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Glutamatergic transmission associated with locomotion-related neurotoxicity to lindane over generations in *Caenorhabditis elegans*

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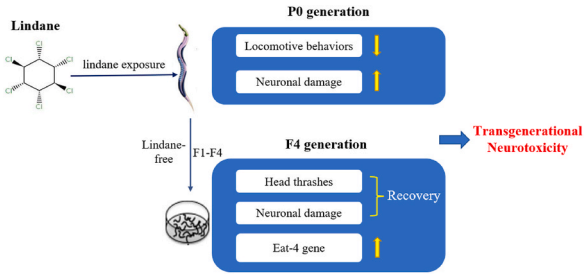
HIGHLIGHTS

- Neuronal damage was observed following exposure to lindane at 10–100 ng/L.
- Lindane exposure could transfer its severe neurotoxicity to offsprings.
- The expression of *eat-4* continued to be regulated from P0 to F4 generation.
- Glutamate transmission may contribute to neurotoxicity of lindane over generations.

GRAPHICAL ABSTRACT



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ABSTRACT

Organochlorine pesticide lindane in the environment and biota results in the potential risks on ecosystem and human health. Lindane can adversely affect the locomotion and nervous system, yet the potential neurotoxicity of lindane over generations remains uncertain. In this study, the neurotoxicity and underlying mechanisms in *Caenorhabditis elegans* (*C. elegans*) were investigated after parental (P0) exposure to lindane at environmentally relevant concentrations over generations. Exposure to lindane at concentrations of 10–100 ng/L significantly decreased body bends and head thrashes in P0 generation. Significant decrease of fluorescence labeled different neurotransmitters, and clear morphological changes by exposure to lindane at 10–100 ng/L suggested that lindane could induce the neuronal damage in *C. elegans*. During the transgenerational process, decreased locomotive behaviors were also observed in F1–F3 generations, and head thrashes returned to normal levels in F4 generation. Moreover, lindane exposure down-regulated the expression of *dat-1*, *dop-1*, *glr-1* and *mod-1*genes, while up-regulated *unc-30* gene in P0 generation, which recovered to normal levels in F4 generation. Interestingly, *eat-4* continued to be regulated from inhibition to stimulation in P0–F4 generations, suggesting that glutamatergic transmission may more contribute to the neurotoxicity of lindane over generations.

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1. Introduction

Organochlorine pesticides (OCPs), such as hexachlorocyclohexane (HCH) and dichloro-diphenyl-trichloroethane, have been produced and used globally as second-generation pesticides since 1990s (Magliano et al., 2014). For example, HCH was regarded as a promising pesticide in fields of pest control and prevention (Li, 1999). However, as the scope of OCPs applications widened, most were discovered to threaten the ecological environment and human health and subsequently added to the list of persistent organic pollutants (POPs) (Stockholm, 2010). Like other POPs, OCPs have unique properties, such as poor aqueous solubility, difficulty of degradation, transportation over a large distance, and high lipophilicity, which result in potential toxicity in the ecosystem (Nandan and Nimila, 2012; Temoka et al., 2016; Madaj et al., 2017; Trukhin and Boyarova, 2019). Epidemiological studies have indicated that OCPs exposure plays an crucial role in the etiology of neurodegenerative diseases, and the neurotoxic effects can influence in subsequent generations (Jurewicz and Hanke, 2008; Saravi and Dehpour, 2016).

Lindane (γ-HCH), a typical OCP, has caused pollution in a variety of environmental matrices, such as water, soil, and sediment (Kathleen Walker et al., 1999; Walker et al., 1999; Fang et al., 2017). Lindane concentrations in different water samples, such as seawater and sewage water-irrigated soil, were in the picomolar to nanomolar range (Lakaschus et al., 2002; Wei He et al., 2012; Wei et al., 2015; Kovacik et al., 2018). As a neurotoxic chemical, toxicological investigations have found that lindane can accumulate in different organs, such as the brain and liver, and damage the central nervous system, which eventually leads to retarded locomotive behaviors, seizures and even death (Parmar et al., 2003; Vucevic et al., 2008; Nandan and Nimila, 2012; Croom et al., 2015; Sharma et al., 2018). To date, neurotoxicity studies have primarily focused on the parental generation; however, low environmental concentrations of lindane may not induce pronounced negative effects, but adversely influence subsequent generations (Wamucho et al., 2019). Therefore, it is of crucial necessity to study transgenerational toxicity in order to explore the influence of accumulation or the adaptive response in the nervous system.

