Environmental Science Nano

PAPER

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Cite this: *Environ. Sci.: Nano*, 2019, **6**, 2591

Neuronal damage induced by nanopolystyrene particles in nematode *Caenorhabditis elegans*†

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Received 25th April 2019,
Accepted 18th June 2019

DOI: 10.1039/c9en00473d

rsc.li/es-nano

The potential adverse effects of nanoplastics have gradually gained significant attention. Herein, we employed *Caenorhabditis elegans* to investigate the possible neurotoxic effects of nanopolystyrene particles on the development and function of D-type GABAergic motor neurons. Nanopolystyrene (1000 μg L^{-1}) induced the neurodegeneration of D-type motor neurons in wild-type nematodes, and nanopolystyrene (≥100 μg L^{-1}) further induced the neurodegeneration phenotype in nematodes with a mutation in *sod-3*, encoding a Mn-SOD or *acs-22*, which governs the functional state of the intestinal barrier. Meanwhile, nanopolystyrene (≥10 μg L^{-1}) decreased head thrash and body bend, and nanopolystyrene (≥100 μg L^{-1}) altered forward and backward movements in wild-type nematodes. Moreover, nanopolystyrene (≥1 μg L^{-1}) decreased head thrash and body bend and nanopolystyrene (≥10 μg L^{-1}) affected forward and backward movements in *sod-3* or *acs-22* mutant nematodes. Along with the neurotoxicity observed in nanopolystyrene-exposed nematodes, nanopolystyrene exposure induced a dynamic autophagy induction. RNAi knockdown of *lgg-1* encoding a key regulator of autophagy induced susceptibility to the neurotoxic effects of nanopolystyrene particles on the development and function of D-type motor neurons, implying the association of dynamic autophagy induction with the neurotoxicity induced by nanopolystyrene particles. Our results highlight the potential neurotoxicity of long-term nanoplastic exposure in organisms.

Environmental significance

The various toxicities due to nanoplastic particles have received increasing attention. However, the neurotoxicity of nanoplastic particles in environmental organisms is still largely unclear. We herein employed an assay based on the D-type GABAergic motor neurons of *Caenorhabditis elegans* to investigate the possible neurotoxic effects of prolonged exposure to nanopolystyrene particles on the development and functions of the nervous system. Our data suggest that prolonged exposure to nanopolystyrene particles has the potential to induce neurodegeneration phenotypes and to alter locomotion behaviors, which is largely due to the reduction in autophagy induction. Additionally, this neurotoxicity from nanopolystyrene particles could be further strengthened by the mutation of *sod-3*, encoding a mitochondrial Mn-SOD, or *acs-22*, which governs the functional state of the intestinal barrier. Our observations highlight the potential of nanoplastics to induce damage to both the development and function of the nervous system after long-term exposure.

Introduction

Microplastics, originating from man-made plastic products through physical, chemical and biological degradation, are an emerging contaminant in the environment. Microplastics are defined as plastic debris or plastics with particle size ≤5 mm.[1,2] The accumulating environmental monitoring data has demonstrated that these microplastics, especially the nanoplastics (microplastics with a nano-size), are pervasive in the environment and potentially threaten both environmental organisms and human health.[3–8] Considering the fact that the microplastics can be further degraded into nanoplastics,[9] the adverse effects of nanoplastics have gradually received increasing attention.[10–14] Exposure to nanoplastics, such as nanopolystyrene, could cause toxicity in organisms in various ways, including through the induction of oxidative stress, reduction in growth and reproduction, and deficiency in development.[10–14] Nevertheless, the potential neurotoxic effects of nanoplastics on environmental organisms is still largely unclear.

Caenorhabditis elegans has been considered a wonderful system for assessing responses to various environmental toxicants, including nanomaterials and nanoplastics.[15–19] Nanopolystyrene exposure has been observed to at least cause intestinal damage, reduction in brood size, and induction of oxidative stress in nematodes.[20–22] *C. elegans* could be further employed to evaluate the possible toxicity of nanopolystyrene particles at predicted environmental concentrations.[23–25] Additionally, the mutation of *sod-3* or *acs-22* could induce a

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9en00473d

susceptibility to the toxic effects of nanopolystyrene particles in nematodes.[23,24]

C. elegans is useful for determining the toxic effects of environmental toxicants on the development and function of neurons.[26] In nematodes, D-type GABAergic motor neurons present a widely used assay system for assessing the neuronal damage of certain environmental toxicants.[27,28] In this study, we employed *C. elegans* to investigate the potential neuronal damage from nanopolystyrene particles on the development and function of D-type GABAergic motor neurons in wild-type and mutant animals. Our results demonstrated that long-term exposure to nanopolystyrene particles could potentially cause neurotoxicity affecting both the development and function of D-type motor neurons in nematodes. Evidence has shown that autophagy induction is helpful for protecting organisms from the neurotoxicity caused by environmental toxicants.[29–31] Additionally, we found that the observed neurotoxicity of nanopolystyrene on the development and function of D-type motor neurons was closely associated with a suppression in autophagy induction. Our data highlight the potential of long-term exposure to nanoplastics to induce neuronal damage in organisms.

Experimental

Characterizations of nanopolystyrene

Nanopolystyrene was obtained from Janus New-Materials Co. (Nanjing, China). The size of the used nanopolystyrene was 102.8 ± 4.5 nm based on analysis by transmission electron microscopy (TEM) (Fig. S1†). Nanopolystyrene particles were well dispersed, and no aggregation was observed in K medium for at least three days. The size of nanopolystyrene particles was confirmed by dynamic light scattering (DLS, 101.6 ± 3.7 nm) using Nano Zetasizer (Malvern Instrument Ltd., Malvern, UK). The zeta potential of nanopolystyrene particles was −9.698 ± 0.966 mV.[26] Working solutions (1, 10, 100, and 1000 μg L^{-1}) of nanopolystyrene particles were prepared by diluting a stock solution (1 mg mL^{-1}) with K medium.

