barrier of mature vascular endothelial cells in mice, thereby regulating tissue homeostasis in the central nervous system (Ben-Zvi and Liebner, 2022). *C. elegans* nhr-76 may regulate fatty acid metabolism by regulating serotonin (Noble et al., 2013). Therefore, it is speculated that crm-1 and nhr-76 may be involved in the regulation of ionizing radiation-induced nervous system dysfunction. PPARD is one of the three known PPARs that are part of the nuclear receptor superfamily of transcription factors (Yang et al., 2021). PPARs negatively regulate the BMP pathway (Zhang et al., 2011). Therefore, *PPARD* is thought to be involved in regulating the BMP pathway, from which it is deduced that the *PPARD* homologous gene nhr-76 may be involved in regulating the BMP pathway. At the same time, CRIM1 is involved in the negative regulation of the BMP pathway (Wilkinson et al., 2003): a classic nervous system developmental regulatory pathway (Wilkinson et al., 2003; Yamamoto and Oelgeschläger, 2004). Therefore, we speculate that both *crm-1* and *nhr-76* may regulate nervous system function by regulating the BMP pathway. It is predicted that *crm-1* may interact with *nhr-76* according to the results of our PPI analysis.

The expression levels of both *crm-1* and *nhr-76* increased after irradiation. To explore whether these two genes are involved in the regulation of ionizing radiation-induced nervous system dysfunction, we knocked down the expression levels of *crm-1* and *nhr-76*, respectively, and examined their
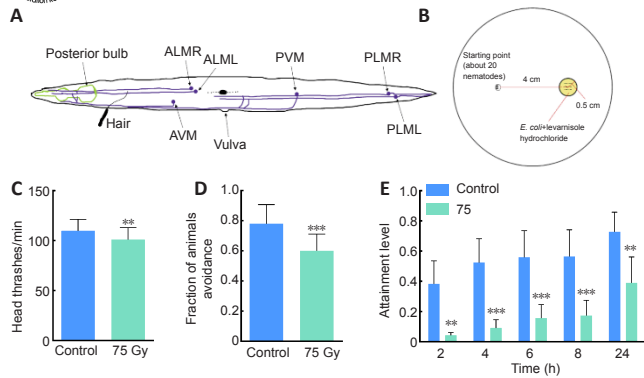
Figure 2 | Schematic diagram of behavioral assays and the effects of ionizing radiation on behavior of *Caenorhabditis elegans* (*C. elegans*).
(A) Schematic diagram of touch avoidance. (B) Schematic diagram of foraging. (C) Effects of ionizing radiation on head thrashes in *C. elegans*. (D) Effects of ionizing radiation on touch avoidance in *C. elegans*. (E) Effects of ionizing radiation on foraging in *C. elegans*. Data are expressed as mean ± SD (n = 30). The experiment was repeated three times. **$P < 0.01$, ***$P < 0.001$ (independent sample t-test). ALML: Anterior lateral microtubule left; ALMR: anterior lateral microtubule right; AVM: anterior ventral microtubule; E. coli: Escherichia coli OP50; PML: posterior lateral microtubule left; PLMR: posterior lateral microtubule right; PVM: posterior ventral microtubule.
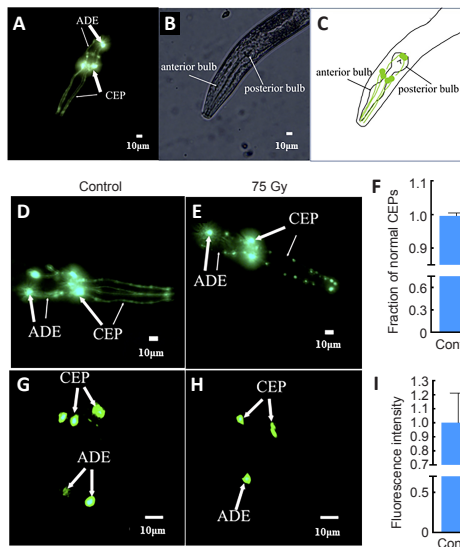
Figure 3 | Visualization of dopamine neurons in the head neurons of *Caenorhabditis elegans* (*C. elegans*) and the effects of ionizing radiation.
(A–C) Dopamine neuron expression in the head. In transgenic strain BZ555, the sodium-dependent dopamine transporter (dat-1) is co-expressed with GFP. (A) Fluorescence picture. Thin and thick arrows indicate dendrites and cell bodies, respectively. (B) Image under bright field. (C) Hand drawn picture. Green lines and dots indicate dendrites and cell bodies, respectively. (D–F) Expression of dopamine neurons in the control and the radiation group. Thin arrows indicate dendrites, and thick arrows indicate cell bodies. (G–I) Cell bodies of cephalic sensilla (CEP) and anterior deirid (ADE) neurons in the control and the radiation group. Thick arrows indicate cell bodies. Scale bars: 10 μm. Data are expressed as mean ± SD (n = 50). The experiment was repeated three times. *$P < 0.05$ (independent sample t-test). ADE: Anterior deirids; CEP: cephalic sensilla.

