Toxicology and Applied Pharmacology 456 (2022) 116283

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Sex-specific behavioral effects of acute exposure to the neonicotinoid clothianidin in mice

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ARTICLE INFO

Editor: Lawrence Lash

Keywords:
Neonicotinoid
Clothianidin
Sex Difference
Behavior
Learning Memory

ABSTRACT

Although neonicotinoids are among the major classes of pesticides that affect mammalian nervous systems, little is known about sex differences in their effects. This study aimed to examine whether the neurobehavioral effects of a neonicotinoid, clothianidin (CLO), differed between sexes. Male and female C57BL/6N mice were orally administered CLO (5 or 50 mg/kg) at or below the chronic no-observed-adverse-effect-level (NOAEL) and subjected to behavioral tests of emotional and learning functions. Changes in neuroactivity in several brain regions and the concentrations of CLO and its metabolites in blood and urine were measured. Acute CLO exposure caused sex-related behavioral effects; decreases in locomotor activities and elevation of anxiety-like behaviors were more apparent in males than in females. In addition, male-specific impairment of short- and long-term learning memory by CLO exposure was observed in both the novel recognition test and the Barnes maze test. Male-dominant increases in the number of c-fos positive cells were observed in the paraventricular thalamic nucleus in the thalamus and in the dentate gyrus in the hippocampus, which are related to the stress response and learning function, respectively. The concentrations of CLO and most metabolites in blood and urine were higher in males. These results support the notion that male mice are more vulnerable than females to the neurobehavioral effects of CLO and provide novel insights into the risk assessment of neonicotinoids in mammalian neuronal function.

1. Introduction

Developed in the 1980s, neonicotinoid pesticides are among the most widely used insecticides in the world to control insects in agriculture, forestry, and households. The main source of exposure to neonicotinoids in human is assumed to be food and drink, and the total concentrations of neonicotinoids in human-derived samples are reportedly on the order of ng/mL worldwide (Zhang and Lu, 2022). Neonicotinoids are structurally similar to nicotine and act selectively as agonists on nicotinic acetylcholine receptors (nAChRs) of insects (Nagata et al., 1998). The affinity of neonicotinoids for mammalian nAChRs is much lower than that for the nAChRs of insects (Tomizawa and Casida, 2005); however,

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https://doi.org/10.1016/j.taap.2022.116283
Received 11 August 2022; Received in revised form 2 October 2022; Accepted 11 October 2022

as with nicotine, neonicotinoids proved to induce intracellular calcium influx via nAChRs in various mammalian neuronal cell models (Kimura-Kuroda et al., 2012; Hirano et al., 2019; Loser et al., 2021). In rodent studies, orally administered neonicotinoids reached the brain in a few hours (Ford and Casida, 2006) and caused behavioral alterations accompanied by changes in neuronal function in several brain regions (Costas-Ferreira and Faro, 2021; Maeda et al., 2021; Hirai et al., 2022). In particular, a recently developed neonicotinoid, clothianidin (CLO), has attracted much attention for its excitatory effects on neurotransmission in the mammalian brain; CLO evoked dopamine release by nAChRs in rat brain (Faro et al., 2019). In our previous study using in vivo Ca^{2+} imaging techniques, CLO activated the firing pattern of neurons in the primary somatosensory cortex (Nishi et al., 2022). Acute exposure to CLO at below the no-observed-adverse-effect level (NOAEL) caused behavioral abnormalities including anxiety-like behavior and excessive stress response in mature male mice in an age-dependent manner (Hirano et al., 2018, 2021).

In neurotoxicity assessments of environmental chemicals, differences in chemical sensitivity should be one of the primary concerns which depend on various factors such as sex. The rates of neurological diseases such as Alzheimer's disease and depression are known to be higher in females than males, and these differences have been attributed partly to sex differences in brain structure and function (Weiss, 2011). For example, tobacco dependence is known to be prevalent in women, and the effects of nicotine on the developing brain are known to differ by sex (Cross et al., 2017). Human PET imaging revealed that the binding level of α4β2 nAChRs was higher in all brain regions in women than in men (Mukherjee et al., 2018). A study of rodents also demonstrated that chronic nicotine exposure induced stronger locomotor activation in males than in females, while anxiogenic effects were observed only in females (Caldarone et al., 2008). In fact, recent epidemiological studies in China found that urinary and blood concentrations of most neonicotinoids were higher in women than in men (Xu et al., 2021), indicating that women have greater exposure to neonicotinoids than men in daily life. In light of these findings, there could be sex-related differences in sensitivities to effects of neonicotinoids by nAChRs in neuronal function. However, only one report showed data comparing the behavioral effects of neonicotinoids between males and females; Burke et al. (2018) showed that fetal and postnatal exposure in mice to a neonicotinoid, imidacloprid, caused sex-related changes in performance in a forced swim test.

Considering that most toxicological studies have used only male animals, further research focusing on sex differences is needed to understand the precise risks of neonicotinoids on neuronal function. In this study, we hypothesized that there were sex-related differences in sensitivity to acute effects of CLO exposure and aimed to compare behavioral consequences of acute CLO exposure between male and female mice using four behavioral tests of emotional status and learning memory. We also analyzed changes in neuroactivity in brain regions related to the stress response and learning memory by using immunohistochemistry, and we measured body concentrations of CLO and its metabolites by using LC-ESI/MS/MS techniques to reveal the mechanisms underlying sex-related differences in the behavioral effects of CLO exposure.

2. Materials and methods

2.1. Experimental animals and procedures

Male and female C57BL/6NCrSlc mice (8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All mice were group-housed (3 mice per ventilated cage of 40.5 × 20.5 × 18.5 cm, Green Line IVC Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2 °C) and humidity (50 ± 10%), and artificial lighting (14-h light, 10-h dark conditions) at the Kobe University Life-Science Laboratory, and were given ad libitum access to a pellet diet (DC-8; CLEA Japan, Tokyo Japan). This study was approved by the Institutional Animal Care and Use Committee (Permission # 26–05-07) of Kobe University and carried out according to the Kobe University Animal Experimental Regulations. Based on the 47.2 mg/kg/day NOAEL for males in an 18-month carcinogenicity test (Food and Agriculture Organization of the United Nations, 2016), male and female mice (9 weeks old) were administered CLO (purity >95%) at a dose of 0, 5, or 50 mg/kg body weight suspended in 0.5% carboxymethylcellulose (10 mL/kg) by oral gavage (Hirano et al., 2018). These groups were defined based on dose, with the control, CLO-5, and CLO-50 groups respectively receiving 0, 5, and 50 mg/kg body weight. Each behavioral test was conducted in the light phase using different sets of mice.

2.2. Open field test

After a single oral administration, mice were acclimated to the testing room conditions (500 lx, 23 ± 2 °C) in their home cage for 1 h prior to the start of the test. The mouse was placed in a corner of an open field (60 × 60 × 40 cm, Tom Products Co., Ltd., Tokyo, Japan) with its nose pointed at the wall and was then free to explore for 10 min. All of the mouse's activities were recorded by a video camera. The total distance and time spent in the center zone (30 × 30 cm) were analyzed using ImageJ software with the ImageOF plugin.

2.3. Elevated plus maze test

After CLO administration, the mice were acclimated to the testing room conditions (10–20 lx, 23 ± 2 °C) in their home cage for 1 h before the start of the test. The test apparatus was a cruciform made of acrylic plate; it consisted of two opposing open arms without walls (30 × 5) and two closed arms with walls (same size, 15 cm wall) and a center zone (5 × 5 cm). The mouse was placed in the center zone with its nose pointed at the open arm and was then free to explore for 10 min. All of the mouse's activities were recorded by a video camera. The total arm entry and time spent in the open arms were analyzed using ImageJ software with the ImageEP plugin.