Strains and maintenance

The used strains contained wild-type N2, mutants of *sod-3*(*gk235*) and *acs-22*(*tm3290*), and transgenic strains of *oxIs12*[*unc-47p::GFP* + *lin-15*(+)] and *adIs2122*[LGG-1::GFP + *rol-6*(*su1006*)]. *oxIs12* is a transgenic strain for visualizing D-type GABAergic motor neurons.[27] LGG-1::GFP is a marker for assessing the autophagosome.[32] The nematodes were normally grown on nematode growth media (NGM) plates fed with a lawn of *Escherichia coli* OP50.[33] The collected gravid nematodes were lysed using a bleaching mixture solution (0.45 M NaOH, 2% HOCl), and the released eggs were used to prepare age synchronous L1-larvae.

Exposure to nanopolystyrene particles

Prolonged exposure to nanopolystyrene particles was performed from L1-larvae to adult day-1 in liquid solutions with the addition of OP50 (~4 × 10^6 colony-forming units (CFUs)).[20] The nanopolystyrene solutions and *E. coli* OP50 were refreshed daily.

Analysis of the development of D-type GABAergic motor neurons

The GABA nervous system contains D-type DD and VD motor neurons (19 neurons) innervating the body muscles in nematodes. To analyze the development of D-type GABAergic motor neurons, the corresponding fluorescence images of *oxIs12* nematodes were captured using fluorescence microscopy (Ti-E, Nikon Co., Japan). The number of both dorsal and ventral nerve cords and neuronal loss were used as endpoints to assess the damage to neuronal development. For each treatment, forty nematodes were examined.

Locomotion behavior assay

Four forms of behavior (head thrash, body bend, forward movement, and backward movement) were used to assess the locomotion behavior in nematodes.[26,34] Locomotion behavior reflects the functional state of motor neurons. Before the locomotion behavior analysis, the examined nematodes were washed with M9 buffer and transferred onto a new NGM agar plate without *E. coli* OP50 to undergo recovery for a 1 min recovery period. A head thrash is defined as a change in the direction of bending at the mid-body. A body bend is counted as a change in the direction of the posterior bulb part along the *y* axis, assuming that the nematodes are traveling along the *x* axis. A forward movement is defined as a sine crawl in the direction of head. A backward movement is defined as the sine crawl in the direction of the tail. For each treatment, forty nematodes were examined.

Analysis of positive autophagy puncta

Fluorescence images of *adIs2122* nematodes were captured using fluorescence microscopy with image-pro insight processing. The number of LGG-1::GFP positive puncta was calculated by measuring at least 15 intestinal cells. For each treatment, thirty nematodes were examined.

Reverse-transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

After total RNA isolation from the nematodes using Trizol and cDNA synthesis, qRT-PCR reactions were carried out in 10 μL volumes using Evagreen in an ABI 7500 real-time PCR system. Reactions were performed on samples in triplicate. The relative quantification of the examined genes in comparison to the reference gene *tba-1* encoding a tubulin was determined. Table S1† shows the detailed primer information.

RNAi assay

The L1-larvae were fed with *E. coli* HT115 (DE3) expressing double-stranded RNA for a certain gene on the RNAi plates to let them develop into gravid adults, as

described.[35] The gravid adults were then transferred onto a fresh RNAi plate to obtain the second generation for toxicity assessment. HT115 bacteria harboring empty vector L4440 were used as RNAi control. RNAi efficiency was confirmed by qRT-PCR (data not shown).

DNA constructs and transformation

The promoter of *hlh-30* was amplified using the whole genome of wild-type nematodes. The purified PCR product was inserted into vector pPD95_77, and the amplified *hlh-30*/