Regarding these limited but important issues, *Caenorhabditis elegans* (*C. elegans*) was applied to evaluate potential toxicity in progeny following parental exposure in this study, owing to its short generation cycle and large numbers of eggs per worm (Leung et al., 2008; Li et al., 2016). *C. elegans* is a common *in vivo* non-parasitic model organism, which inhabits water and soil environments, provided with *Escherichia coli OP50* (*E. coli OP50*) for survival (Brenner, 1974). A series of studies demonstrated that the adverse effects of environmental contaminants could be transferred to the offspring in *C. elegans,* using brood size, body length, locomotion behaviors, oxidative stress, and gene expression as endpoints (Chen et al., 2019; Li et al., 2020; Liu et al., 2020). *C. elegans* individuals contain 302 neurons, which have been fully described in terms of their neuronal lineage. The nervous system of this species is mainly adjusted by neurotransmission systems, such as serotonin, γ-aminobutyric acid (GABA), glutamate, and dopamine (I-Ling Tseng et al., 2013). Thus, *C. elegans* is considered to be an appropriate organism for extrapolating the possible neurotoxicity of lindane to higher animals.

In the present study, the transgenerational neurotoxic effect of lindane exposure was investigated in *C. elegans*. After exposure of the parental generation (P0) to environmentally relevant lindane concentration (0–100 ng/L), nematodes were exposed to lindane-free conditions to acquire offspring of F1–F4 generations. At a physiological level, the frequency of body bend and head thrash was measured. Neuronal degeneration and loss were estimated using transgenic nematodes labeled with green fluorescent protein. In addition, the expression of genes related to neurotransmission was analyzed to determine the underlying genetic mechanisms. The results of *in vivo* experiments will provide insight into neuronal damage induced by lindane over generations.

2. Materials and methods

2.1. Purchase and growth of C. elegans strain

The wild-type N2 and transgenic strains were obtained from the *Caenorhabditis* Genetics Center. The transgenic strains included BZ555 [*dat-1p::GFP*], labeling dopaminergic neurons; EG1285 [*unc-47p::GFP + lin-15(+)*], labeling GABAergic neurons; GR1366 [*tph-1::GFP + rol-6 (su1006)*], labeling serotonergic neurons; and DA1240 [*eat-4::sGFP + lin-15(+)*], labeling glutamatergic neurons. According to the standard protocol, the populations of *C. elegans* were grown on aseptic nematode growth medium agar plates (NGM), fed with *E. coli OP50* in a biochemical incubator at 20 °C (Brenner, 1974). To acquire nematodes of same larval phase, nematodes and NaClO/NaOH solution were added to tubes, sufficiently mixed, and then centrifuged at 3050 rpm for 2.5 min (Donkin and Williams, 1995). Subsequently, the eggs were placed on NGM with adequate food and incubated for 48 h to obtain nematodes at L4 larval stage for use in the toxicity study (Williams and Dusenbery, 1990).

2.2. Lindane exposure and transgenerational design

All experimental chemicals (analytical grade) were purchased from Aladdin Corporation (Shanghai, China), including lindane and dimethyl sulfoxide (DMSO). Lindane was dissolved in DMSO to obtain the stock solutions (0, 0.0001, 0.001, 0.01, 0.1, 1 g/L), then diluted with sterile K medium to prepare the environmentally relevant concentrations (0, 0.01, 0.1, 1, 10, 100 ng/L). Nematodes at L4 larval stage were exposed to each concentration of lindane in 6-well cell culture plates for 24 h, which were considered as the P0 generation. Nematodes were exposed by adding adequate *E. coli OP50* (killed by the ultraviolet irradiation before fed) every day and incubating at 20 °C. After acute exposure, one-third of the nematodes in each concentration group were used for subsequent toxicity evaluation, and two-thirds were washed three times with sterile K medium for the subsequent transgenerational study.

In the transgenerational study, unexposed offspring generations (F1–F4) were examined under lindane-free conditions. Washed nematodes from P0 were lysed with NaClO/NaOH solution to collect the subsequent offspring and transferred to new NGM plates to acquire F1 generation. Subsequent generations were obtained using the same approach, and one-third of the nematodes were used for the toxicity evaluation (Fig. 1). Three independent experiments were performed per generation.

