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FULL PAPER

Toxicology

Neurotoxicity and behavioral disorders induced in mice by acute exposure to the diamide insecticide chlorantraniliprole

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ABSTRACT. Diamide insecticides activate ryanodine receptors expressed in lepidopteran skeletal muscle and promote Ca^{2+} release in the sarcoplasmic reticulum, causing abnormal contractions and paralysis, leading to death of the pest. Although they had been thought not to act on nontarget organisms, including mammals, adverse effects on vertebrates were recently reported, raising concerns about their safety in humans. We investigated the neurotoxicity of the acute no-observed-adverse-effect level of chlorantraniliprole (CAP), a diamide insecticide, in mice using clothianidin (CLO), a neonicotinoid insecticide, as a positive control. The CLO-administered group showed decreased locomotor activities, increased anxiety-like behaviors, and abnormal human-audible vocalizations, while the CAP-administered group showed anxiety-like behaviors but no change in locomotor activities. The CAP-administered group had greater numbers of c-fos–immunoreactive cells in the hippocampal dentate gyrus, and similar to the results in a CLO-administered group in our previous study. Blood corticosterone levels increased in the CLO-administered group but did not change in the CAP-administered group. Additionally, CAP was found to decreased 3-Methoxytyramine and histamine in mice at the time to maximum concentration. These results suggest that CAP-administered mice are less vulnerable to stress than CLO-administered mice, and the first evidence that CAP exposure increases neuronal activity and induces anxiety-like behavior as well as neurotransmitter disturbances in mammals.

KEYWORDS: anxiety-like behavior, chlorantraniliprole, diamide insecticides, monoamine neurotransmitters, neurobehavioral toxicity

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Diamide pesticides are novel insecticides that target the lepidopteran ryanodine receptor (RyR), and two skeletal formulas, phthalic acid and anthranilic diamides, have been developed [27, 54]. The RyR of the target pest is activated by the binding of diamide pesticides and releases Ca^{2+}, which induces abnormal muscle contraction and paralysis, resulting in the death of the pest [7, 10, 26]. Currently, flubendiamide for phthalic acid diamides and chlorantraniliprole (CAP), cyantraniliprole, cyclaniliprole, and tetraniliprole for anthranilic diamides are deployed in the global market.

Neonicotinoid pesticides are insecticides with strong agonist effects on nicotinic acetylcholine receptors, which were first marketed in 1993 [30, 62]. Neonicotinoids have high binding affinity to insect nicotinic acetylcholine receptors and were originally considered

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safe for mammals [22, 55]. Later, their toxicity to higher vertebrates was reported [8, 17, 18, 20, 21, 23, 25, 33, 49, 50, 59]. Their use is now regulated in the EU and the United States.

In contrast, diamide insecticides are considered highly selective due to the low sensitivity of nontarget insects and vertebrates to RyR [10, 13, 44, 47]. They are attracting attention as alternatives to organophosphorus and neonicotinoid pesticides, which have been disputed as toxic. However, CAP has been reported to cause genotoxic, behavioral, and endocrine effects in aquatic organisms and vertebrates [9, 28, 34, 37, 40, 48, 61], and the binding site of CAP has been identified in mammalian RyR1 [32].

RyR is a voltage-gated Ca^{2+} release channel expressed mainly in the sarcoplasmic reticulum of muscle cells and neurons. Insects have one isoform [43, 52], while mammals have three, which are found in various tissues; RyR1 is primarily expressed in skeletal muscle, RyR2 primarily in cardiac muscle, and RyR3 primarily in the brain [15, 38, 41, 53, 63]. Because RyR plays a vital role in Ca^{2+} signaling in the body, there is concern about its effects on mammals. *In vivo* studies in mammals have found pathological and genetic influences [39]. However, its neurotoxicological effects have not been addressed.

In this study, we examined behavioral and neural activity changes in male mice exposed to acute oral administration of the diamide insecticide CAP. We compared the results with those of mice exposed to clothianidin (CLO), a neonicotinoid insecticide.