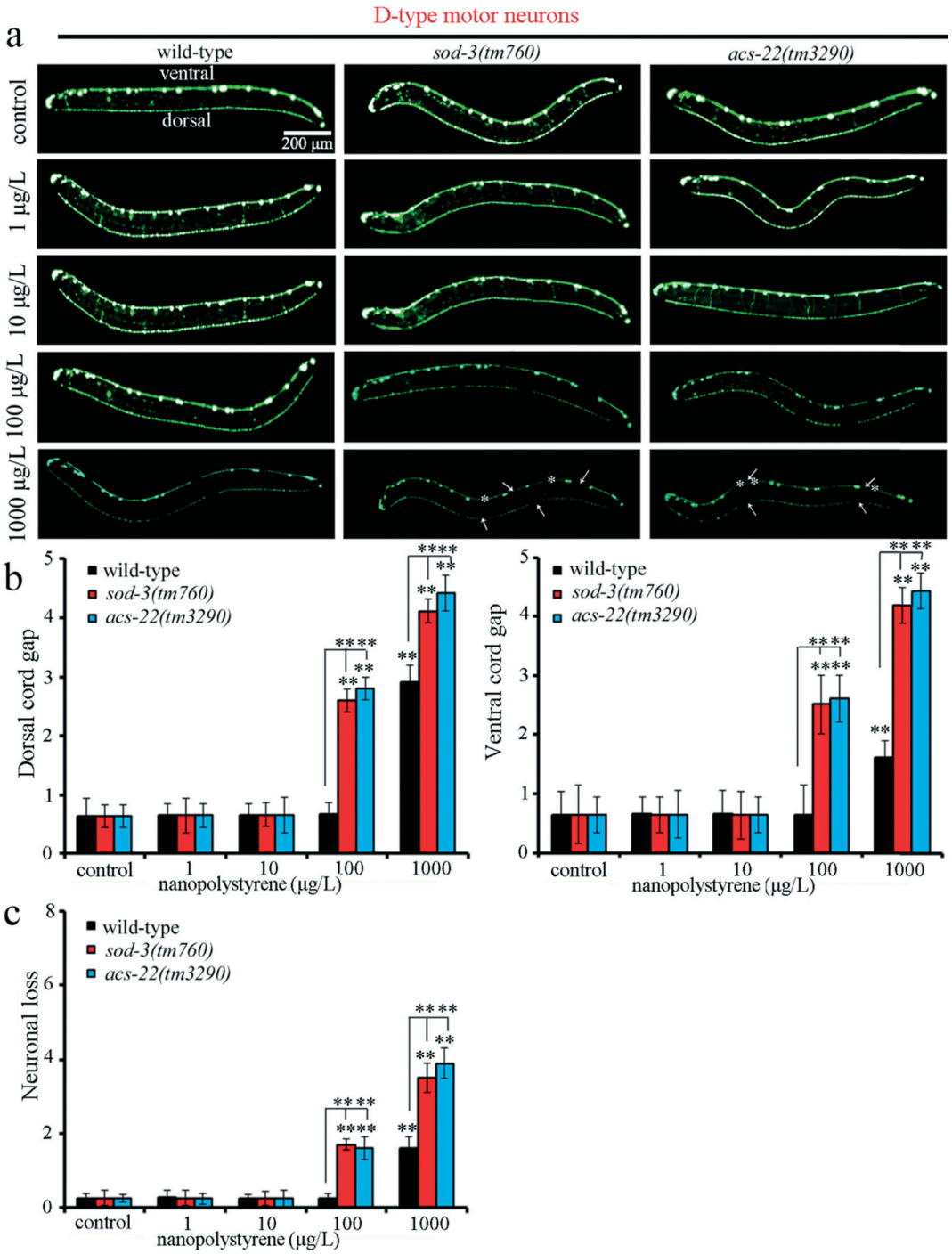

Fig. 1 Neuronal damage to the development of D-type GABAergic motor neurons by nanopolystyrene particles. (a) Images showing the development of D-type motor neurons in nematodes exposed to nanopolystyrene particles. Asterisks indicate the neuronal loss, and arrowheads indicate the gap formation on the ventral or dorsal cord. (b) Comparison of gap formation in both the ventral and dorsal cords of D-type motor neurons. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). (c) Comparison of neuronal loss of D-type motor neurons. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). The exposure concentrations of nanopolystyrene particles were 1–1000 μg L^{-1}. Prolonged exposure was performed from L1-larvae to adult day-1.

W02C12.3 cDNA was further inserted into the vector pPD_95_77 carrying *hlh-30* promoter. Germline transformation was conducted by coinjecting a testing DNA (10–40 μg mL^{-1}) and a marker DNA of P*dop-1::rfp* (60 μg mL^{-1}) into the gonad.[36] Primer information for DNA constructions is shown in Table S2.†

Statistical analysis

Statistical analysis was performed by SPSS 12.0 (SPSS Inc., Chicago, USA). All parameters used were continuous variables and the Agostino *D* test was used to check normality before the parameter statistics. Differences between groups were tested using one-way analysis of variance (ANOVA), and the differences were checked using a *post hoc* multiple comparison. For the multiple factor comparison, two-way ANOVA analysis was performed. Probability levels of 0.05 and 0.01 were considered statistically significant.

Results

Neuronal damage affecting the development of D-type GABAergic motor neurons caused by nanopolystyrene particles

Using transgenic strain *oxIs12* with the D-type motor neurons labeled, we first investigated the effects of prolonged exposure to nanopolystyrene particles on the development of D-type motor neurons. Under the wild-type background, only a high concentration of nanopolystyrene particles could noticeably affect the development of D-type motor neurons. Among the examined concentrations, only prolonged exposure to nanopolystyrene particles (1000 μg L^{-1}) caused the severe formation of gaps in both the ventral and dorsal cords and neuronal loss under the wild-type background (Fig. 1).

In nematodes, the mutation of *sod-3* encoding a mitochondrial Mn-SOD protein induced susceptibility to the toxicity of nanopolystyrene particles.[24] Additionally, *acs-22* mutant nematodes, which have a deficit in the intestinal barrier, also showed susceptibility to the toxicity of nanopolystyrene particles.[23] We further found that the mutation of *sod-3* or *acs-22* induced susceptibility to the neurotoxicity of nanopolystyrene particles affecting the development of D-type motor neurons. On the one hand, we observed a severe formation of gaps on both the ventral and dorsal cords and neuronal loss in *sod-3* or *acs-22* mutant nematodes exposed to nanopolystyrene particles at concentrations of both 100 μg L^{-1} and 1000 μg L^{-1} (Fig. 1). On the other hand, the formation of more severe gaps on both the ventral and dorsal cords and neuronal loss could be detected under exposure to nanopolystyrene particles (1000 μg L^{-1}) in *sod-3* or *acs-22* mutant nematodes compared with those observed under the wild-type background (Fig. 1).

Neuronal damage to locomotion behaviors by nanopolystyrene particles

The functional state of motor neurons are reflected by different locomotion behaviors in animals.[25] The head thrash, body bend, forward movement, and backward movement were used to determine the possible effects of nanopolystyrene exposure on locomotion behavior in nematodes. In wild-type nematodes, prolonged exposure to nanopolystyrene at concentrations ≥10 μg L^{-1} could significantly decrease both the head thrash and the body bend (Fig. 2a). Additionally, in wild-type nematodes, prolonged exposure to nanopolystyrene at concentrations ≥100 μg L^{-1} could significantly decrease the forward movement frequency and increase the backward movement frequency (Fig. 2b). Therefore, nanopolystyrene particles also potentially damage the functional state of motor neurons.

Moreover, we found that the mutation of *sod-3* or *acs-22* also induced susceptibility to the neurotoxic effects of nanopolystyrene particles on locomotion behaviors. On the one hand, we detected the significant decrease in both the head thrash and body bend in *sod-3* or *acs-22* mutant nematodes exposed to nanopolystyrene particles at concentrations ≥1 μg L^{-1} (Fig. 2a). Additionally, we detected a significant decrease in the forward movement frequency and increase in the backward movement frequency in *sod-3* or *acs-22* mutant nematodes exposed to nanopolystyrene particles at concentrations ≥10 μg L^{-1} (Fig. 2b). On the other hand, a more severe decrease in both the head thrash and body bend could be observed upon exposure to nanopolystyrene particles (10–1000 μg L^{-1}) in *sod-3* or *acs-22* mutant nematodes compared with those in wild-type nematodes (Fig. 2a). Additionally, a more severe decrease in the forward movement frequency and increase in backward movement frequency were found upon exposure to nanopolystyrene particles (100–1000 μg L^{-1}) in *sod-3* or *acs-22* mutant nematodes compared with those in wild-type nematodes (Fig. 2b).