2.4. Novel object recognition test

All mice were acclimated to the testing room conditions (10–20 lx, 23 ± 2 °C) in their home cage for 1 h prior to start of the test. The experimental design of the test according to the previous protocol with some modification is illustrated in Fig. 2A (Bevins and Besheer, 2006). The task procedure consisted of three phases: habituation, familiarization, and test. In the habituation phase, the mouse was allowed to freely explore an open field (60 × 60 × 40 cm) in the absence of objects for 5 min and then was moved to its home cage. The mouse was administered CLO 24 h after the end of the habituation phase. At 1 h after CLO administration, in the familiarization phase, the mouse was allowed to explore freely in the open field with four black bottles (6.5 × 6.5 × 11.2 cm) for 5 min and then was moved to its home cage. In the test phase, which began 30 min after the end of the familiarization phase, the mouse was allowed to explore freely for 5 min in the same field, in which one of the familiar bottles used in the familiarization phase was replaced by a tower of Lego blocks (4.8 × 4.8 × 11.3 cm) as a novel object. All of the mouse's activities were recorded by a video camera, and the mouse's trajectory was analyzed with ImageJ software. Touching behavior, in which the mouse touches objects with its nose, was defined as exploration. The percentage of explorations of the new object (the Lego tower) among explorations of all objects was calculated.

2.5. Barnes maze test

After CLO administration, all mice were acclimated to the testing room conditions (800 lx, 23 ± 2 °C) in their home cage for 1 h prior to start of the test. The Barnes maze (90 cm in diameter) was made of white acrylic plates with 20 holes (5 cm in diameter) and brightly illuminated (1800 lx). One hole, designated as the target, led to an escape box (20 × 9 × 9 cm) formed from sections of black acrylic plate (Fig. 3A). The experimental schedule of the test is illustrated in Fig. 4B as described by Rosenfeld and Ferguson (2014) with some modifications. The mouse was placed in a cylinder made of black acrylic (diameter 8 cm, height 12.5 cm) which was then placed on the center of the maze. After 8 s, the trial was started by removing only the cylinder, leaving the mouse on the center of the plate. If the mouse did not enter the escape box within 3 min, the mouse was gently guided to it and allowed to stay in it for 2 min. The training was repeated four times a day at 5 min intervals for 5 days and recorded by a video camera. The distance traveled and the latency to enter the escape box were analyzed with ImageJ software.

2.6. *Immunohistochemical analysis*

Immunohistochemical analyses using c-fos as a neuroactivity marker were performed according to a previous report (Hirano et al., 2018). After 2 h of behavioral tests, all mice were deeply anesthetized with isoflurane and transcardially perfused with 0.9% normal saline, followed by perfusion with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was sliced into 3 mm coronal section using a brain slicer (Muromachi Kikai, Tokyo, Japan), fixed by immersion in PFA for 6 h at 4 °C, cryoprotected in ascending solutions of sucrose (10%, 20%, 30%) in 0.1 M PB overnight, and frozen in an embedding solution consisting of Tissue-Tek® OCT compound (Sakura Finetek, Tokyo, Japan). The prepared frozen blocks were sliced into 10-μm-thick sections on a cryostat (CM1950; Leica Microsystems, Wetzlar, Germany) and the sections were mounted on slide glasses. The brain sections were washed in 0.01 M phosphate-buffered saline with 0.05% Tween-20 (Wako Pure Chemical Industries, Osaka, Japan) (PBST; pH 7.4) and incubated with Blocking OneHisto (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature (RT). The sections were then incubated with rabbit polyclonal anti-c-fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz CA, USA) diluted 1:2000 in PBST for 18 h at 4 °C. After washing in PBST, the sections were reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision+; Dako, Glostrup, Denmark) for 1 h at RT. Immunoreactivity was detected by incubation with DAB (3,3′-diaminobenzidine-tetrachloride) solution (EnVision+ kit/HRP[DAB]; Dako). The sections were coverslipped with Eukitt mounting medium (O. Kindler, Freiburg, Germany) and photographed with a BX61 microscope equipped with a DP-70 digital camera (Olympus Japan, Tokyo, Japan). The number of c-fos–immunopositive cells per area was calculated using ImageJ software for at least three sections per mouse.

2.7. *Measurement of CLO and metabolites from blood and urine samples*

At 2 h after the last behavioral test, mice in the CLO-50 group were weighed and sacrificed under deep anesthesia with isoflurane. Blood and urine samples were collected in 1.5 mL tubes and stored at −80 °C until use. The concentrations of CLO and metabolites (1-methyl-3-nitroguanidine (MNG), desmethyl-CLO (dm-CLO), desmethyl-desnitro-CLO (dm-dn-CLO), desnitro-CLO (dn-CLO), and CLO-urea) from blood and urine samples were measured using an LC-ESI/MS/MS (Agilent 6495B, Agilent Co., CA, USA) as described previously (Ohno et al., 2020).

2.8. *Statistical analysis*

All experimental results are expressed as the mean ± standard error (SE), and statistical analyses were performed with Excel Statistics 2012 for Windows (SSRI version 1.00; SSRI, Tokyo, Japan). All behavioral and immunohistochemical data were analyzed by two-way ANOVA (CLO × sex) followed by the Tukey's post hoc test. The sex differences in the blood and urine concentrations of CLO were analyzed using the Mann–Whitney *U* test. The results were considered significant when the *p*-value was <0.05.

3. Results

3.1. *Effects of CLO on locomotive and anxiety-like behavior*

To examine the sex-related effects of CLO on the emotional behavior of mice in a novel environment, we first conducted an open field test. The trajectory maps in Fig. 1A summarize the exploratory behaviors of mice in the open field for 10 min. The two-way ANOVA showed that CLO had a significant main effect on the total distance traveled [$F(2,30) = 5.508$, $p < 0.01$], while the main effects of sex [$F(1,30) = 3.936$, $p = 0.057$] and the interaction [$F(2, 30) = 3.089$, $p = 0.060$] were close to significant. The post hoc comparisons revealed that 50 mg/kg of CLO significantly decreased the total distance compared to the control group only in males [$p < 0.05$] (Fig. 1B). As shown in Fig. 1C, CLO and sex did not significantly affect the time spent in the center zone (two-way ANOVA: main effect of CLO [$F(2, 30) = 0.724$]; main effect of sex [$F(1, 30) = 0.153$]; interaction [$F(2, 30) = 0.459$]).

The sex-related effects of CLO on the anxiety-like behavior of mice in a novel environment were evaluated by an elevated plus maze test. The trajectory maps showed that mice in the CLO-administered groups tended to stay in the open arms (Fig. 1D). The total number of arm entries, an index of locomotor activity, was significantly decreased by CLO (two-way ANOVA: main effect of CLO [$F(2, 30) =4.142$, $p < 0.05$]; main effect of sex [$F(1, 30) = 0.555$]; interaction [$F(2, 30) = 1.060$]), and there was a significant difference between the control and the CLO-50 group only in males (Fig. 1E). As shown in Fig. 1F, the time spent in the open arms was significantly decreased by CLO (two-way ANOVA: main effect of CLO [$F(2, 30) =11.744$, $p < 0.01$]; main effect of sex [$F(1, 30) = 0.316$]; interaction [$F(2, 30) = 1.405$]). The post hoc comparisons showed that, for both males and females, the CLO-50 group spent significantly less time in the open arms than the control group. For the CLO-5 group, on the other hand, only males spent significantly less time in the open arms compared to the control.

3.2. *Effects of CLO on recognition memory*

To evaluate the sex-related effects of CLO on object recognition memory in a short time, mice were subjected to s novel recognition test (Fig. 2A). The trajectory maps showed the exploratory behaviors of mice in the test phase for 5 min (Fig. 2B). As shown in Fig. 2C, the quantitative data revealed no significant changes in total exploration (two-way ANOVA: main effect of CLO [$F(2, 30) =3.014$]; main effect of sex [$F(1, 30) = 2.931$]; interaction [$F(2, 30) = 1.664$]). The two-way ANOVA showed significant main effects of CLO and interaction in the percentage of explorations of the novel object as an index of object recognition memory (main effect of CLO [$F(2, 30) =5.618$, $p < 0.01$]; main effect of sex [$F(1, 30) = 1.528$]; interaction [$F(2, 30) = 5.371$, $p < 0.05$] (Fig. 2D). The post hoc comparisons showed that the percentage of exploration of the novel object was significantly lower in the CLO-5 and CLO-50 groups than in the control group among males, while no difference was observed among females.