MATERIALS AND METHODS

Experimental animals

Male C57BL/6NCrSlc mice (7 to 8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and acclimated for at least 1 week. All mice were maintained in 40.5 × 20.5 × 18.5 cm individually ventilated cages (Green Line IVC Sealsafe Plus Mouse 2GM140; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2°C) and humidity (50 ± 10%) conditions and on a 14-hr/10-hr light/dark cycle at the Kobe University Life-Science Laboratory. They were fed *ad libitum* access to a pellet diet (DC-8; CLEA Japan, Tokyo, Japan) and tap water. This study was approved by the Institutional Animal Care and Use Committee (Permission number: #30-01-01) and carried out according to the Kobe University Animal Experimentation Regulations.

Chemical administration

CAP was purchased from the chlorantraniliprole standard (CAP 98.0+%; Wako Pure Chemical Industries, Osaka, Japan), and CLO was purified from the water-soluble agent Dantotsu® (containing 16% CLO; Sumitomo Chemical, Tokyo, Japan) (95% purity; [17]). Based on the no-observed-adverse-effect level (NOAEL; CLO: 47.2 mg/kg body weight, CAP: 158 mg/kg body weight) determined in an 18-month carcinogenicity test in male mice [3, 11], the compounds were administered by oral gavage at a dose of CLO 0 or 50 mg/kg body weight or of CAP 0 or 160 mg/kg body weight suspended in 0.5% carboxymethylcellulose (10 mL/kg body weight) [18]. They were defined based on the time to maximum concentration and were named CLO-administered group (CLO-0, CLO-50) and CAP-administered group (CAP-0, CAP-160). All chemicals were orally administered to mice under isoflurane anesthesia to prevent stress.

Behavioral analysis

Figure 1 is a schematic of the behavioral tests used in this study. The CLO-administered group was tested 1 hr after chemical administration, and the CAP-administered group was tested 5 hr after chemical administration, calibrated to the time to maximum concentration of each compound (Fig. 1A) [3, 11]. All behavioral tests were conducted during the light period (9:00 a.m.–12:00 p.m.).

Open field test

After the 1 hr (CLO-administered group) or 5 hr (CAP-administered group) single oral administration, the mice were acclimated to the testing room conditions (500 lux, 23 ± 2°C) in their home cages for 1 hr prior to the start of the test, which evaluated locomotor activity and anxiety-like behavior of the mice as described in our previous study [25]. Briefly,