2.3. Evaluation of physiological indicators

To evaluate the physiological effects of lindane, locomotion behaviors were used as the endpoints in each exposure group (0, 0.01, 0.1, 1, 10, 100 ng/L) for each generation. Based on a previous study (Xu et al., 2017), head thrashes and body bends were chosen to assess the locomotion behaviors of *C. elegans*, which were counted under a dissecting microscope. Head thrashes were defined as the frequency of changes in the direction of bending at the middle body. The nematodes were placed in K medium on the food-free NGM plates, and the counting was done for 1 min. Body bends were defined as the frequency of changes in nematodes in the direction corresponding to the posterior bulb of the pharynx along the y axis. The washed nematodes were placed on NGM plates and counted for 20s after stabilization. In the experiments, independent experiments were replicated 4 times and each exposure group contained at least 8 nematodes.

2.4. Evaluation of neurotransmitters

In transgenic strains, GFP can be co-expressed with specific

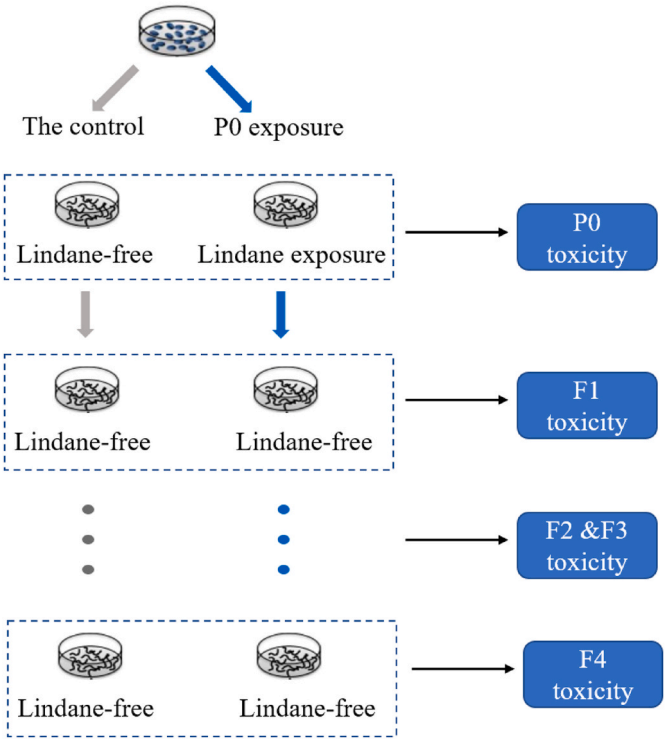

Fig. 1. The experimental design following P0 exposure to lindane in *C. elegans*.

exogenous genes, resulting in the generation of green fluorescence. To evaluate the effects on the nervous system, four transgenic strains of neurons were selected, including EG1285 (GABA), BZ555 (dopamine), DA1240 (glutamate), and GR1366 (serotonin). **Following acute exposure to lindane at concentrations of 0–100** ng/L**,** transgenic **nematodes were** placed on 2% agar pads with anesthetics. Fluorescence images were obtained using a fluorescence microscope (Nikon Eclipse 80i). The results are presented in terms of morphology of labeled neurons, the number of neuronal losses and the relative fluorescence intensity. With 4 independent experiments performed, 40 nematodes were picked out per treatment.

2.5. Evaluation of gene expression

Nematodes (0 and 100 ng/L) of the P0–F4 generations were pretreated with K medium. The purified whole RNA was extracted from *C. elegans* using RNA isolater reagent (Vazyme, China), and the concentrations were measured by Nano-drop (Thermo Fisher Scientific, USA). The cDNA was completed by reverse transcription, and qRT-PCR was used to test expression levels of neurotransmitter-related genes. Primers specific for neurotransmitter-related genes are shown in the Supplementary Data (Table S1). All gene expression values were normalized using *tba-1* gene. The results were analyzed using the $2e^{\triangle\triangle Ct}$ method, and are expressed as relative gene expression. With 4 independent experiments performed, three technical replicates were performed per concentration.

2.6. Statistical analyses

The final results are presented as the mean ± standard error of the mean. The fluorescence intensity was analyzed by ImageJ software. To explore significant variations between concentrations, one-way analysis of variance was performed, followed by Tukey post-hoc tests using SPSS 24.0 software. The Pearson correlation analysis was performed to evaluate between measured parameters. Statistical significance was presented by p value < 0.05 or $p < 0.01$. Graphs were generated using Origin 8.0 (USA).