Figure 4 | Analysis of differentially expressed genes induced by ionizing radiation in *Caenorhabditis elegans* (*C. elegans*).
(A) Heatmap analysis results. Red indicates highly expressed genes while blue indicates a lower level of expression. The darker the color, the higher the fold change. (B) Differentially expressed genes volcano plot. Grey indicates a non-significant difference, red and green a significant difference. Red indicates highly expressed genes while green indicates a lower level of expression. (C) Differentially expressed genes pie chart. (D) KEGG enrichment bubble plot. (E) GO enrichment bubble diagram. acd-1: Acid-sensitive degenerin; acdh-5: acyl coa dehydrogenase; arf-1.1: ADP-ribosylation factor 1-like 1; B0281.3: ring-type domain-containing protein; ceh-24: homeobox protein ceh-24; crm-1: cysteine rich motor neuron protein homolog; C28G1.5: uncharacterized protein; C33F10.8: uncharacterized protein; C34F11.5: tyrosine-protein kinase; C52A10.1: uncharacterized protein; FC: fold change; F01G10.5: uncharacterized protein; F15A8.6: uncharacterized protein; F47E1.2: solute carrier organic anion transporter family member; F56F4.3: transporter; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; K07F5.6: uncharacterized protein; M05B5.1: uncharacterized protein; R09B5.11: facilitated glucose transporter homolog; skr-5: Skp1-related protein; srp-8: serpin domain-containing protein; TGF-beta: transforming growth factor-beta; T22C1.8: tyrosine-protein phosphatase; ZK1240.1: ring-type domain-containing protein; ZK418.7: g_protein_recep_f1_2 domain-containing protein; ZK622.1: tyrosine-protein kinase.
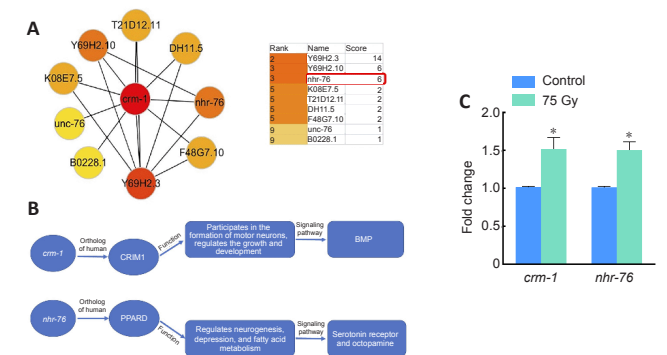
Figure 5 | Interaction analysis of *crm-1*, functions, and expression level changes of *crm-1* and *nhr-76* in *Caenorhabditis elegans* (*C. elegans*) using PPI analysis.
(A) Interacting gene analysis of *crm-1*. The red box indicates the target gene, which plays a prominent role. (B) Functions of *crm-1* and *nhr-76*. (C) Expression level changes of *crm-1* and *nhr-76* in *C. elegans* induced by ionizing radiation by qPCR. Data are expressed as mean ± SD. The experiment was repeated at least three times. *$P < 0.05$ (independent sample t-test). BMP: Bone morphogenetic protein; B0228.1: uncharacterized protein; CRIM1: cysteine rich transmembrane BMP regulator 1; *crm-1*: cysteine rich motor neuron protein homolog; DH11.5: C2 domain-containing protein; F48G7.10: uncharacterized protein; K08E7.5: uncharacterized protein; *nhr-76*: nuclear hormone receptor family; PPARD: peroxisome proliferator activated receptor delta; T21D12.11: uncharacterized protein; *unc-76*: uncoordinated; Y69H2.3: uncharacterized protein; Y69H2.10: uncharacterized protein; qPCR: quantitative polymerase chain reaction.

association. Then behavioral measurement was conducted. In this study, the expression level of nhr-76 in *C. elegans* was measured after knocking down *crm-1* using RNAi technology. After knocking down *nhr-76*, the expression level of *crm-1* was assessed in wild-type strain N2 by qPCR, and then in transgenic strain PHX5872 *crm-1* (syb5872) by an assessment of changes in fluorescence intensity. These experiments showed that nhr-76 could positively regulate the expression level of *crm-1* at the gene level.