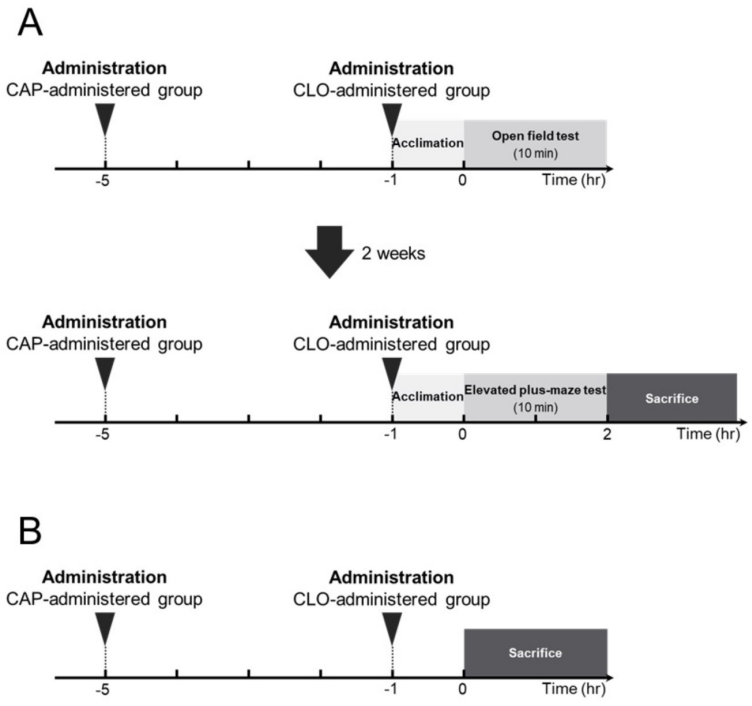

Fig. 1. Schematic diagram representing the experimental design of the present study. (**A**) The experimental schedule of the groups subjected to the behavioral tests at the time to maximum concentration of each compound. All mice were acclimated to the laboratory conditions for 1 hr, and behavioral tests were conducted for 10 min. The open field test was conducted first, followed by the elevated plus-maze test 2 weeks later. All mice were euthanized 2 hr after completing the elevated plus-maze test. (**B**) Experimental schedule of groups euthanized at the time to maximum concentration of each compound.

the mouse was placed in a corner of an open field (60 × 60 × 40 cm, Tom Products Co., Tokyo, Japan) with its nose pointed at the wall and was then free to explore. All of the mouse's activities were recorded by a video camera for the subsequent 10 min, and the total distance traveled was used as an indicator of locomotor activity, and the time spent in the center zone (30 × 30 cm) was used as an indicator of anxiety-like behavior, were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with the ImageOF plugin.

Elevated plus-maze test

After the 1 hr (CLO-administered group) or 5 hr (CAP-administered group) single oral administration, anxiety-like behavior of mice in a stressful environment was examined by an elevated plus-maze test, which was conducted as described elsewhere [16]. Briefly, the mice were acclimated to laboratory conditions (10–20 lux, 23 ± 2°C) in their home cages for 1 hr prior to the start of the test. The behavioral test apparatus consisted of two opposite open arms (30 × 5 cm) without walls and two opposite enclosed arms (the same size, with walls 15 cm high) extending from a common center platform (5 × 5 cm) made of white acrylic panels (Tom Products Co.). The mouse was placed on the center zone facing an open arm, and all its activities were recorded for the subsequent 10 min. The total distance traveled was used as an indicator of locomotor activity, the time spent in the open arms, the percentage of open arms entries [number of open arms entries / number of total arms entries], and the time spent in the center zone were used as indicators of anxiety-like behavior, were scored by ImageJ software with the ImageEP plugin.

Vocalization analysis

During all behavioral tests in all groups, mouse vocalizations were recorded with a pulse code modulation recorder (96 kHz sampling rate, 24 bit depth) to visualize the chirp-like vocalizations emitted by CLO-administered mice in the human-audible range (≤20 kHz). Frequency spectra (FFT length=256, frame size=100%, overlap=75%, cutoff frequency <3 kHz) were generated with SAS Lab software (Avisoft Bioacoustics, Berlin, Germany). Mice usually communicate socially by vocalizing at high frequencies (>20 kHz) and do not vocalize in the human-audible range (≤20 kHz) except when restrained by instruments or under conditions of pain or fear [14]. We previously reported that CLO single-dose mice produce a new class of abnormal vocalizations consisting of overtones in the human-audible range below 20 kHz under novel environmental conditions [18]. In the present study, we used the abnormal vocalizations of mice as an indicator of stress vulnerability.