3. Results and discussion

3.1. Physiological effects of lindane on the parental generation of *C. elegans*

Locomotion behavior is a basic adaptive response of organism, including humans, to environmental neurotoxins (Kannan et al., 2003). To evaluate the physiological effects of lindane, locomotion behaviors were investigated in P0 nematodes. After acute exposure to lindane at environmentally relevant concentrations, the frequency of head thrashes reduced from 92.3% to 85.3%, which represented a significant decrease compared to the untreated group (Fig. 2A). Different from this, the influence of lindane on body bends was not clear following exposure to 0.01 and 1 ng/L (Fig. 2B). Moreover, body bends were significantly inhibited in 0.1, 10, and 100 ng/L exposure groups, with relative frequencies of 89.7%, 79.8% and 64.1%, respectively.

Consistent with the above results, different behavioral changes, such as respiratory frequency, anxious movements and aggressive behaviors, were exhibited by *Etroplus maculatus* fish after exposure to sub-lethal concentrations of lindane (Nandan and Nimila, 2012). In addition, some studies have suggested that adverse physiological effects of lindane are associated with neurotoxicity in animals (Saravanan et al., 2011; Zhang et al., 2020a). Paralysis and convulsions were detected after lindane exposure and resulted in central nervous system hyperexcitability (Mladenovic et al., 2010). In this study, exposure to lindane at concentrations as low as 0.01 ng/L significantly decreased the physiological indicators, which caused the neurotoxicity in *C. elegans*. Notably, the lowest adverse effect concentration on head thrashes was less than body bends in nematodes. Compared to former researches evaluating the toxicity of lindane, *C. elegans* presented greater sensitivity than other organisms, such as fish (Pesce et al., 2008; Croom et al., 2015). Lindane mainly accumulated in the head region containing the nerve nets, which is likely to result in neurotoxic effects (Fleming et al., 1994). Therefore, the corresponding attention should be centered on lindane-induced neuronal damage in *C. elegans*.

3.2. Neurotransmitter changes of lindane after parental exposure in *C. elegans*

In nervous system of *C. elegans*, neurotransmitters play an critical role in modulating the locomotion behaviors and other motor functions, such as γ-aminobutyric acid (GABA), dopamine, serotonin, and glutamate (Si and Song, 2018). Herein, the adverse changes of neurons were investigated using different transgenic strains in P0 generation. The GABA receptor is involved in the locomotion function, including seizures (Treiman, 2001). Compared with the control group, lindane at 10–100 ng/L triggered a significant decrease in fluorescence intensity in the EG1285 strains, and obvious fracture of ciliated dendrites were observed in the treated groups (Fig. 3A and B). Neurons associated with dopamine system are mainly located in head and tail (Sawin et al., 2000). Exposure to lindane above 0.1 ng/L significantly reduced fluorescence intensity in the BZ555 strains compared to the control (Fig. 3A and B). Moreover, the percentage of neuronal losses in the 100 ng/L group was 25% higher than the control group (Fig. 3C), which was in agreement with the results that organophosphorus flame retardants (e.g., tris[2-chloroethyl] phosphate) resulted in a clear reduction and neuronal losses in the tail (Xu et al., 2017). Additionally, serotonin and glutamate are common neurotransmitters associated with locomotion in *C. elegans* (Estevez et al., 2006; Mano et al., 2007). Exposure to lindane at concentrations as low as 0.1 ng/L did cause a notable decrease in fluorescence intensity in transgenic DA1240, while a marked reduction was observed at doses of 10–100 ng/L in GR1366 strains (Fig. 3A and B). Furthermore, Pearson correlation analysis showed that the correlation order between head thrashes and neurotransmitters was serotonin >