RNAi technology was used to knock down the expression of *crm-1*. Changes in head thrash, touch avoidance and foraging behaviors were observed to verify whether *crm-1* was involved in the regulation of nervous system function. We found that down-regulating the expression level of *crm-1* gene could effectively alleviate the effects of ionizing radiation-induced head thrashes and touch avoidance. Therefore, it may regulate the function of the nervous system. Because the expression level of *crm-1* gene was shown to be regulated by *nhr-76* in a previous experiment, we conclude that both *nhr-76* and *crm-1* are involved in the regulation of ionizing radiation-induced nervous system dysfunction in *C. elegans*.

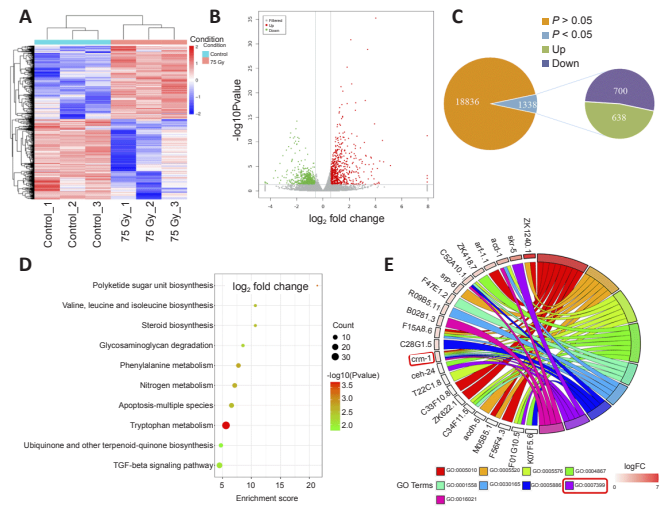In the present study, the change in expression level of *nhr-76* was not

statistically significant after knockdown of *crm-1*. However, the standard deviation is large, which may be because of the interference efficiency of *crm-1*. It may be that *crm-1* and *nhr-76* have other regulatory roles. In future studies, we aim to knock out *crm-1*, and then measure the expression level of *nhr-76*. Additionally, we will insert a reporter gene into *nhr-76*, creating a new transgenic animal to further study the role of this gene.

In conclusion, ionizing radiation exposure can inhibit behaviors such as head thrashes, touch avoidance, and foraging in *C. elegans*, as well as induce degeneration of dopamine neurons. Ionizing radiation exposure also induced
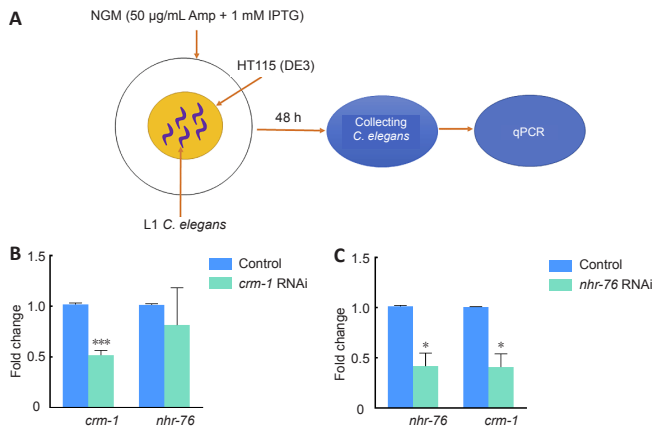
Figure 6 │ Interaction and RNAi efficiency between *crm-1* and *nhr-76* in *Caenorhabditis elegans* (*C. elegans*).