Dynamic autophagy induction in nematodes exposed to nanopolystyrene particles

Autophagy induction has an important function in the response of nematodes to environmental stresses.[37,38] Along with the induction of neurotoxicity in nematodes exposed to nanopolystyrene particles, we observed a dynamic autophagy induction in nematodes exposed to nanopolystyrene particles. LGG-1::GFP is a marker of autophagy induction,[32] and LGG-1 is expressed in multiple tissues.[39] We observed a significant increase in the number of LGG-1::GFP positive puncta of wild-type nematodes exposed to nanopolystyrene particles (10 μg L^{-1}) compared with the controls (Fig. 3a). In contrast, the observed increase in the number of LGG-1::GFP positive puncta in nanopolystyrene (10 μg L^{-1})-exposed nematodes was significantly inhibited by exposure to higher levels of nanopolystyrene particles (1000 μg L^{-1}) (Fig. 3a).

In nematodes, LGG-1, LGG-2, ATG-18, and BEC-1 are key regulators of autophagy induction.[32,40] *lgg-1* encodes an ortholog of human GABARAP and GABARAPL1; *lgg-2* encodes an ortholog of human MAP1LC3A and MAP1LC3B; *atg-18* encodes an ortholog of human WIPI1 and WIPI2; and *bec-1* encodes an ortholog of human BECN-1. Similarly, we detected a

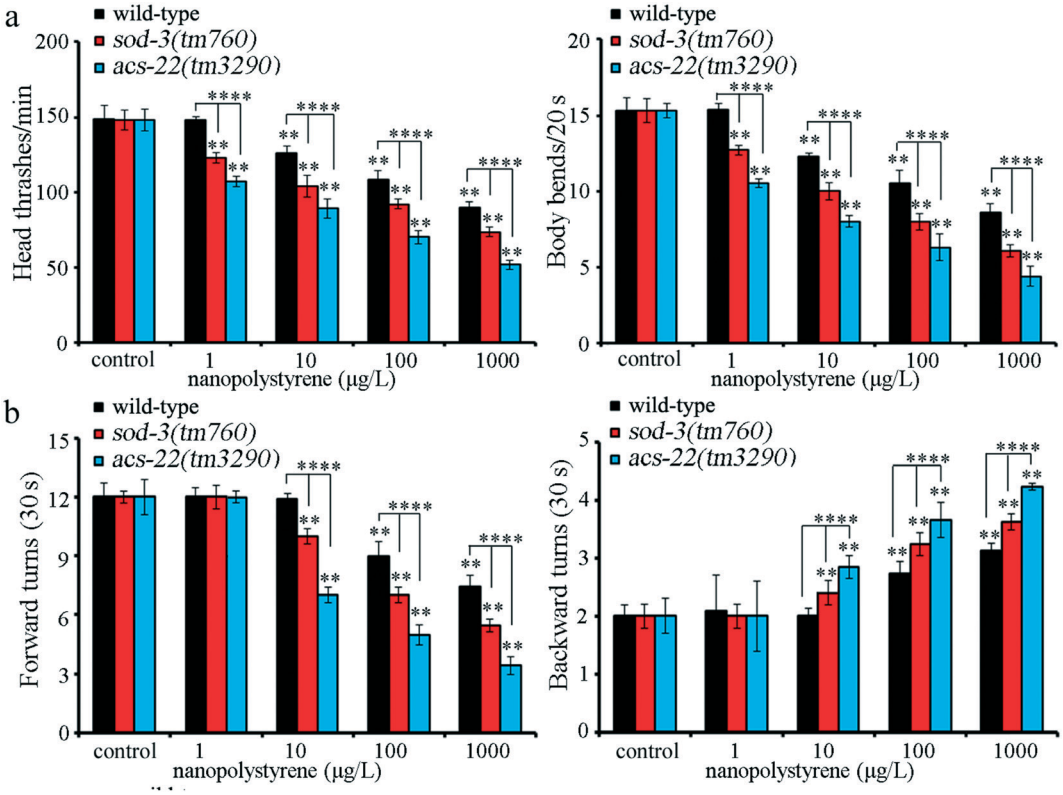

Fig. 2 Neuronal damage to locomotion behaviors by nanopolystyrene particles in wild-type and mutant nematodes. (a) Effects of nanopolystyrene exposure on head thrash and body bend in wild-type and mutant nematodes. (b) Effects of nanopolystyrene exposure on the forward movement and backward movement in wild-type and mutant nematodes. Exposure concentrations of nanopolystyrene particles were 1–1000 μg L^{-1}. Prolonged exposure was performed from L1-larvae to adult day-1. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated).

significant increase in the expression of *lgg-1*, *lgg-2*, *atg-18*, and *bec-1* in wild-type nematodes exposed to nanopolystyrene particles (10 μg L^{-1}) compared with the controls (Fig. 3b). However, the increase in the expression of these four genes in nanopolystyrene (10 μg L^{-1})-exposed nematodes was further significantly suppressed by exposure to higher concentrations of nanopolystyrene particles (1000 μg L^{-1}) (Fig. 3b).

Mutation of *sod-3* or *acs-22* caused a more severe decrease in autophagy induction in nematodes exposed to nanopolystyrene

As introduced above, nanopolystyrene particles at high concentrations, such as 1000 μg L^{-1}, could induce both neuronal damage on the development and function of D-type motor neurons and a decrease in autophagy induction. We next examined the effects of a *sod-3* or *acs-22* mutation on autophagy induction in nematodes exposed to nanopolystyrene (1000 μg L^{-1}). We observed a more severe decrease in the expression of *lgg-1*, *lgg-2*, *atg-18*, and *bec-1* in nanopolystyrene (1000 μg L^{-1})-exposed *sod-3* or *acs-22* mutant nematodes compared with those in nanopolystyrene (1000 μg L^{-1})-exposed wild-type nematodes (Fig. 3c). Additionally, exposure to nanopolystyrene particles (1000 μg L^{-1}) induced a more severe decrease in the number of LGG-1::GFP positive puncta in *sod-3* or *acs-22* mutant nematodes compared with that in wild-type nematodes (Fig. 3d). Therefore, the mutation of *sod-3* or *acs-22* resulted in a more severe decrease in autophagy induction in nematodes exposed to nanopolystyrene (1000 μg L^{-1}).