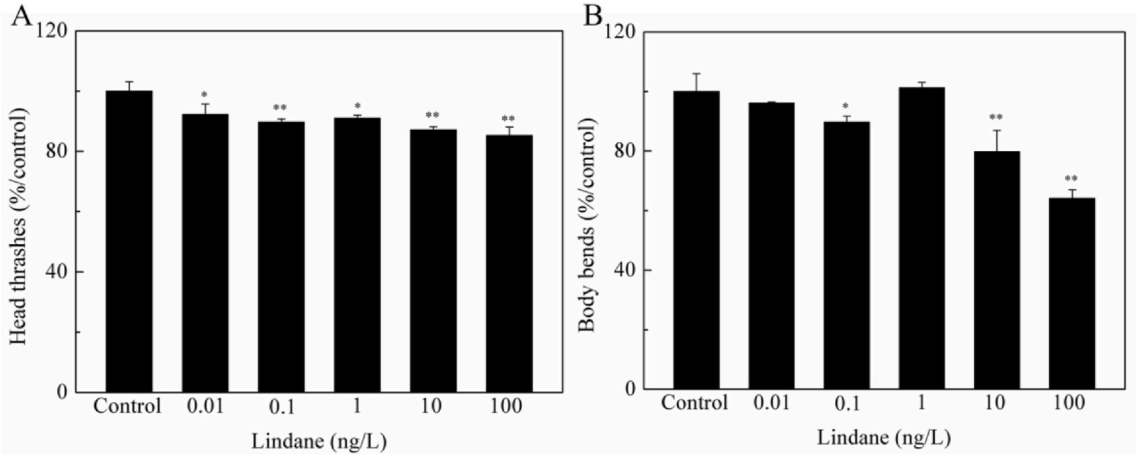

Fig. 2. Physiological effects of lindane in parental generation (P0) of *C. elegans* after acute exposure. (A) Comparison of head thrashes of *C. elegans*. (B) Comparison of body bends of *C. elegans*. Data (mean ± SEM) are expressed as the percentage value compared to the untreated group. The asterisks above indicate significant differences between the exposure and corresponding untreated groups. $*p < 0.05$, $**p < 0.01$.