(A) RNAi workflow. (B) Changes in the expression levels of *crm-1* and *nhr-76* after interfering with *crm-1*. (C) Changes in the expression levels of *nhr-76* and *crm-1* after interfering with *nhr-76*. Data normalized by control group are expressed as mean ± SD. The experiment was repeated at least three times. *$P < 0.05$, ***$P < 0.001$ (independent sample *t*-test). Amp: Ampicillin; *crm-1*: cysteine rich motor neuron protein homolog; HT115(DE3): *Escherichia coli* HT115(DE3); IPTG: isopropyl-beta-D-thiogalactopyranoside; NGM: nematode growth media; *nhr-76*: nuclear hormone receptor family; qPCR: quantitative polymerase chain reaction; RNAi: ribonucleic acid interference.
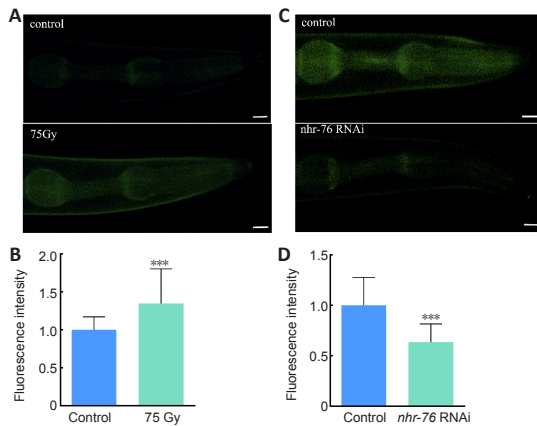

Figure 7 │ Fluorescence intensity changes after irradiation or knockdown of *nhr-76* in transgenic strain PHX5872 *crm-1* (syb5872).

(A, B) Fluorescence intensity changes in control group and radiation group. (C, D) Fluorescence intensity changes in control group and interference group. Scale bars: 10 μm. Data are expressed as mean ± SD (*n* = 30). The experiment was repeated at least three times. ***$P < 0.001$ (independent sample *t*-test). *nhr-76*: Nuclear hormone receptor family; RNAi: ribonucleic acid interference.

increases in expression of both crm-1 and *nhr-76* genes. After knocking down the expression of *crm-1* gene, the ionizing radiation-induced changes in head thrash and touch avoidance behaviors were improved. Because the expression level of *crm-1* gene is regulated by the nuclear receptor gene *nhr-76*, we conclude that the *nhr-76/crm-1* pathway may be involved in the regulation of ionizing radiation-induced nervous system dysfunction in *C. elegans*.

Author contributions: *NC and YT conceived and designed the experiment. HQL, SQH and JFH performed the experiments. JG analyzed the data. HQL wrote the article. HQL, JG, SQH and JFH contributed equally to this work. All authors approved the final version of this paper.*

Conflicts of interest: *The authors declare that there is no conflict of interests.*
Availability of data and materials: *All data generated or analyzed during this study are included in this published article and its supplementary information files.*
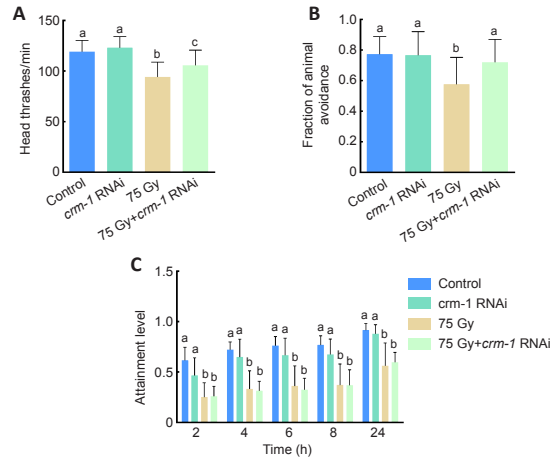
Figure 8 │ The effects of knockdown of *crm-1* gene expression on behavior of *Caenorhabditis elegans* (*C. elegans*).

(A) Head thrashes after *crm-1* knockdown and irradiation in *C. elegans* (*n* = 30). (B) Touch avoidance after knockdown of *crm-1* and irradiation in *C. elegans* (*n* = 30). (C) Foraging after knockdown of *crm-1* and irradiation in *C. elegans* (*n* = 20). Data are expressed as mean ± SD. The experiment was repeated at least three times. Letters a, b, c indicate the results of one-way analysis of variance followed by least significant difference *post hoc* test (head thrashes and foraging) or Dunnett's T3 *post hoc* test (touch avoidance). The same letter indicates that there was no statistical difference between the two samples, while different letters indicate that there was a statistical difference between the two samples. The significance level was *P* < 0.05. Foraging behavior was only compared at the same time point. *crm-1*: Cysteine rich motor neuron protein homolog; RNAi: ribonucleic acid interference.

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Additional file: *Open peer review reports 1 and 2.*

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