RNAi knockdown of *lgg-1* induced susceptibility to neurotoxicity from nanopolystyrene particles

To determine the association between autophagy induction and the neurotoxicity of nanopolystyrene particles, we performed an RNAi knockdown of *lgg-1* in a transgenic strain of *oxIs12*. Without nanopolystyrene exposure, the RNAi knockdown of *lgg-1* could not induce gap formation on the ventral and dorsal cords or neuronal loss (Fig. 4a). However, RNAi knockdown of *lgg-1* induced the severe formation of gaps on both the ventral and dorsal cords and neuronal loss in nanopolystyrene (100 g L^{-1})-exposed nematodes; however, no obvious damage was observed in the development of D-type motor neurons in nanopolystyrene (100 g L^{-1})-exposed wild-type nematodes (Fig. 4a). Therefore, the RNAi knockdown of *lgg-1* induced a susceptibility to neurotoxic effects from nanopolystyrene particles on the development of D-type motor neurons.

Moreover, we found that RNAi knockdown of *lgg-1* resulted in a more severe decrease in the head thrash, body bend, and forward movement frequency and an increase in the backward movement frequency compared with nematodes without *lgg-1*

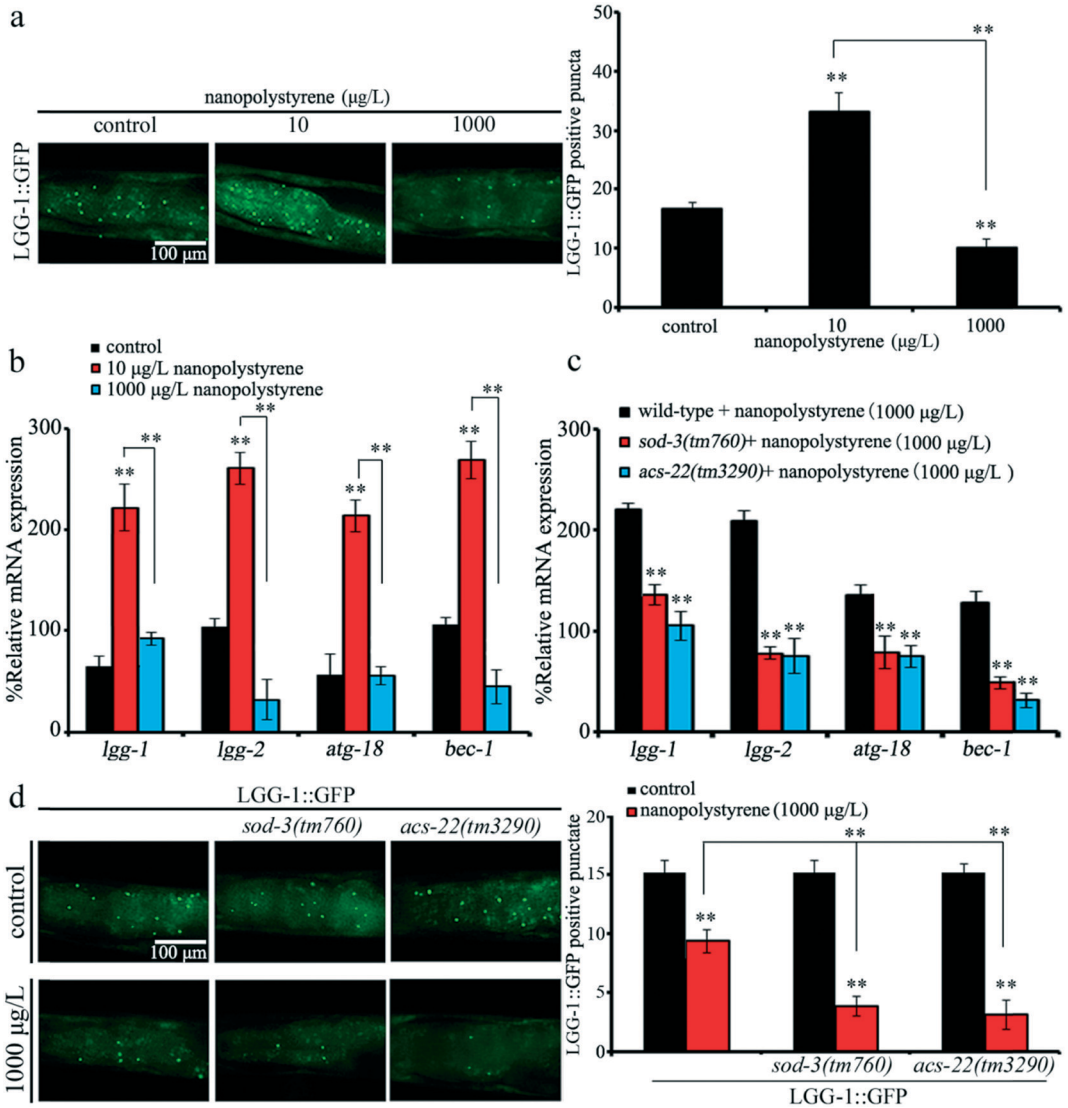

Fig. 3 Dynamic autophagy induction in wild-type and mutant nematodes exposed to nanopolystyrene particles. (a) LGG-1::GFP positive puncta in intestinal cells of nematodes exposed to nanopolystyrene particles. Exposure concentrations of nanopolystyrene particles were 10 and 1000 μg L^{-1}. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control. (b) Effects of nanopolystyrene exposure on the expression of several genes required for autophagy control. Exposure concentrations of nanopolystyrene particles were 10 and 1000 μg L^{-1}. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). (c) Expression of several genes required for autophagy control in wild-type and mutant nematodes exposed to nanopolystyrene particles. Exposure concentration of nanopolystyrene particles was 1000 μg L^{-1}. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* wild-type. (d) LGG-1::GFP positive puncta in intestinal cells of *sod-3* or *acs-22* mutant nematodes exposed to nanopolystyrene particles. Exposure concentration of nanopolystyrene particles was 1000 μg L^{-1}. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). Prolonged exposure was performed from L1-larvae to adult day-1.