3.3. *Effects of CLO on spatial learning memory*

The sex-related effects of 50 mg/kg of CLO on long-term spatial learning memory were further evaluated by the Barnes maze test for 5 days, with the distance traveled and the latency to the escape box serving as performance indexes (Fig. 3A and B). The distance traveled to the escape box decreased by the day in both males (Fig. 3C) (two-way ANOVA in males: main effect of day [$F(4, 45) =2.898$, $p < 0.05$]; main effect of CLO [$F(1, 45) = 8.105$, $p < 0.01$]; interaction [$F(4, 45) = 0.888$]) and females (Fig. 3D) (two-way ANOVA in females: main effect of the day [$F(4, 49) =3.417$, $p < 0.05$]; main effect of CLO [$F(1, 49) =$

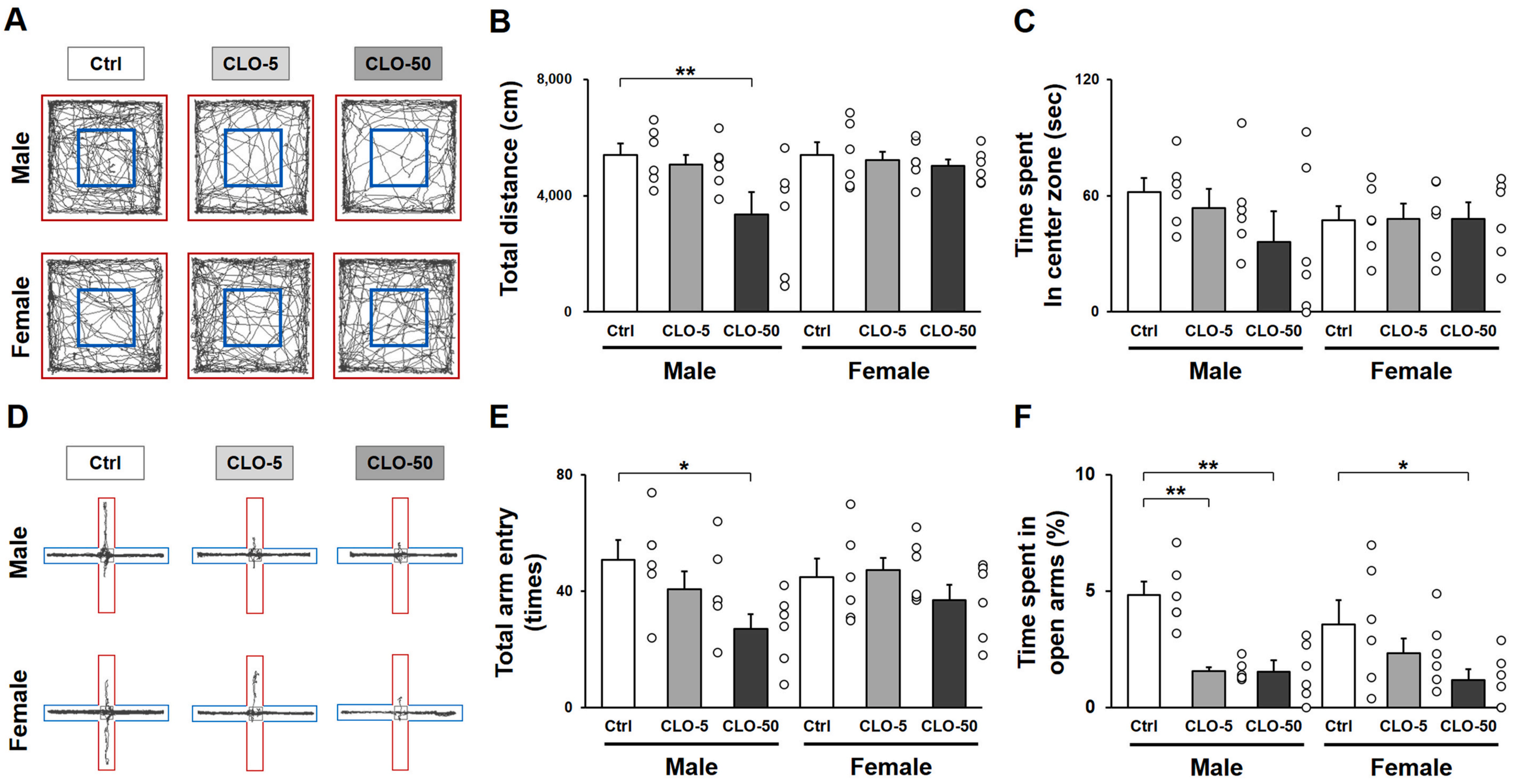

Fig. 1. Sex differences in behavioral effects of acute CLO on locomotor behavior in an open field test and an elevated plus maze test. (A) Representative trajectory maps of exploratory behaviors of male and female mice in an open field (60 × 60 cm). Graphs present (B) total distance and (C) time in center zone (30 × 30 cm, blue square) of vehicle control and CLO-administered mice. (D) Representative trajectory maps of exploratory behaviors of male and female mice in the elevated plus maze with open (vertical) arms and closed (horizontal) arms. Graphs present (E) total arm entry and (F) time spent in open arms of vehicle control and CLO-administered mice. Columns and circles show group mean ± SE and individual values of mice (Ctrl: $n = 6$, CTD-5: n = 6, CTD-50: n = 6); $*p < 0.05$, $**p < 0.01$ vs. control groups (two-way ANOVA followed by Tukey's post-hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