Immunohistochemical analysis

Immunohistochemical analyses using c-fos as a neuroactivity marker were performed according to our previous report [18]. After 2 hr of the elevated plus-maze test, all mice were deeply anesthetized with isoflurane using an inhalation anesthesia apparatus (BS-400T; Brain Science Idea, Osaka, Japan), and euthanized by whole blood collection from the heart (Fig. 1A). The brains were removed and immersed in 0.9% saline solution. A brain slicer (Muromachi Kikai, Tokyo, Japan) was used to make 3-mm-thick slices (around bregma −1.70 mm), and the slices were immersed and fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) (20 hr, 4°C). Following this period, the slices were cryoprotected in ascending solutions of sucrose (10%, 20%, 30%) in 0.1 M phosphate buffer overnight and then frozen quickly in liquid nitrogen in an embedding solution consisting of Tissue-Tek® O.C.T. compound (Sakura Finetek, Tokyo, Japan). The prepared frozen blocks were sliced into 30-µm-thick coronal sections on a cryostat (CM1950; Leica Biosystems, Wetzlar, Germany), and the sections were placed in 12-well inserts filled with 0.01 M phosphate-buffered saline with 0.05% Tween-20 (Wako Pure Chemical Industries) (PBST). Using the free-floating method, the sections were washed three times with PBST, and endogenous peroxidase was removed with 100% methanol (30 min, room temperature) and 0.5% hydrogen peroxide (light-shielded, 30 min, room temperature). After the sections were washed twice with distilled water and once with PBST, they were reacted with Blocking One Hist (Nacalai Tesque, Kyoto, Japan) (1 hr, room temperature). Next, rabbit polyclonal anti-c-fos antibody (sc-52; Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:10,000 in PBST was reacted (18 hr, 4°C) and washed four times with PBST. The sections were then reacted with HRP-labeled dextran polymer conjugated anti-rabbit IgG goat antibody (EnVision$^+$; Dako, Glostrup, Denmark) (1 hr, room temperature). After the sections were washed three times with PBST, they were replaced with 50 mM Tris buffer (pH 7.6) and incubated with DAB (3,3′-diaminobenzidine-tetrachloride) solution (EnVision$^+$ kit/HRP[DAB]; Dako). The sections were washed once with distilled water and twice with PBST, mounted onto glass slides, and air-dried overnight. The sections were counterstained with Meyer's hematoxylin solution. The sections were dehydrated in ethanol (70%, 80%, 90%, 99%, 100%), permeabilized with xylene, and coverslipped with Eukitt mounting medium (O. Kindler, Freiburg, Germany).

The slide-mounted sections were photographed with a BX61 microscope equipped with a DP-70 digital camera (Olympus Japan, Tokyo, Japan). The number of c-fos–immunoreactive cells per area (per 1 mm^2) was calculated using ImageJ software and by fixing the threshold value. We averaged the number of c-fos–immunoreactive cells in both cerebral hemispheres using at least three sections per mouse. The right or left hemisphere was quantified if the tissue was torn and could not be measured.

Endocrinological analysis

The plasma endocrine substances analyzed were monoamines (dopamine, 3-Methoxytyramine, histamine and serotonin), and steroid hormones (testosterone and corticosterone). When the time to maximum concentration of each compound was reached, all mice were euthanized by whole blood collection from the heart (Fig. 1B). Blood collected in this procedure and after the elevated plus-maze test was centrifuged (3,000 rpm, 10 min, 4°C) to obtain plasma. For the analysis of the above substances, Liquid chromatography–electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) was performed. The analytes were detected by electrospray ionization (ESI) in positive ion mode.

LC-ESI/MS/MS measurement conditions of monoamines

The analytical method for monoamines was performed according to our previously reported method [16]. In brief, 50 µL of the plasma was thoroughly mixed with 20 µL of IS mix (1 µg/mL in 70% methanol containing 0.1% formic acid). Then, 50 µL of ice-cold acetonitrile containing 0.05% formic acid was added, and the mixture was centrifuged (10,000 × *g* , 10 min, 25°C). Subsequently, 10 µL of the supernatant was added 10 µL of the 2,4-diphenyl-pyranylium (DPP) mix, and the derivatization reaction was carried out in a thermal cycler (4 hr, 60°C). After completion of the reaction, 80 µL of distilled water was added, and the target substances were measured by LC-ESI/MS/MS. An Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for LC, and an Agilent 6495B triple quadrupole mass spectrometer (Agilent Technologies) was used for MS as described elsewhere [16].