RNAi knockdown after nanopolystyrene (100 g L^{-1}) exposure (Fig. 4b). Therefore, RNAi knockdown of *lgg-1* also induced susceptibility to the neurotoxic effects of nanopolystyrene particles on the function of D-type motor neurons.

Overexpression of HLH-30 induced resistance to the neurotoxic effects of nanopolystyrene particles

In nematodes, HLH-30/TFEB is a MiT transcription factor. The transcription factor HLH-30 governs autophagy induction.[41–44] Under conditions of stress, the overexpression of HLH-30 can maintain a high level of autophagy induction, including high expression of LGG-1::GFP.[41] Under normal conditions without nanopolystyrene exposure, the overexpression of HLH-30 did not affect the development of D-type motor neurons or locomotion behaviors (head thrash, body bend, forward movement, and backward movement) (Fig. 5). HLH-30 overexpression effectively suppressed the neurodegeneration of D-type motor neurons under exposure to nanopolystyrene (1000 μg L^{-1}) in *Is*(P*hlh-30-hlh-30*) nematodes (Fig. 5a). Additionally, HLH-30 overexpression also effectively inhibited the damage of nanopolystyrene (1000 μg L^{-1}) to locomotion behaviors, such as the decrease in head thrash, body bend, and forward movement

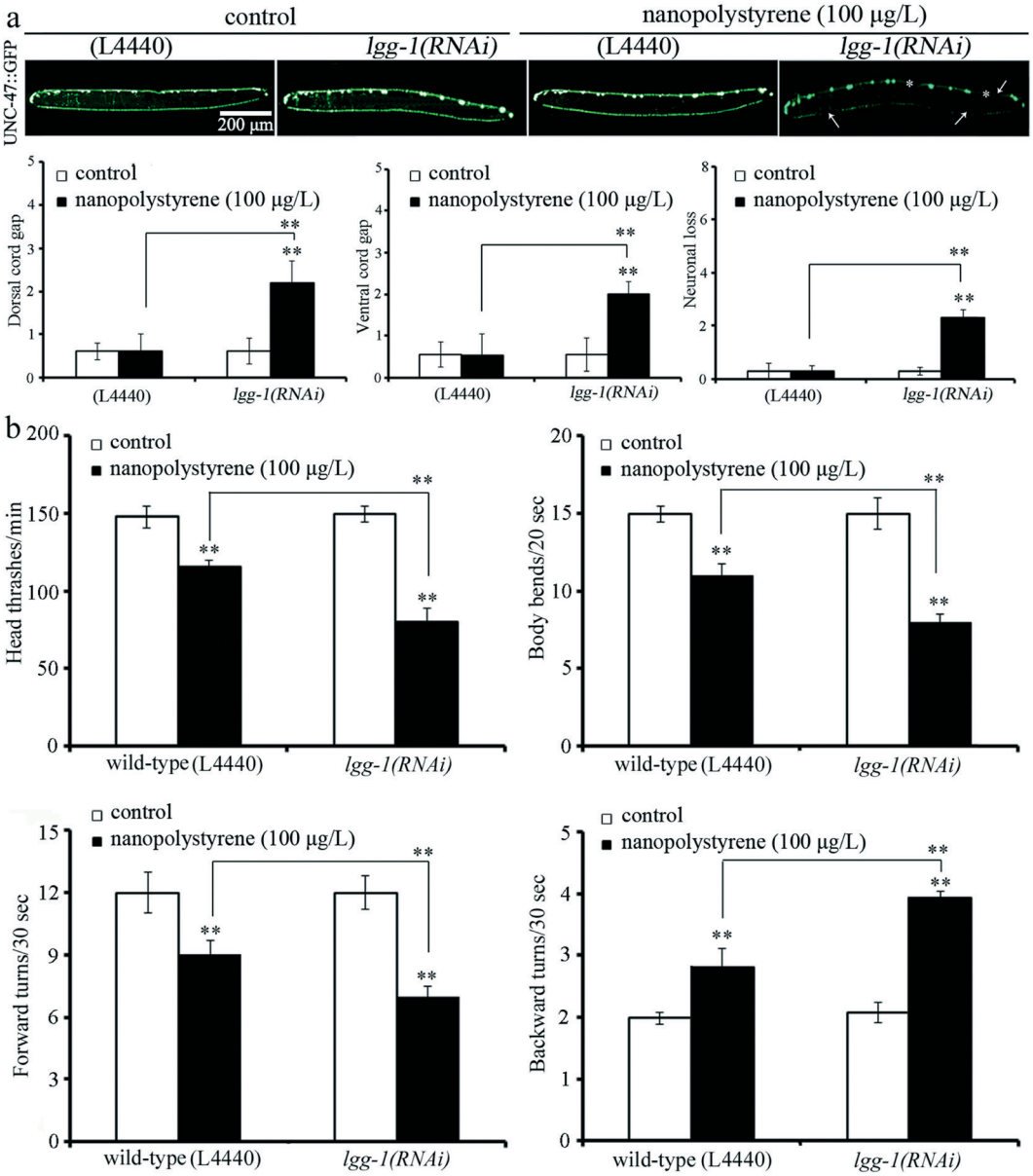

Fig. 4 Susceptibility to the neurotoxic effects of nanopolystyrene particles on the development and function of D-type GABAergic motor neurons in *lgg-1*(*RNAi*) nematodes. (a) Effect of RNAi knockdown of *lgg-1* on the development of D-type motor neurons in nanopolystyrene-exposed nematodes. Asterisks indicate the neuronal loss, and arrowheads indicate the gap formation on the ventral or dorsal cord. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). (b) Effect of RNAi knockdown of *lgg-1* on locomotion behaviors in nanopolystyrene-exposed nematodes. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). Exposure concentration of nanopolystyrene particles was 100 μg L^{-1}. Prolonged exposure was performed from L1-larvae to adult day-1.

frequency and the increase in backward movement frequency in *Is*(P*hlh-30-hlh-30*) nematodes (Fig. 5b). In nanopolystyrene (1000 μg L^{-1})-exposed nematodes, overexpression of HLH-30 clearly prevented the decrease in LGG-1::GFP expression (data not shown).