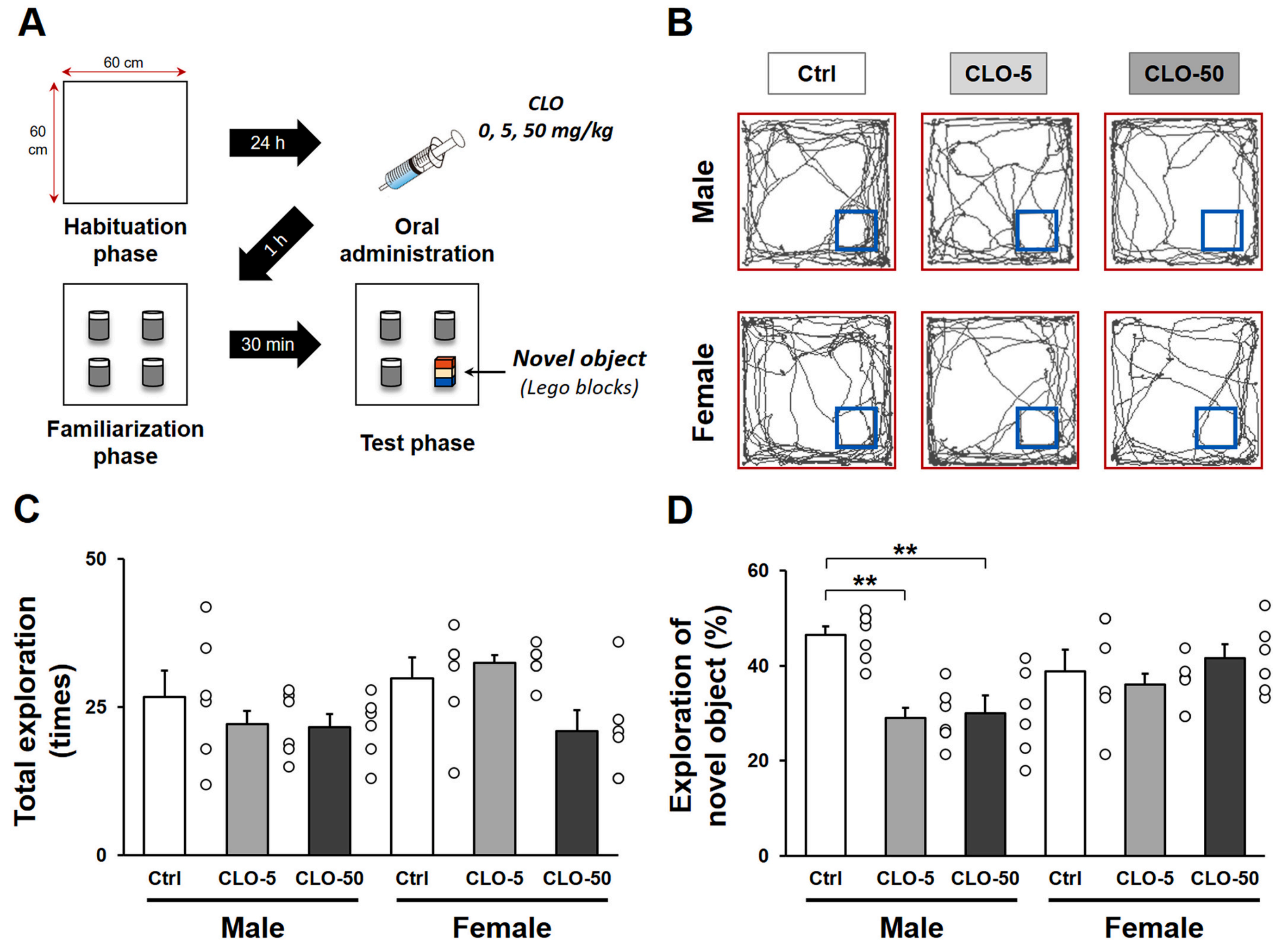

Fig. 2. Sex differences in behavioral effects of acute CLO on recognition memory in a novel recognition test. (A) Schematic diagram illustrating the experimental design of the test. Following a 24 h habituation phase, mice were orally administered CLO and subjected to a familiarization phase at 1 h after, followed by a test phase at 30 min after. (B) Representative trajectory maps of exploratory behaviors of male and female mice in the test phase of the novel recognition test. Graphs present (C) total exploration and (D) exploration of the novel object (blue squares). Columns and circles show group mean ± SE and individual values of mice (Ctrl: n = 6, CTD-5: n = 6, CTD-50: n = 6); $^{**}p < 0.01$ vs. control groups (two-way ANOVA followed by Tukey's post-hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.357]; interaction [F(4, 49) = 1.126]). CLO had a main effect only in males, and the difference between the CLO-50 and control groups reached significance at day 3. As shown in Fig. 3E and F, the latency to the escape box also decreased by the day, and the two-way ANOVA showed that the main effect of the day was observed only in females (females: main effect of day [F(4, 49) =3.268, $p < 0.05$]; main effect of CLO [F(1, 49) = 0.241]; interaction [F(4, 49) = 0.310]). On the other hand, there CLO had a main effect only in males (males: main effect of day [F(4, 45) =1.023]; main effect of CLO [F(1, 45) = 12.343, $p < 0.01$]; interaction [F(4, 45) = 0.489]), and the post hoc comparison revealed that the latency to the escape box was significantly higher in the CLO-50 group than in the control group at day 3.

3.4. Effects of CLO on c-fos expression induced by the behavioral tests

Our previous reports demonstrated that the behavioral effects of CLO were accompanied by changes in neuroactivities in several brain regions, including the paraventricular thalamic nucleus (PVT) in the thalamus and the dentate gyrus (DG) in the hippocampus (Hirano et al., 2018). To explore the mechanism underlying marked sex-related behavioral effects of CLO, the neuronal activities of mice during the elevated plus maze test and the Barnes maze test were analyzed by immunohistochemistry to visualize the c-fos, an activated neuron marker. As shown in Fig. 4A, the immunoreactivities of c-fos in PVT were increased in both male and female mice subjected to the elevated plus maze test. The two-way ANOVA showed the significant main effects of CLO (main effect of CLO [F(2, 24) =12.100, $p < 0.01$]; main effect of sex [F(1, 24) = 3.960]; interaction [F(2, 24) = 0.501), and a post hoc comparison revealed that the number of immunoreactive cells in PVT was significantly higher in the CLO-5 and CLO-50 groups than in the control group among males, whereas among females the number was higher only in the CLO-5 group (Fig. 4B). Fig. 5A shows the immunoreactivities of c-fos in the DG of mice subjected to the Barnes maze test. CLO increased the number of immunoreactive cells in the DG (two-way ANOVA; main effect of CLO [F(1, 19) =16.292, $p < 0.01$]; main effect of sex [F(1, 19 = 3.550]; interaction [F(1, 19) = 13.124, $p < 0.01$]), and post hoc analyses showed a significant difference between the CLO-50 and control groups only among males (Fig. 5B). There was no marked sex-related difference of c-fos immunoreactivities in DG of mice subjected to the elevated plus maze test and PVT of mice subjected to the Barnes maze test (data not shown).

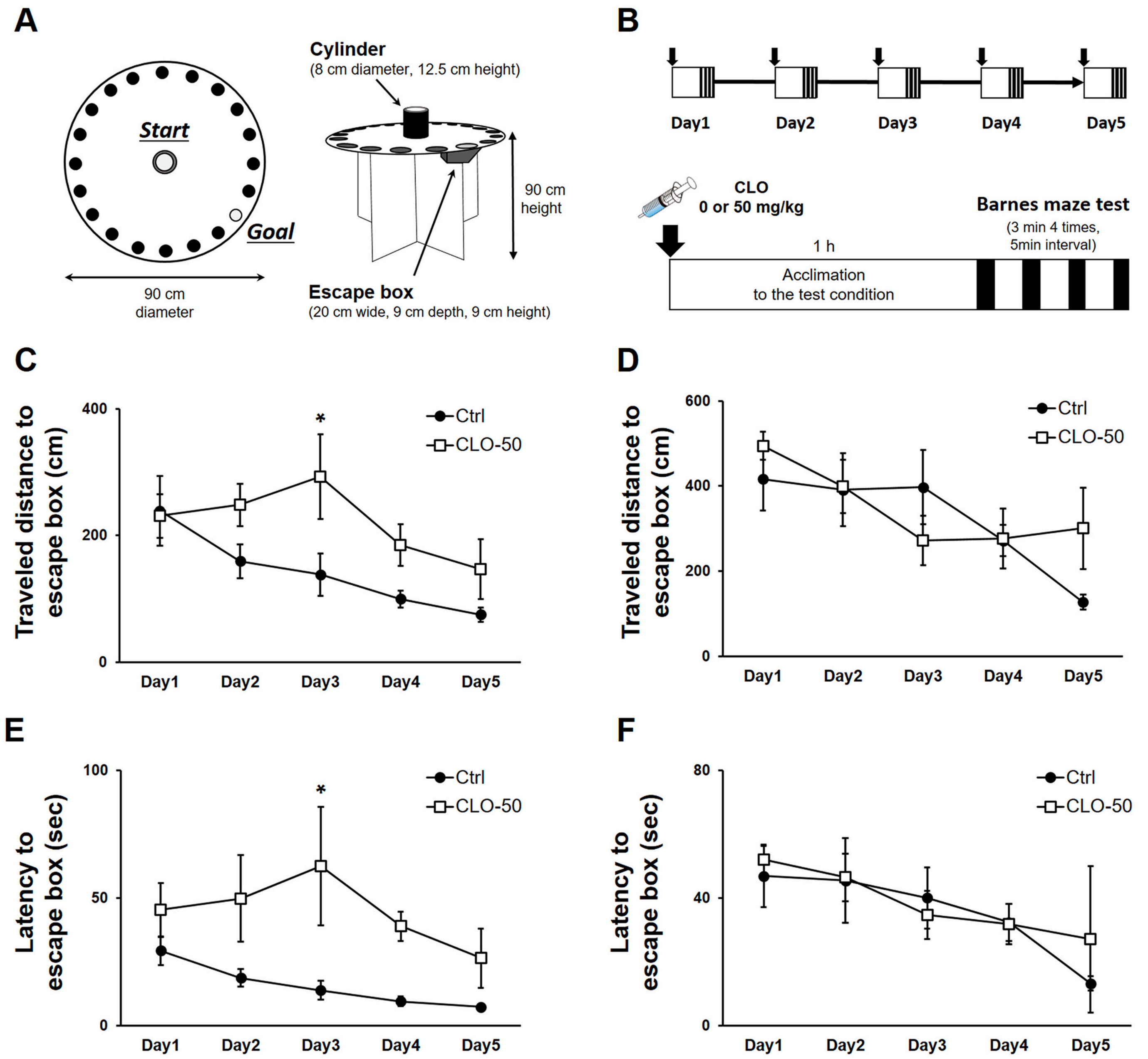

Fig. 3. Sex differences in behavioral effects of acute CLO on spatial memory in a Barnes maze test. (A) Brief overview of apparatus used for the test. (B) Schematic diagram illustrating the test's experimental design. At 1 h after oral administration of CLO, mice were subjected to three training trials with 5 min intervals for 5 consecutive days. Learning curves show daily changes in performance in the distance traveled and in the latency to the escape box by male (C and E) and female (D and F) mice (Ctrl male: $n = 5$, CTD-50 male: n = 6, Ctrl female: n = 6, CTD-50 female: n = 6). Plots show group mean ± SE; $^{*}p < 0.05$ vs. control groups (two-way ANOVA followed by Tukey's post-hoc test).