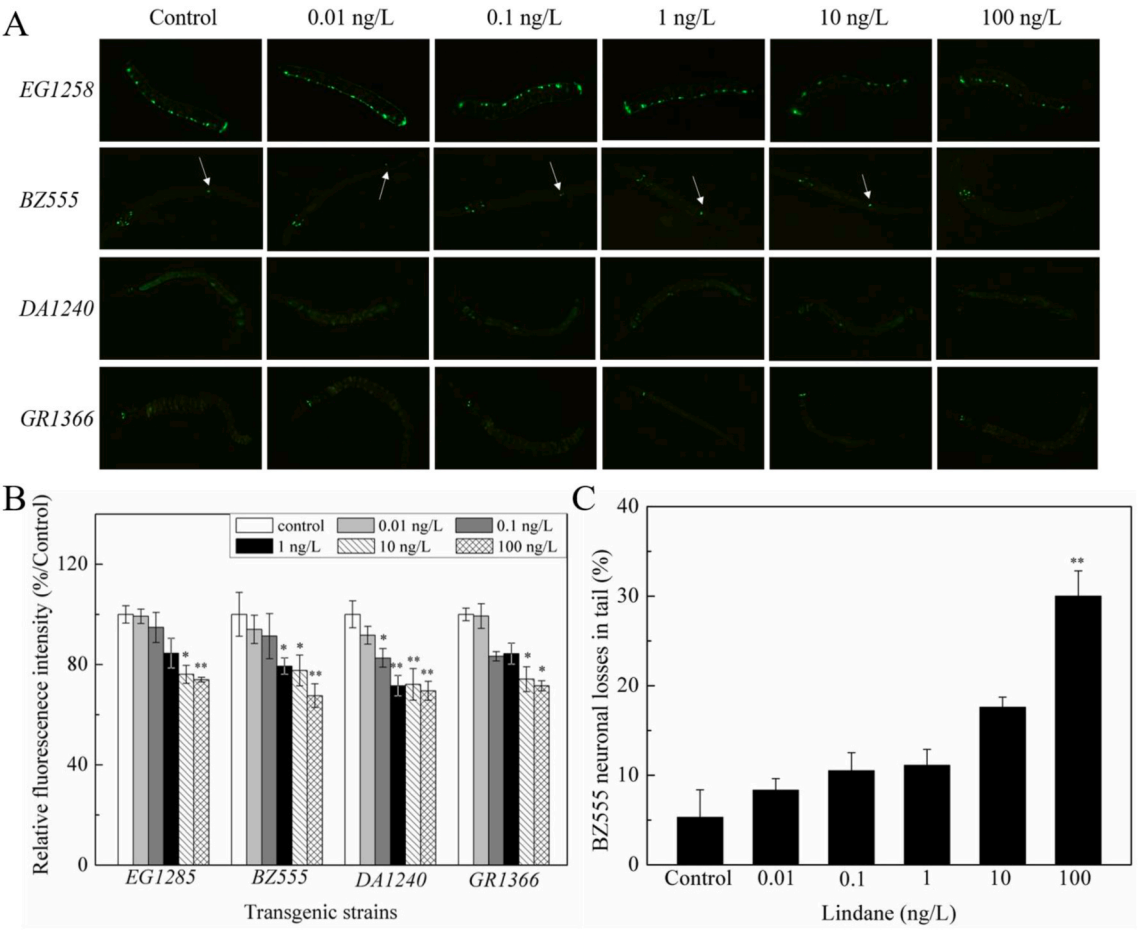

Fig. 3. Neurotransmitters changes of lindane in parental generation (P0) of *C. elegans*. The GABA, dopamine, glutamate and serotonin systems were visualized using the EG1285, BZ555, DA1240 and GR1366 strains. (A) Morphology changes of GABA, dopamine, glutamate and serotonin in nematodes. (B) Comparison of relative fluorescent intensities in EG1285, BZ555, DA1240 and GR1366 strains. (C) Comparison of the percentage of neuronal losses in the tail of BZ555 strains. Data (mean ± SEM) are expressed as the percentage value compared to the control group. White arrowheads indicate the neuron of tail in BZ555 strains. The asterisks indicate significant differences between the exposure and control groups. $*p < 0.05$, $**p < 0.01$.

glutamate > dopamine (Supplemental Data, Table S3).

Previous studies have used different transgenic strains to assess the neuronal damage of xenobiotic pollutants in organisms (Li et al., 2017; Kim et al., 2019). Following 28 days of exposure to lindane at 2.4 μg/g soil dry weight, glutamate and GABA levels were declined sharply, which induced locomotion changes in earthworms (Xu et al., 2020). Similarly, higher concentrations (10–100 ng/L) of lindane significantly decreased the fluorescence intensity of different neurotransmitters and

induced neuronal losses, resulting in the retardation of locomotion behaviors on nematodes after P0 exposure. Pearson correlation analysis indicated that head thrashes were correlated with dopamine, glutamate and serotonin neurotransmitters. Furthermore, changes in neurotransmitters also are characteristic of the neurotoxicity of pollutants. Previous *in vivo* research has demonstrated that exposure to graphene oxide decreases dopamine, GABA, and serotonin levels, which induced its neurotoxic potential (Kim et al., 2019). These results indicated that P0 exposure to lindane could cause neuronal damage by influencing neurotransmitter levels and induce neurotoxicity.

3.3. Physiological effects of lindane exposure in F1–F4 generations of *C. elegans*

To investigate the effects of lindane exposure on offspring, body bends and head thrashes were scored in F1–F4 generations of *C. elegans*. Exposure to lindane at a concentration of 0.01 ng/L did not significantly affect head thrashes in the F1–F4 generations (Fig. 4A). In the F1 and F3 generations, there was a clear reduction after parental exposure to 0.1–10 ng/L of lindane. Subsequently, the adverse effects were eliminated in the F2 (0.1 ng/L) and F4 generations (0.1–100 ng/L).

Consistent with the results of head thrashes, exposure to 0.01 ng/L lindane did not influence body bends in the F1–F4 generations (Fig. 4B). Additionally, P0 exposure to lindane at concentration of 0.1 ng/L significantly restricted body bends in the F1 and F3 generations but recovered in the F2 and F4 generations. Moreover, there was a clear decrease in body bends in nematodes of the F1–F3 generations, which was similar to head thrashes. Notably, P0 exposure to lindane at a dose of 100 ng/L consistently induced a significant reduction in body bends in the F1–F4 generations, with relative frequencies of 65.7%, 66.1%, 75.2%, and 83.0%, respectively, indicating a notable recovery. Considering that 100 ng/L of lindane resulted in more severe neurotoxicity, 100 ng/L was used to explore the mechanism of lindane in transgenerational study.