LC-ESI/MS/MS measurement conditions of steroid hormones

The analytical method for steroid hormones was according to our previously reported method [40]. In brief, the 25 µL of plasma was thoroughly mixed with 10 µL of IS mix and 90 µL of acetonitrile containing 1% formic acid, and the mixture was centrifuged (10,000 × *g*, 10 min, 25°C). After that, 100 µL of the supernatant was loaded into the MonoSpin phospholipid column (GL Science, Tokyo, Japan). The eluate was dried at 60°C using the speed vacuum evaporation system (CVE-2000D; EYELA, Bohemia, NY, USA). Then, 100 µL of 50% methanol-double distilled water containing 0.1% formic acid was added, and the target substances were measured by LC-ESI/MS/MS. An Agilent 1260 Infinity II HPLC system (Agilent Technologies) was used for LC, and an Agilent 6495B triple quadrupole mass spectrometer (Agilent Technologies) was used for MS as described elsewhere [40].

Statistical analysis

Statistical analyses were performed with BellCurve for Excel (Version 3.23; SSRI, Tokyo, Japan). Welch's *t*-test was employed to detect any significant differences in the behavioral assessments, endocrinological measurements and quantitative immunohistological results between the control and administration groups. Correlations between behavioral measures and plasma testosterone were assessed using Spearman's rank correlation coefficient and the test of no correlation.

RESULTS

Behavioral analysis

Behavioral effects of CLO- or CAP-exposed mice in open field test: To evaluate locomotor activity and anxiety-like behavior induced by CLO or CAP, an open field test was first conducted (Fig. 2). The trajectory map in Fig. 2A shows the exploratory behavior of mice in the open field test for 10 min. The total distance traveled was significantly decreased in the CLO-50 group ($P<0.01$), but there was no significant difference in the CAP-administered group (Fig. 2B). The time spent in the center zone was significantly decreased in the CLO-50 group ($P<0.05$), but there was no significant difference in the CAP-administered group (Fig. 2C).

Behavioral effects of CLO- or CAP-exposed mice in the elevated-plus maze test: To evaluate locomotor activity and anxiety-like behaviors induced by CLO or CAP, an elevated plus-maze test was conducted. The trajectory map in Fig. 3A shows the exploratory behavior of mice in the elevated plus-maze test for 10 min. The total distance traveled was significantly decreased in the CLO-50 group ($P<0.05$). However, there was no significant difference in the CAP-administered group (Fig. 3B). The time spent in the open arms and the percentage of open arms entries were not different in the CLO-administered group, although there were decreasing trends in the CAP-160 group ($P=0.104$, $P=0.058$, Fig. 3C and 3D). The time spent in the center zone trended toward a decrease in the CLO-50 group ($P=0.065$) and significantly decreased in the CAP-160 group ($P<0.01$, Fig. 3E).

Mouse vocalization analysis during behavioral tests: In each behavioral test, human-audible vocalizations of mice were observed only in the CLO-50 group and not in the CLO-0, CAP-0 or CAP-160 group (Fig. 3F and 3G). The vocalizations of mice were observed in 6 of 10 cases in the open field test and in 9 of 10 cases in the elevated plus-maze test. Voice analysis of recorded mouse vocalizations demonstrated frequencies of 4 and 8 kHz (Fig. 3G).

Neuronal activity in CAP-exposed mice after behavioral test: To identify brain regions potentially associated with behavioral effects observed in the elevated plus-maze test in CAP-administered mice, c-fos expression, a marker of neural activity, was visualized in the CAP-0 and CAP-160 groups through immunohistochemistry. The hippocampal dentate gyrus (DG) granule cell layer, where RyR1 and RyR2 are highly expressed in mice, was analyzed (Fig. 4A) [12]. Both groups exhibited c-fos–immunoreactive cells. Compared with the CAP-0 group, the number of c-fos–immunoreactive cells significantly increased in the CAP-160 group ($P<0.01$, Fig. 4B and 4C).