3.5. *Concentrations of CLO and its metabolites in blood and urine*

To compare the differences in the metabolic fate of CLO between male and female mice in the CLO-50 group, the concentrations of CLO and its six metabolites in blood and urine samples were measured using LC-ESI/MS/MS (Table 1). The concentrations of CLO and most of its metabolites in blood and urine were higher in males than in females, and there were statistical differences between the sexes for the parent compound in blood and urine (blood, $p < 0.05$; urine, $p < 0.01$). Moreover, the concentrations of dm-CLO and dm-CLO-urea in urine were significantly higher in males than in females ($p < 0.05$), whereas blood samples showed no sex-related differences in the concentrations of the metabolites.

4. Discussion

In the present study, we investigated whether the behavioral effects of the chronic NOAEL level of CLO differed between male and female mice and examined the mechanisms underlying those differences by focusing on the changes in neuroactivities and metabolic fates. In summary, our findings revealed that male mice showed anxiety-like behavior and learning impairment by acute exposure to CLO, which were not observed in female mice (see Fig. 6). These results were consistent with a previous report showing anxiety-like behavior in the elevated plus maze test caused by CLO in male mice (Hirano et al., 2018). However, there has been no study about the effects of CLO in female animals. The present study is the first report to demonstrate sex-

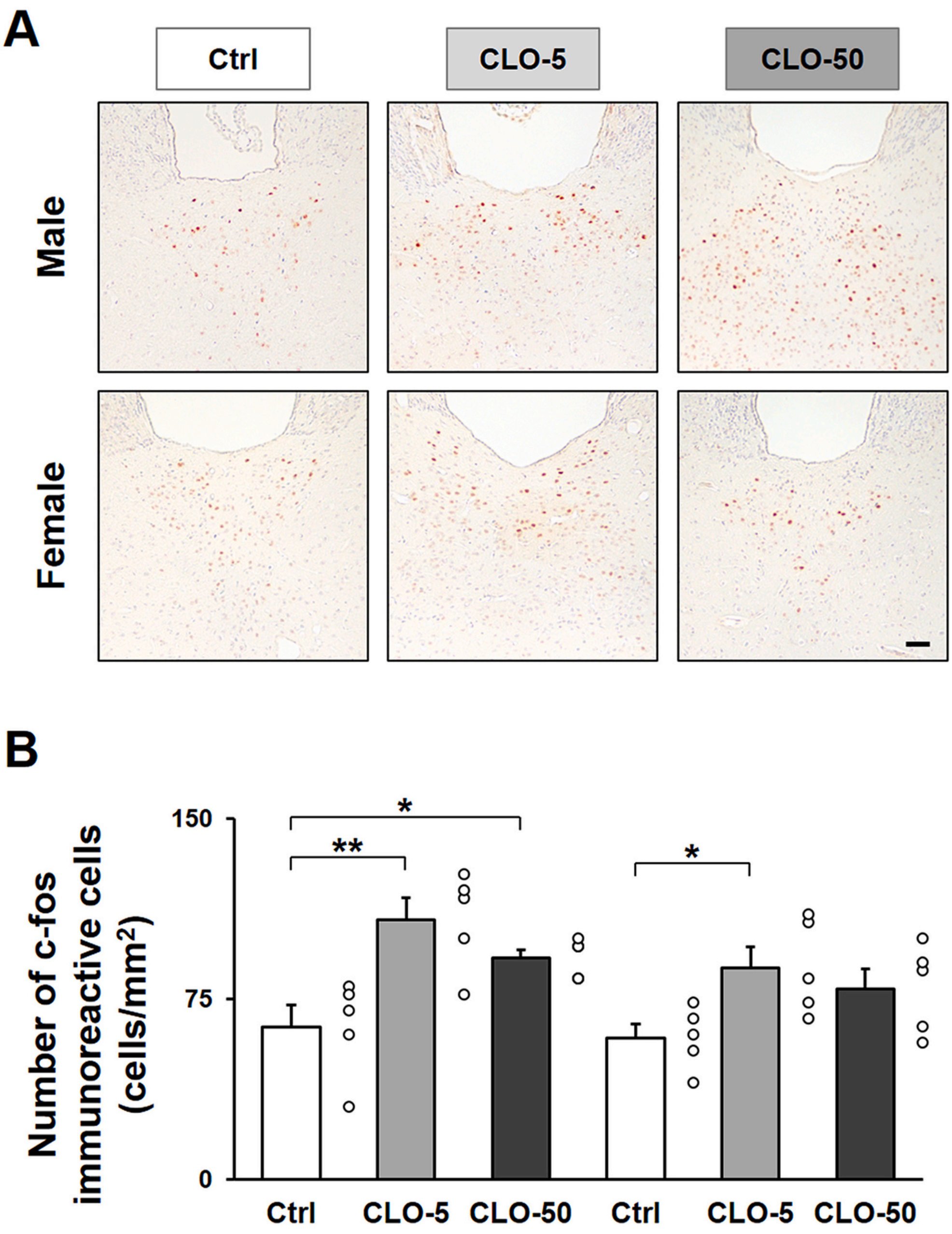

Fig. 4. Sex differences in effects of acute CLO on neuroactivity in paraventricular thalamic nucleus (PVT) in an elevated plus maze test. (A) Representative images of immunoreactivity of c-fos in the PVT in male and female mice. (B) Quantitative data on the number of immunoreactive cells per area in the PVT analyzed using at least three sections per mouse. Bar = 100 μm. Columns and circles show group mean ± SE and individual values of mice (Ctrl: n = 6, CTD-5: n = 6, CTD-50: n = 6); $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control groups (two-way ANOVA followed by Tukey's post-hoc test).

related differences in neurobehavioral effects of CLO. These differences provide a novel base of knowledge on which to understand sex-related differences in the risk of neonicotinoids on neuronal function.

The most interesting findings of the present study were that CLO acutely affected emotional behaviors in a novel environment and that these effects differed by sex. The locomotor activities in both the open field test and the elevated plus maze test were decreased only in the male CLO-50 group, which was consistent with a previous report showing that female mice exhibited less sensitivity than males to the locomotor suppression caused by acute nicotine (Hatchell and Collins, 1980). In addition, the decrease in time spent in the open arms in the elevated plus maze test was observed in both sexes in the CLO-50 group, whereas only males showed a decrease in the CLO-5 group. As with nicotine, an earlier neonicotinoid, acetamiprid, induced anxiety-like behavior in male mice in the elevated plus maze test (Hirai et al., 2022). On the other hand, in adult rats nicotine induced anxiogenic effects in the elevated plus maze test only among females (Elliott et al., 2004; Cao et al., 2010). This consistency implied species-specific differences in sensitivity to the behavioral effects of nicotinic agonists. Taken together, these previous and present results indicated that CLO at the chronic NOAEL level influenced locomotor activities and anxiety-like behaviors in mice of both sexes and that male mice were more vulnerable than females to the novel stressful environment.