Nanopolystyrene particles did not accumulate in D-type motor neurons after exposure

To assess the co-localization of nanopolystyrene particles with D-type motor neurons, we employed nanopolystyrene (100 nm) with a fluorescent dye (rhodamine B, red signal) enveloped in the particles. The related physicochemical properties of this polystyrene has been described in a previous report.[20] After the exposure of the transgenic strain *oxIs12* to this nanopolystyrene (1000 μg L^{-1}), we did not observe any obvious co-localization between the red and green signals (Fig. S2†), suggesting that the nanopolystyrene particles were not accumulating in the D-type motor neurons after exposure. In the transgenic strain *oxIs12* with RNAi knockdown of *sod-3* or *acs-22*, we also failed to observe any obvious co-localization between the red and green signals

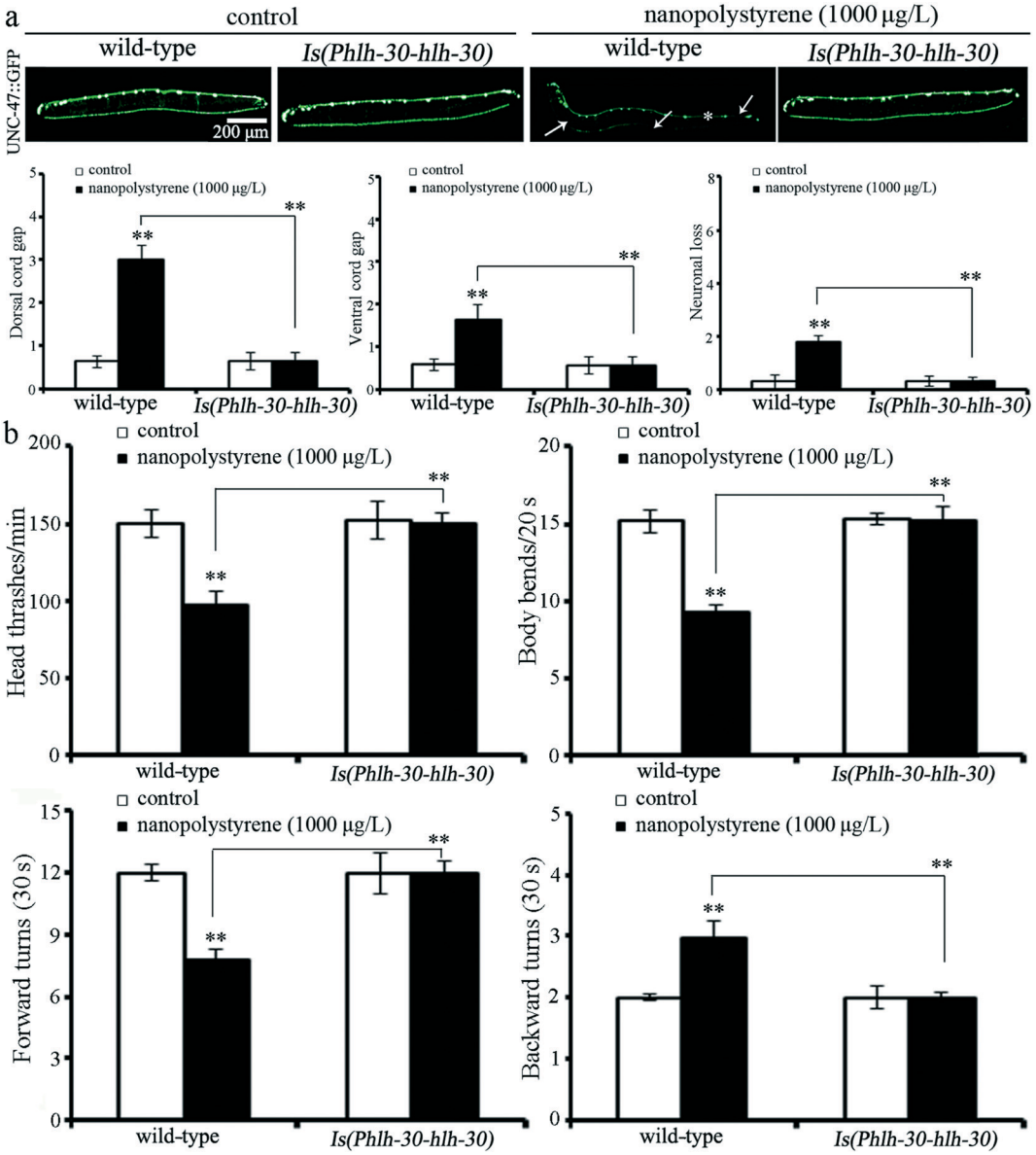

Fig. 5 Resistance to the neurotoxic effects of nanopolystyrene particles on the development and function of D-type GABAergic motor neurons in nematodes overexpressing HLH-30. (a) Effect of HLH-30 overexpression on the development of D-type motor neurons in nanopolystyrene exposed nematodes. Asterisks indicate the neuronal loss, and arrowheads indicate the gap formation on the ventral or dorsal cord. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specifically indicated). (b) Effect of HLH-30 overexpression on locomotion behaviors in nanopolystyrene-exposed nematodes. Bars represent means ± SD. $^{**}P < 0.01$ *vs.* control (if not specially indicated). Exposure concentration of nanopolystyrene particles was 1000 μg L^{-1}. Prolonged exposure was performed from L1-larvae to adult day-1.

after exposure to nanopolystyrene (1000 μg L^{-1}) (data not shown).