Endocrinological analysis

Changes in plasma endocrine levels in CLO- or CAP-exposed mice after behavioral test: 3-Methoxytyramine and serotonin were not significantly different in all groups after the elevated plus-maze test (Fig. 5A and 5C). Histamine was significantly increased in the CLO-50 group ($P<0.05$), but there was no significant difference in the CAP-administered group (Fig. 5B). Dopamine was not detected in any of the groups. Corticosterone was significantly increased in the CLO-50 group ($P<0.05$), but there was no significant difference in the CAP-administered group (Fig. 5D). There were little correlations between any of the behavioral parameters and testosterone in the CAP-0, CAP-160, CLO-50 groups (Fig. 5E, Supplementary Fig. 1). In the CLO-0 group, there was a positive correlation between each of the behavioral parameters and testosterone, but the testosterone levels in these mice were shown to be bipolar, which suggest that the behavior of the two out of eight high-testosterone (>10 ng/mL) mice reinforced the correlation (Supplementary Fig. 1).

Changes in plasma endocrine levels in CLO- or CAP-exposed mice at the time to maximum concentration: There were no significant differences in 3-Methoxytyramine, histamine, or serotonin in the CLO-administered group (Fig. 6A–C). 3-Methoxytyramine showed

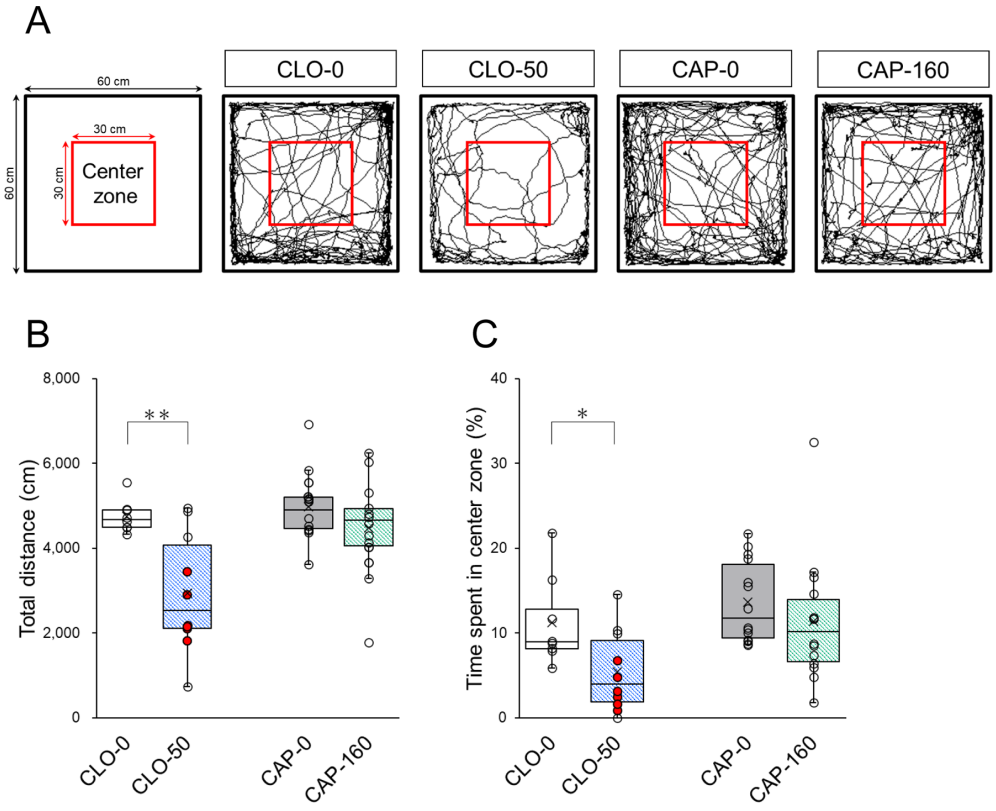

Fig. 2. Behavioral evaluation of CLO- or CAP-administered mice in the open field test. (**A**) Representative trajectory maps of exploratory behaviors in the open field test. The red square indicates the center zone. (B) The total distance and (**C**) the time spent in the center zone are shown in box-and-whisker plots. The CLO-50 group show a significant decrease in the total distance and the time spent in the center zone, but the CAP-administered group showed no difference. Circles show the values for individual mice, and red circles show abnormal vocalizations in mice (CLO-0: n=8, CLO-50: n=10, CAP-0: n=14, CAP-160: n=14), *: $P<0.05$, **: $P<0.01$ vs. each control group (Welch's *t*-test). ×: the mean value.