In fact, there are sex-related differences in learning performance in mice; males have advantages in working memory, while females have advantages in reference memory (Frick and Gresack, 2003; Jonasson, 2005). Learning abilities were known to be impaired by other classes of pesticides such as organochlorides and pyrethroids in a male-specific manner in rats (Gómez-Giménez et al., 2017). Subchronic exposure to the earlier neonicotinoid imidacloprid or to CLO impaired spatial learning memory in young male rats (Ozdemir et al., 2014; Kara et al., 2015). Our results showed that acute CLO impaired working memory, resulting in decreased exploration behavior of a novel object, only in male mice. In accordance with the present study, Kenney et al. (2011) demonstrated that male mice acutely exposed to nicotine exhibited deficits in novel object recognition tasks. In the present study, in

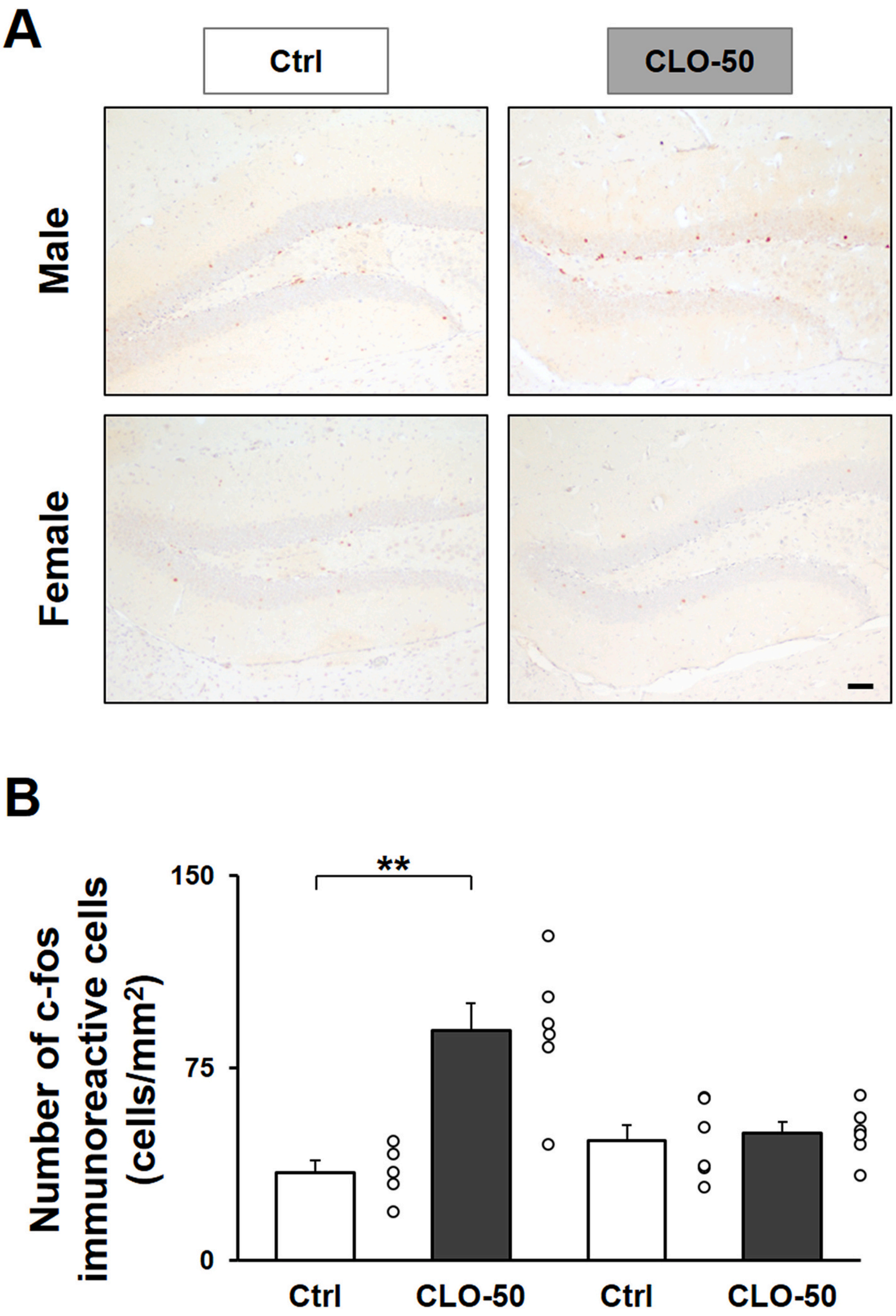

Fig. 5. Sex differences in effects of acute CLO on neuroactivity in the dentate gyrus (DG) in the hippocampus in a Barnes maze test. (A) Representative images of immunoreactivity of c-fos in the DG in male and female mice. (B) Quantitative data on the number of immunoreactive cells per area in the DG analyzed using at least three sections per mouse. Bar = 100 μm. Columns and circles show group mean ± SE and individual values of mice (Ctrl male: n = 5, CTD-50 male: n = 6, Ctrl female: n = 6, CTD-50 female: n = 6); $^{**}p < 0.01$ vs. control groups (two-way ANOVA followed by Tukey's post-hoc test).

Table 1
Blood and urine concentrations of CLO and its metabolites, in ppm.

	Blood			Urine		
	Male	Female	*p*-value	Male	Female	*p*-value
CLO	43.9 ± 4.615	25.1 ± 3.35	*0.0284	437 ± 10.8	337 ± 11.0	**0.00394
dm-CLO	43.6 ± 5.57	31.5 ± 3.38	0.201	578 ± 38.3	439 ± 17.1	*0.0104
dm-dn-CLO	0.287 ± 0.143	0.338 ± 0.00750	0.855	13.9 ± 2.33	10.2 ± 2.06	0.423
dn-CLO	0.538 ± 0.222	0.303 ± 0.0520	0.584	20.3 ± 3.62	18.0 ± 3.44	0.749
CLO-urea	0.123 ± 0.079	0.0368 ± 0.0240	0.304	20.9 ± 2.29	14.5 ± 2.39	0.150
dm-CLO-urea	ND	ND	–	3.90 ± 0.439	1.94 ± 0.395	*0.0163
MNG	0.598 ± 0.270	0.186 ± 0.0253	0.100	13.6 ± 2.24	12.2 ± 3.67	0.337

ND: not detected. $^{*}p < 0.05$, $^{**}p < 0.01$, Mann–Whitney *U* test.
dm-CLO: desmethyl-CLO; dm-dn-CLO: desmethyl-desnitro-CLO; dn-CLO: desnitro-CLO; dm-CLO-urea: desmethyl-CLO-urea; MNG: 1-methyl-3-nitroguanidine.

		Male		Female	
		CLO-5	CLO-50	CLO-5	CLO-50
OF	Total distance	—	⬇	—	—
	Time in center zone	—	—	—	—
EPM	Total arm entry	—	—	—	—
	Time spent in open arm	⬇	⬇	—	⬇
	Neuroactivity in PVT	⬆	⬆	⬆	—
NOR	Total exploration	—	—	—	—
	Exploration of novel object	⬇	⬇	—	—
BM	Latency to escape box		⬆		—
	Traveled distance to escape box		⬆		—
	Neuroactivity in DG		⬆		—

Fig. 6. Summary of sex differences in behavioral effects of acute CLO observed in this study. Arrows show significant differences compared to the control group.

contrast to the results of the open field test, no marked effects of CLO were observed in the total times of exploration, which is an index of locomotor activity, in the novel object recognition test. This discrepancy may be explained by habituation to the apparatus, suggesting that the locomotor suppression caused by CLO may have been apparent in a novel environment. Our results also demonstrated that consecutive exposure to CLO at day3 interfered with spatial learning in the Barnes maze test in male mice but not in females. These findings suggested that CLO acutely decreased recognition memory in a short time in a male-specific manner, resulting in the impairment of the acquisition learning of spatial memory for a long time.

Emerging evidence has presented the various structural and functional differences between the male and female mammalian brains, which could be factors in sensitivity to exogenous chemicals (Weiss, 2011). A series of studies at the cellular level proved that the excitatory effects of neonicotinoids are mediated by various types of mammalian nAChRs, such as α7 (Li et al., 2011), α4β2 (Xiang et al., 2020), and α3β4 (Hirano et al., 2019), which play crucial roles in neurotransmission and synapse plasticity. In both rats and mice, the basal expression of α4β2 nAChRs was higher in most brain regions in females than in males, while the upregulation of nAChRs by repeated nicotine treatment was greater in males than in females (Koylu et al., 1997; Mochizuki et al., 1998). In the present study, to further understand the mechanisms underlying sex-related differences in behavioral outcomes, we analyzed changes in neuroactivities in several brain regions measured by c-fos expression 2 h after the behavioral test. In the elevated plus maze test, male-dominant upregulation by acute CLO exposure was observed in the PVT, which governs emotional responses to fear, anxiety, and stress (Hsu et al., 2014). These results agreed with those of a previous study in which administration of α4β2 nAChR agonists and nicotine increased the number of c-fos–positive cells in the PVT (Jacobsen et al., 2012). A recent study showed that sex-related differences in PVT activation properties contributed to acute and chronic stress responses (Rowson and Pleil, 2021), suggesting that PVT may be involved in sex-related differences in the effects of CLO on emotional behaviors. In addition, our results showed that the neuroactivities of DG, a brain region involved in the learning process in the Barnes maze test (Bott et al., 2016), were male-specifically activated by CLO during the behavioral test. Knockout of β2 nAChRs eliminated the male advantage in performance on the radial arm maze test in mice, suggesting that β2 nAChRs play crucial roles in sex differences in spatial learning and memory (Levin et al., 2009). The α7 nAChRs in the DG contributed to the spatial discrimination task in male but not female mice (Otto and Yakel, 2019). These observations supported the idea that the male-only learning impairment by CLO in mice maybe arise from sex-related responses of the DG. Given that ovarian hormones were also known to modulate nAChR function (Cross et al., 2017), differences in the expression profiles and responses of nAChRs in several brain regions underlie the differences in neurobehavioral effects of CLO between the sexes.