According to above results, P0 exposure to lindane transferred the adverse physiological effects on subsequent generations. Similarly, lindane exposure adversely impairs learning and other motor functions in the offspring, especially in simple organisms (Gupta et al., 1999; Axmon et al., 2006; Johri et al., 2007). Previous studies have indicated that exposure to xenobiotic pollutants can induce changes in locomotive behaviors in *C. elegans* (Zhao et al., 2014; Liu et al., 2015). After exposure to arsenic, head thrashes in the F1 generation decreased in a dose-dependent relation from 24 h, while recovered to some extent in the F2 generation; body bends decreased from 48 h in both the F1 and F2 generations (Zhang et al., 2020b). Additionally, locomotion behaviors could be regulated by the nervous system, reflecting the neurotoxicity of toxicants in the offspring of *C. elegans* (Tsalik and Hobert, 2003; Li et al., 2018). After P0 exposure to tetrabromobisphenol A (TBBPA) at concentrations of 100–1000 μg/L, nematodes of the filial generation (G2) were restricted in aspects of locomotion behaviors, indicating that TBBPA could restrict neurobehavioral development and induce neurotoxicity (Liu et al., 2020). In the present study, locomotion behaviors were inhibited in the parents but recovered in the F1–F4 generations, especially at the highest concentration. Taken together, results demonstrated that lindane induced transgenerational neurotoxicity following P0 exposure in *C. elegans*.

3.4. Effects of lindane on gene expression of neurotransmitters in P0–F4 generations of *C. elegans*

To further validate the roles of neurotransmitters in the transgenerational neurotoxicity of lindane, the expression of neurotransmitters-related genes was measured in P0–F4 nematodes. In the dopamine system of *C. elegans*, *dat-1* gene encodes the presynaptic dopamine transporter; *dop-1* gene encodes the D1-like receptor acting in the ventral motor neurons (Jayanthi et al., 1998; Chase et al., 2004). In GABA system, the *unc-30* gene was associated with the synthesis and transportation of the GABA receptor (Jin et al., 1994). Glutamate (e.g., *eat-4*, *glr-1 and mgl-1*) is an excitatory and inhibitory modulator of neural cells. Moreover, the *mod-1* gene encodes the serotonin-gated chloride channel inhibiting locomotion behaviors (Ranganathan et al., 2001).

At the P0 generation, the expression of *dat-1*, *dop-1*, *eat-4*, *glr-1*, and *mod-1* was significantly downregulated, while that of *unc-30* was upregulated compared to the control (Fig. 5). Moreover, the expression of *dat-1*, *unc-30*, and *mod-1* was significantly influenced in the F1–F3 generations, whereas their expression recovered to normal levels in the F4 generation. Additionally, *dop-1* expression was obviously influenced in the F1 and F3 generations, but quickly recovered to the control level in the F2 and F4 generations, which may contribute to the atavism. The reason for this phenomenon may be a cumulative potential of lindane on target organs affecting the subsequent generations. Similarly, parental tebuconazole exposure significantly increased the abnormalities in F2 generation, and reduced in F3 abnormalities in reproductive system (Lu et al., 2020). Interestingly, the expression of *eat-4* continued to be influenced until the F4 generation, indicating the important role of glutamate in the transgenerational neurotoxicity of lindane.

Various neurotransmitters are likely to be involved in the mechanisms underlying lindane neurotoxicity (Mladenovic et al., 2010). For example, exposure to pentylenetetrazol caused marked changes in *unc-30* expression, which triggered abnormal behavior in nematodes