a decreasing trend ($P=0.081$, Fig. 6A), and histamine significantly decreased in the CAP-160 group ($P<0.05$, Fig. 6B). However, there was no significant difference in serotonin (Fig. 6C). Dopamine was not detected in any of the groups. Corticosterone tended to increase in the CLO-50 group ($P=0.055$), but there was no significant difference in the CAP-administered group (Fig. 6D).

DISCUSSION

Diamide insecticides are thought to primarily affect lepidopteran RyR without affecting nontarget organisms including mammals. This was proposed due to the specific amino acid sequence of lepidopteran RyR, which has less than 50% amino acid sequence homology with mammalian RyR [29, 43]. There have been reports of point mutations in lepidopteran RyR that cause insecticide resistance [45, 56, 58]. However, recent reports have raised concerns about the adverse effects of diamide insecticides on mammals [6, 9, 28, 32, 34, 39, 48, 61]. In this study, we investigated the behavioral effects, neuronal activity, and endocrinological changes in male mice after a single administration of the NOAEL level of CLO or CAP and found that CAP affects the nervous system of mice.

Previous studies have suggested that testosterone has anxiolytic-like effects in mice and rats [2, 19]. Our data showed fluctuations in plasma testosterone levels in mice subjected to behavioral tests, which led to concerns about the validity of anxiety evaluation in mice with high testosterone levels. However, plasma testosterone levels showed little correlation with behavioral parameters in the elevated plus-maze test. Therefore, we considered the results of the behavioral analysis of all mice to be valid.

Acute CLO administration in mice decreased locomotor activity, increased anxiety-like behavior, and caused abnormal vocalizations. These results are consistent with a previous report in which mice were subjected to the elevated plus-maze test after acute CLO administration [18]. To our knowledge, CAP has not been reported to affect neurological function in mammals. Interestingly, the present study revealed that CAP induces anxiety-like behavior in mice. The CLO-50 group showed significantly decreased locomotor activity in the open field and the elevated plus-maze tests, suggesting that the increase in anxiety-like behavior may be a result of motor dysfunction. However, abnormal human-audible vocalizations were observed in the CLO-50 group but not in the CAP-160 group. This is supported by the significant increase in plasma corticosterone in the CLO-50 group after the behavioral test, in contrast with the absence of change in the CAP-administered group. Additionally, the CLO-50 group showed an increasing trend in plasma corticosterone concentration at the time to maximum concentration, suggesting that CLO administration is stressful for mice even in