Neurotoxic effects of polystyrene on nematodes is size-dependent

We further employed head thrash, body bend, forward movement frequency, and backward movement frequency as endpoints to compare the neurotoxicity of polystyrene with different sizes. We found that polystyrene (10 or 100 μm) exhibited less neurotoxicity on the head thrash, body bend, and forward/backward movements of nematodes compared with nanopolystyrene (100 nm) (Fig. S3†). Exposure to polystyrene (10 μm) at concentrations ≥1 mg L^{-1} and 10 mg L^{-1} polystyrene (100 μm) caused a significant decrease in both the head thrash and body bend (Fig. S3a and b†). Exposure to 10 mg L^{-1} polystyrene (10 μm) resulted in a significant decrease in the forward movement frequency and increase in the backward movement frequency, and exposure to polystyrene (100 μm) at the examined concentrations did not affect either the forward movement or backward movement (Fig. S3c and d†). Therefore, the neurotoxicity of polystyrene on nematodes was size-dependent. The properties of polystyrene (10 or 100 μm) are shown in Fig. S4.†

Discussion

Previous studies have suggested that exposure to microplastics, including nanopolystyrene, could alter some forms of behavior, such as feeding, ingestion, excretion, and predation.[45–48] In this study, we found that prolonged exposure to nanopolystyrene also altered some forms of locomotion behaviors, such as head thrash, body bend, forward movement, and backward movement (Fig. 2). The observed decrease in forward movement and increase in backward movement implies a possible avoidance tendency or deficit in choice in nanopolystyrene-exposed nematodes.[26,49] More importantly, we observed that prolonged exposure to nanopolystyrene particles had the potential to induce neurodegeneration phenotypes, such as gap formation in both the ventral and dorsal cords and neuronal loss (Fig. 1). Therefore, long-term exposure to nanoplastic particles potentially not only alters behaviors including locomotion but also induces a deficit in neuronal development, such as neurodegeneration. Considering polystyrene with a μm size showed less neurotoxicity to nematodes compared with nanopolystyrene (Fig. S3†), we herein mainly focused on nanopolystyrene (100 nm) to determine its neurotoxicity in nematodes.

The predicted environmentally relevant concentrations of nanoplastic particles (100 nm) are in the range of ≤1 μg L^{-1}.[50] Our data suggest that prolonged exposure to nanopolystyrene (1 μg L^{-1}) could cause a significant decrease in head thrash and body bend in *sod-3* or *acs-22* mutant nematodes (Fig. 2a). Nevertheless, prolonged exposure to nanopolystyrene (1 μg L^{-1}) could not obviously affect the forward and backward movements (Fig. 2b). These observations indicate that prolonged exposure to nanopolystyrene at the predicted environmentally relevant concentration (1 μg L^{-1}) can potentially cause neurotoxic effects on the function of D-type motor neurons in *sod-3* or *acs-22* mutant nematodes, and this neurotoxicity can be detected with relatively sensitive locomotion behavior endpoints. Meanwhile, prolonged exposure to nanopolystyrene (1 μg L^{-1}) did not noticeably affect the development of D-type motor neurons (Fig. 1), suggesting that prolonged exposure to nanopolystyrene at predicted environmentally relevant concentrations (1 μg L^{-1}) is not enough to induce neurotoxicity in the development of D-type motor neurons.

In nematodes, the dynamics of autophagy induction is closely associated with the stress response, and autophagy induction is widely considered as a protective response to environmental stresses.[37,38] In this study, along with the neurotoxicity of nanopolystyrene, we observed the corresponding dynamics of autophagy induction. In nanopolystyrene (1000 μg L^{-1})-exposed wild-type nematodes, we observed severe neurotoxic effects on the development and function of D-type motor neurons and a significant reduction in autophagy induction (Fig. 3a and b). Additionally, in nanopolystyrene (1000 μg L^{-1})-exposed *sod-3* or *acs-22* mutant nematodes, we observed more severe neurotoxic effects on the development and function of D-type motor neurons and the reduction in autophagy induction compared with wild-type nematodes (Fig. 3c and d). Moreover, RNAi knockdown of *lgg-1* encoding a key regulator of autophagy induced susceptibility to the neurotoxic effects of nanopolystyrene particles on the development and function of D-type motor neurons (Fig. 4). Especially, the overexpression of HLH-30 could suppress the damage to the development and functions of D-type motor neurons (Fig. 5) and a decrease in LGG-1::GFP in nanopolystyrene-exposed nematodes. Therefore, our results imply that the observed severe neurotoxic effects of nanopolystyrene on the development and function of D-type motor neurons is largely due to a reduction in autophagy induction in nematodes.

In nematodes exposed to nanopolystyrene at the examined concentrations (1–1000 μg L^{-1}), we did not observe obvious translocation or accumulation of nanopolystyrene particles in D-type motor neurons (Fig. S2†), suggesting that the observed neurotoxicity may not be directly due to the accumulation of nanopolystyrene particles in D-type motor neurons. This result implies that some still unknown toxic compounds or metabolites may be released and accumulate in the D-type motor neurons in nematodes exposed to nanopolystyrene at high concentrations. However, our previous study indicated that the leachate of nanopolystyrene (1000 μg L^{-1}) could not cause obvious toxic effects on locomotion behavior.[20] Thus, the release and accumulation of still unknown toxic metabolites may be closely associated with the observed neurotoxicity in nanopolystyrene-exposed nematodes. The dysregulation of autophagy induction may activate or enhance the release of still unknown toxic metabolites in nanopolystyrene-exposed nematodes.

In nanopolystyrene (10 μg L^{-1})-exposed wild-type nematodes, we observed both a significant decrease in the head thrash and body bend and obvious autophagy induction (Fig. 3a and b), which suggests that prolonged exposure to nanopolystyrene (10 μg L^{-1}) activates the autophagy induction-mediated protective response. Nevertheless, the autophagy induction-mediated protective response might not be sufficient to counteract the neurotoxic effects of nanopolystyrene (10 μg L^{-1}) on head thrash and body bend in nematodes.

Conclusions

In this study, we employed the animal model of *C. elegans* to examine the possible neurotoxicity of prolonged exposure to nanopolystyrene particles affecting the development and function of D-type motor neurons. Our results demonstrate that exposure to nanopolystyrene particles could potentially cause neuronal damage to both the development and function of D-type motor neurons in nematodes. Additionally, the observed neurotoxic effects of nanopolystyrene particles on inducing neurodegeneration and altering locomotion behaviors were further enhanced by *sod-3* or *acs-22* mutation. Moreover, the observed neurotoxicity of nanopolystyrene particles was largely due to the dynamic alteration in autophagy

induction in nanopolystyrene-exposed nematodes. Our data imply the potential neurotoxicity of nanoplastic particles after long-term exposure in organisms.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was supported by the grant of Scientific Research Foundation of Graduate School of Southeast University (YBPY1945).

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