Another question in the present study was whether there were sex-related differences in the metabolism of CLO, so we compared the blood and urine concentrations of CLO and its metabolites between males and females 2 h after the behavioral tests. Previous studies showed that human metabolic enzymes, including CYP3A4, 2C19, and 2A6, in the liver contributed to the metabolism of CLO to dm-CLO (Shi et al., 2009), the main metabolite in both mice and humans (Ohno et al., 2020; Wrobel et al., 2022). In the present study, the blood and urine concentrations of CLO and its metabolites were consistently higher in males than in females. The female-predominant expression of the CYP3A family was reported in both mice and humans (Waxman and Holloway, 2009), and the enzyme activity of coumarin 7-hydroxylase, which is mouse CYP2A5 corresponding to human CYP2A6, was higher in female than in male mice (van Iersel et al., 1994). Our data also showed the ratio of dm-CLO/CLO in the blood in females (1.25) was greater than in males (0.99), suggesting that the metabolic capacity for CLO were higher in female mice compared to male mice. Ford and Casida (2006) demonstrated that orally administered CLO was excreted into urine within an hour of its half-life and that dm-CLO tends to stay in the brain longer than the parent compound. These results suggest that male mice would take longer than females to carry CLO and its metabolites out of the body, leading to male-specific neurobehavioral effects of CLO.

5. Conclusion

The results of the present study demonstrated that acute administration of CLO at the chronic NOAEL dose resulted in sex-specific behavioral effects on locomotor activity, anxiety-like behavior, and spatial learning in mice. Our data also revealed male-dominant changes in neuroactivities in several brain regions, including the PVT and the DG, in addition to greater tissue concentrations of CLO and its metabolites, which could be associated with sex-related differences in behavioral consequences. These results highlight for the first time that sex is a significant factor in sensitivities to behavioral effects of neonicotinoids. To understand the effects of neonicotinoids on neuronal function, further research is needed to assess the effects of the environmental relevant exposure level of neonicotinoids.

Funding

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, awarded to T. Hirano (No. JP19K19406 and JP22K17342), N. Hoshi (No. JP21K19846 and JP22H03750), and Y. Ikenaka (No. JP18H04132 and JP22K18425). We also acknowledge financial support from the Environmental Research Projects from Sumitomo Foundation, Nihon Seimei Foundation, act beyond trust, and Triodos Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Shizuka Kubo: Conceptualization, Methodology, Investigation, Writing – original draft. **Tetsushi Hirano:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Funding acquisition. **Yuka Miyata:** Methodology, Validation. **Shuji Ohno:** Methodology, Validation. **Kanoko Onaru:** Methodology, Validation. **Yoshinori Ikenaka:** Investigation, Supervision, Writing – review & editing, Funding acquisition. **Shouta M.M. Nakayama:** Supervision, Writing – review & editing. **Mayumi Ishizuka:** Supervision, Writing – review & editing. **Youhei Mantani:** Supervision, Writing – review & editing. **Toshifumi Yokoyama:** Supervision, Writing – review & editing. **Nobuhiko Hoshi:** Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

References

Bevins, R.A., Besheer, J., 2006. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat. Protoc. 1, 1306–1311.

Bott, J.B., Muller, M.A., Jackson, J., Aubert, J., Cassel, J.C., Mathis, C., Goutagny, R., 2016. Spatial reference memory is associated with modulation of theta-gamma coupling in the dentate gyrus. Cereb. Cortex 26, 3744–3753.

Burke, A.P., Niibori, Y., Terayama, H., Ito, M., Pidgeon, C., Arsenault, J., Camarero, P.R., Cummins, C.L., Mateo, R., Sakabe, K., Hampson, D.R., 2018. Mammalian susceptibility to a neonicotinoid insecticide after fetal and early postnatal exposure. Sci. Rep. 8, 16639.

Caldarone, B.J., King, S.L., Picciotto, M.R., 2008. Sex differences in anxiety-like behavior and locomotor activity following chronic nicotine exposure in mice. Neurosci. Lett. 439, 187–191.

Cao, J., Belluzzi, J.D., Loughlin, S.E., Dao, J.M., Chen, Y., Leslie, F.M., 2010. Locomotor and stress responses to nicotine differ in adolescent and adult rats. Pharmacol. Biochem. Behav. 96, 82–90.

Costas-Ferreira, C., Faro, L.R.F., 2021. Neurotoxic effects of neonicotinoids on mammals: what is there beyond the activation of nicotinic acetylcholine receptors?—a systematic review. Int. J. Mol. Sci. 22, 8413.

Cross, S.J., Linker, K.E., Leslie, F.M., 2017. Sex-dependent effects of nicotine on the developing brain. J. Neurosci. Res. 95, 422–436.

Elliott, B.M., Faraday, M.M., Phillips, J.M., Grunberg, N.E., 2004. Effects of nicotine on elevated plus maze and locomotor activity in male and female adolescent and adult rats. Pharmacol. Biochem. Behav. 77, 21–28.

Faro, L.R.F., Tak-Kim, H., Alfonso, M., Durán, R., 2019. Clothianidin, a neonicotinoid insecticide, activates α4β2, α7 and muscarinic receptors to induce in vivo dopamine release from rat striatum. Toxicology 426, 152285.

Food and Agriculture Organization of the United Nations, 2016. FAO specifications and evaluations for agricultural pesticide clothianidin. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Specs/Clothianidin2011.pdf.

Ford, K.A., Casida, J.E., 2006. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. Chem. Res. Toxicol. 19, 1549–1556.

Frick, K.M., Gresack, J.E., 2003. Sex differences in the behavioral response to spatial and object novelty in adult C57BL/6 mice. Behav. Neurosci. 117, 1283–1291.

Gómez-Giménez, B., Llansola, M., Hernández-Rabaza, V., Cabrera-Pastor, A., Malaguarnera, M., Agusti, A., Felipo, V., 2017. Sex-dependent effects of developmental exposure to different pesticides on spatial learning. The role of induced neuroinflammation in the hippocampus. Food Chem. Toxicol. 99, 135–148.

Hatchell, P.C., Collins, A.C., 1980. The influence of genotype and sex on behavioral sensitivity to nicotine in mice. Psychopharmacology 71, 45–49.

Hirai, A., Sugio, S., Nimako, C., Nakayama, S.M.M., Kato, K., Takahashi, K., Arizono, K., Hirano, T., Hoshi, N., Fujioka, K., Taira, K., Ishizuka, M., Wake, H., Ikenaka, Y., 2022. Ca^{2+} imaging with two-photon microscopy to detect the disruption of brain function in mice administered neonicotinoid insecticides. Sci. Rep. 12, 5114.

Hirano, T., Yanai, S., Takada, T., Yoneda, N., Omotehara, T., Kubota, N., Minami, K., Yamamoto, A., Mantani, Y., Yokoyama, T., Kitagawa, H., Hoshi, N., 2018. NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. Toxicol. Lett. 282, 57–63.

Hirano, T., Minagawa, S., Furusawa, Y., Yunoki, T., Ikenaka, Y., Yokoyama, T., Hoshi, N., Tabuchi, Y., 2019. Growth and neurite stimulating effects of the neonicotinoid pesticide clothianidin on human neuroblastoma SH-SY5Y cells. Toxicol. Appl. Pharmacol. 383, 114777.