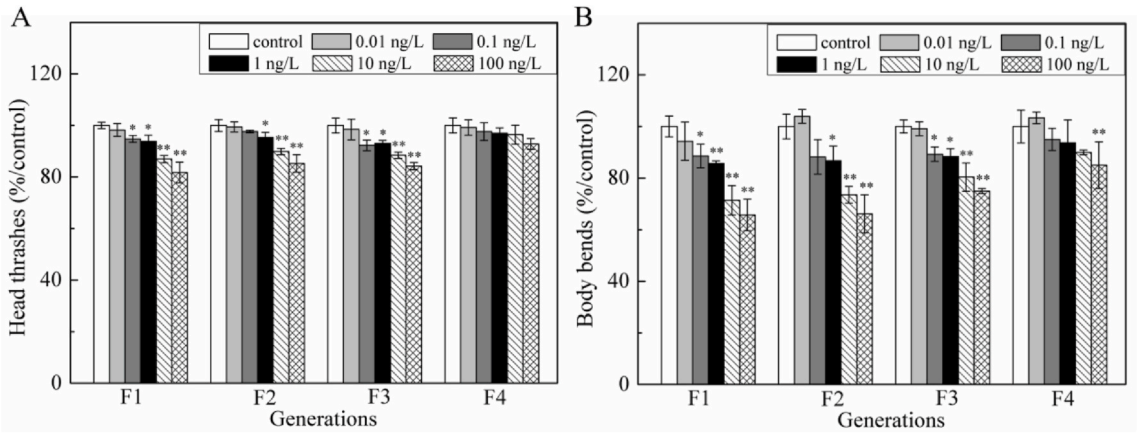

Fig. 4. Physiological effects of lindane in F1 – F4 generations of *C. elegans*. The nematodes from F1 to F4 generations were exposed under lindane-free condition. (A) Comparison of head thrashes. (B) Comparison of body bends. Data (mean ± SEM) are expressed as the percentage value compared to the untreated group. The asterisks above indicate significant differences between the exposure and corresponding untreated groups. *$p < 0.05$, **$p < 0.01$.

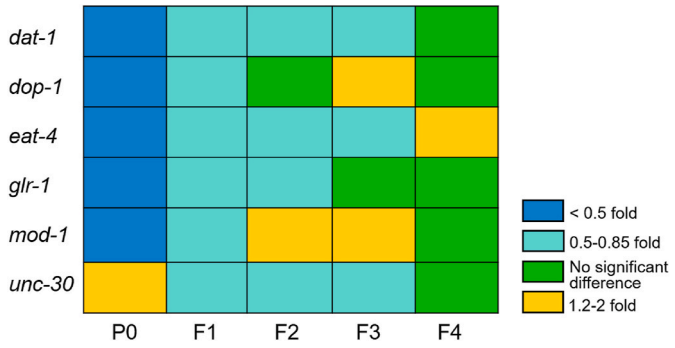

Fig. 5. Gene expression required for neurotransmitters in nematodes of P0–F4 generations exposed to 100 ng/L of lindane. Values of gene expressions were normalized using *tba*-1 mRNA and represented means (n = 3) relative to the control.

(Camara et al., 2019). The expression of *mod-1* decreased notably following the exposure of L4 larval nematodes to nonylphenol at 10–200 μg/L, indicating the damage to neurons and the neurotoxicity (Cao et al., 2019). As a neurotoxin, lindane could cause neuronal damage in both parent and the offspring. In this study, P0 exposure to lindane at a concentration of 100 ng/L resulted in non-monotonic changes of genes in P0–F4 generations, and *eat-4* gene played an important role in the nervous system. The results showed that the gene expression of neurotransmitters was significantly suppressed in P0, whereas recovered to normal levels in the F2–F4 generations, elucidating the mechanism of lindane-induced transgenerational neurotoxicity. Consistently, previous studies have illustrated that lindane could directly influence the function of GABA receptor, inducing neurotoxicity in mammals (Sunol et al., 1989, 1998; Damgaard et al., 1999). Therefore, taken together, these data support the hypothesis that lindane causes neurotoxicity via neuronal damage induced by neurotransmitters, and glutamate plays a vital role in the offspring.

4. Conclusion

The adverse effects of P0 exposure to lindane were observed by evaluating locomotion behaviors in *C. elegans*. At the P0 generation, exposure to lindane notably influences the levels of GABA, dopamine, serotonin, and glutamate neurotransmitters, which suggests the severe neuronal damage in nematodes. The correlation order between locomotion and neurotransmitters was serotonin > glutamate > dopamine. Additionally, the toxicity of lindane was demonstrated to be transferred to subsequent generations (F1–F4), which was accompanied with the change of locomotive behaviors. Moreover, P0 exposure to lindane regulated the expression of neurotransmissions-related genes in subsequent generations, and *eat-4* gene played an important role in the nervous system. Thus, lindane induced the transgenerational neurotoxicity through mechanisms involving in the glutamatergic transmission in *C. elegans*.

Credit author statement

Yunjiang Yu: Conceptualization, Methodology. Xin Hua: Writing-Original draft, Investigation, Formal analysis. Haibo Chen: Data curation, Writing- Review and Editing. Zhengdong Wang: Validation. Yajing Han: Validation. Xichao Chen: Validation. Yue Yang: Validation. Mingdeng Xiang: Validation

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.133360.

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