Hirano, T., Miyata, Y., Kubo, S., Ohno, S., Onaru, K., Maeda, M., Kitauchi, S., Nishi, M., Tabuchi, Y., Ikenaka, Y., Ichise, T., Nakayama, S.M.M., Ishizuka, M., Arizono, K., Takahashi, K., Kato, K., Mantani, Y., Yokoyama, T., Hoshi, N., 2021. Aging-related changes in the sensitivity of behavioral effects of the neonicotinoid pesticide clothianidin in male mice. Toxicol. Lett. 342, 95–103.

Hsu, D.T., Kirouac, G.J., Zubieta, J.K., Bhatnagar, S., 2014. Contributions of the paraventricular thalamic nucleus in the regulation of stress, motivation, and mood. Front. Behav. Neurosci. 8, 73.

Jacobsen, J., Hansen, H.H., Kiss, A., Mikkelsen, J.D., 2012. The α4β2 nicotine acetylcholine receptor agonist ispronicline induces c-Fos expression in selective regions of the rat forebrain. Neurosci. Lett. 515, 7–11.

Jonasson, Z., 2005. Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. Neurosci. Biobehav. Rev. 28, 811–825.

Kara, M., Yumrutas, O., Demir, C.F., Ozdemir, H.H., Bozgeyik, I., Coskun, S., Eraslan, E., Bal, R., 2015. Insecticide imidacloprid influences cognitive functions and alters learning performance and related gene expression in a rat model. Int. J. Exp. Pathol. 96, 332–337.

Kenney, J.W., Adoff, M.D., Wilkinson, D.S., Gould, T.J., 2011. The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice. Psychopharmacology 217, 353–365.

Kimura-Kuroda, J., Komuta, Y., Kuroda, Y., Hayashi, M., Kawano, H., 2012. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. PLoS One 7, e32432.

Koylu, E., Demirgören, S., London, E.D., Pöğün, S., 1997. Sex difference in up-regulation of nicotinic acetylcholine receptors in rat brain. Life Sci. 61, 185–190.

Levin, E.D., Petro, A., Rezvani, A.H., Pollard, N., Christopher, N.C., Strauss, M., Avery, J., Nicholson, J., Rose, J.E., 2009. Nicotinic alpha7- or beta2-containing receptor knockout: effects on radial-arm maze learning and long-term nicotine consumption in mice. Behav. Brain Res. 196, 207–213.

Li, P., Ann, J., Akk, G., 2011. Activation and modulation of human α4β2 nicotinic acetylcholine receptors by the neonicotinoids clothianidin and imidacloprid. J. Neurosci. Res. 89, 1295–1301.

Loser, D., Hinojosa, M.G., Blum, J., Schaefer, J., Brüll, M., Johansson, Y., Suciu, I., Grillberger, K., Danker, T., Möller, C., Gardner, I., Ecker, G.F., Bennekou, S.H., Forsby, A., Kraushaar, U., Leist, M., 2021. Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons. Arch. Toxicol. 95, 2081–2107.

Maeda, M., Kitauchi, S., Hirano, T., Ikenaka, Y., Nishi, M., Shoda, A., Murata, M., Mantani, Y., Tabuchi, Y., Yokoyama, T., Hoshi, N., 2021. Fetal and lactational exposure to the no-observed-adverse-effect level (NOAEL) dose of the neonicotinoid pesticide clothianidin inhibits neurogenesis and induces different behavioral abnormalities at the developmental stages in male mice. J. Vet. Med. Sci. 83, 542–548.

Mochizuki, T., Villemagne, V.L., Scheffel, U., Dannals, R.F., Finley, P., Zhan, Y., Wagner Jr., H.N., Musachio, J.L., 1998. Nicotine induced up-regulation of nicotinic receptors in CD-1 mice demonstrated with an in vivo radiotracer: gender differences. Synapse 30, 116–118.

Mukherjee, J., Lao, P.J., Betthauser, T.J., Samra, G.K., Pan, M.L., Patel, I.H., Liang, C., Metherate, R., Christian, B.T., 2018. Human brain imaging of nicotinic acetylcholine α4β2* receptors using $[^{18}F]$Nifene: selectivity, functional activity, toxicity, aging effects, gender effects, and extrathalamic pathways. J. Comp. Neurol. 526, 80–95.

Nagata, K., Song, J.H., Shono, T., Narahashi, T., 1998. Modulation of the neuronal nicotinic acetylcholine receptor-channel by the nitromethylene heterocycle imidacloprid. J. Pharmacol. Exp. Ther. 285, 731–738.

Nishi, M., Sugio, S., Hirano, T., Kato, D., Wake, H., Shoda, A., Murata, M., Ikenaka, Y., Tabuchi, Y., Mantani, Y., Yokoyama, T., Hoshi, N., 2022. Elucidation of the neurological effects of clothianidin exposure at the no-observed-adverse-effect level (NOAEL) using two-photon microscopy in vivo imaging. J. Vet. Med. Sci. 84, 585–592.

Ohno, S., Ikenaka, Y., Onaru, K., Kubo, S., Sakata, N., Hirano, T., Mantani, Y., Yokoyama, T., Takahashi, K., Kato, K., Arizono, K., Ichise, T., Nakayama, S.M.M., Ishizuka, M., Hoshi, N., 2020. Quantitative elucidation of maternal-to-fetal transfer of neonicotinoid pesticide clothianidin and its metabolites in mice. Toxicol. Lett. 322, 32–38.

Otto, S.L., Yakel, J.L., 2019. The α7 nicotinic acetylcholine receptors regulate hippocampal adult-neurogenesis in a sexually dimorphic fashion. Brain Struct. Funct. 224, 829–846.

Ozdemir, H.H., Kara, M., Yumrutas, O., Uckardes, F., Eraslan, E., Demir, C.F., Bal, R., 2014. Determination of the effects on learning and memory performance and related gene expressions of clothianidin in rat models. Cogn. Neurodyn. 8, 411–416.

Rosenfeld, C.S., Ferguson, S.A., 2014. Barnes maze testing strategies with small and large rodent models. J. Vis. Exp. 84, e51194.

Rowson, S.A., Pleil, K.E., 2021. Influences of stress and sex on the paraventricular thalamus: implications for motivated behavior. Front. Behav. Neurosci. 15, 636203.

Shi, X., Dick, R.A., Ford, K.A., Casida, J.E., 2009. Enzymes and inhibitors in neonicotinoid insecticide metabolism. J. Agric. Food Chem. 57, 4861–4866.

Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.

van Iersel, M., Walters, D.G., Price, R.J., Lovell, D.P., Lake, B.G., 1994. Sex and strain differences in mouse hepatic microsomal coumarin 7-hydroxylase activity. Food Chem. Toxicol. 32, 387–390.

Waxman, D.J., Holloway, M.G., 2009. Sex differences in the expression of hepatic drug metabolizing enzymes. Mol. Pharmacol. 76, 215–228.

Weiss, B., 2011. Same sex, no sex, and unaware sex in neurotoxicology. Neurotoxicology 32, 509–517.

Wrobel, S.A., Bury, D., Hayen, H., Koch, H.M., Brüning, T., Käfferlein, H.U., 2022. Human metabolism and urinary excretion of seven neonicotinoids and neonicotinoid-like compounds after controlled oral dosages. Arch. Toxicol. 96, 121–134.

Xiang, D., Xu, X., Zhou, Q., Yan, R., Chen, M., Guo, Y., Zhu, G., 2020. The expression of soluble functional α7-nicotinic acetylcholine receptors in E. coli and its high-affinity binding to neonicotinoid pesticides. Pestic. Biochem. Physiol. 164, 237–241.

Xu, M., Zhang, Z., Li, Z., Kan, S., Liu, Z., Wang, D., Liu, Q., Zhang, H., 2021. Profiles of neonicotinoid insecticides and characteristic metabolites in paired urine and blood samples: partitioning between urine and blood and implications for human exposure. Sci. Total Environ. 773, 145582.

Zhang, D., Lu, S., 2022. Human exposure to neonicotinoids and the associated health risks: a review. Environ. Int. 163, 107201.