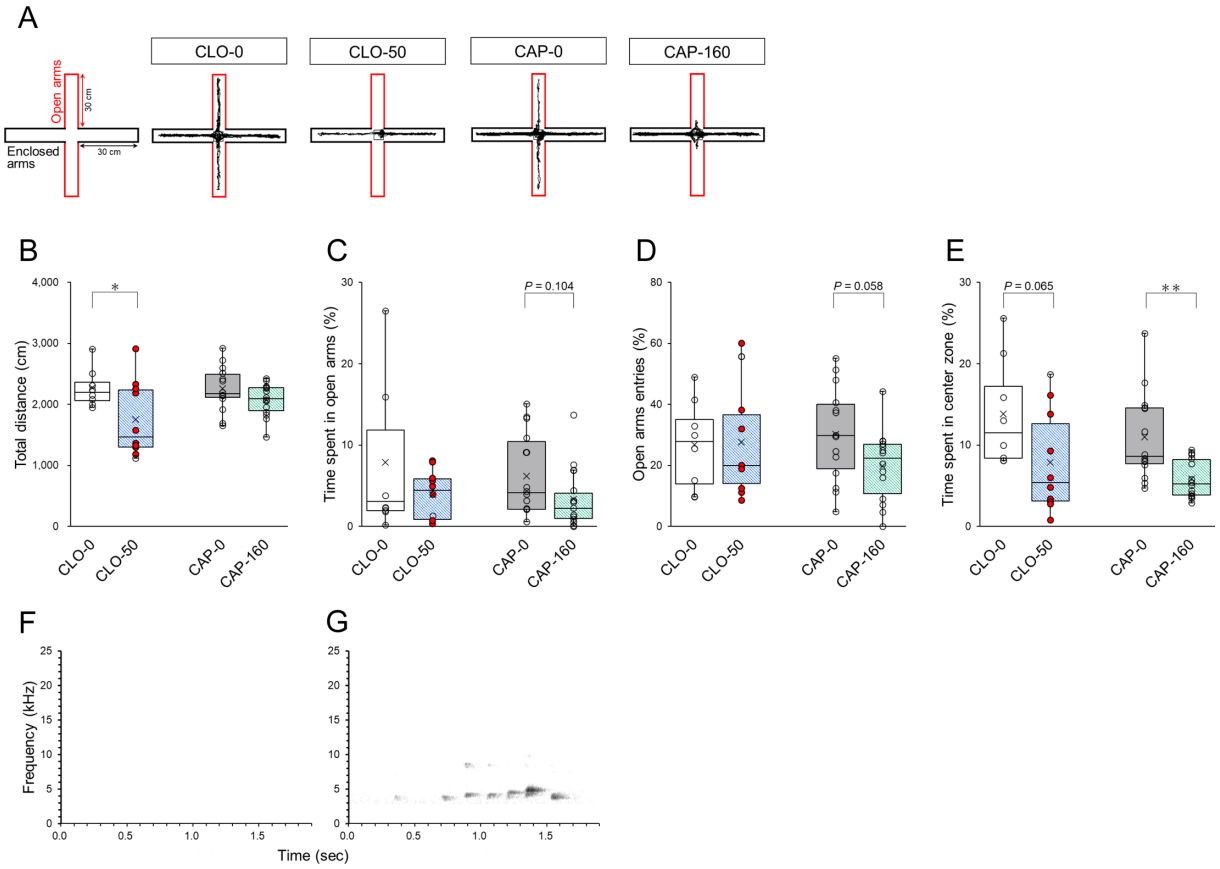

Fig. 3. Behavioral evaluation of CLO- or CAP-administered mice in the elevated plus-maze test. (**A**) Representative trajectory maps of exploratory behaviors in the elevated plus-maze test. The upper and lower red lines indicate open arms, and the left and right black lines indicate enclosed arms. (**B**) the total distance, (**C**) the time spent in the open arms, (**D**) the open arms entries, and (**E**) the time spent in the center zone are shown in box-and-whisker plots. Total distance significantly decreased in the CLO-50 group, but there was no difference in the CAP-administered group. There was a trend toward a decrease in the time spent in the center zone in the CLO-50 group. There were trends toward a decrease in the time spent in the open arms and the open arms entries in the CAP-160 group, and the time spent in the center zone significantly decreased in the CLO-50 group. Circles show the values for individual mice, and red circles show abnormal vocalizations in mice (CLO-0: n=8, CLO-50: n=10, CAP-0: n=14, CAP-160: n=14), *: $P<0.05$, **: $P<0.01$ vs. each control group (Welch's *t*-test). ×: the mean value. Representative spectrograms of mouse vocalizations in (**F**) the CLO-0 group and (**G**) the CLO-50 group during the behavioral tests. The abnormal vocalizations observed were at the frequencies of 4 and 8 kHz.

the absence of novel environmental conditions.

Diamide insecticides enhance Ca^{2+} release from RyR expressed in the muscles of lepidopterans, leading to sustained muscle contraction. The acute administration of CAP (160 mg/kg) did not seem to affect skeletal muscle contraction in mice, since there was no change in locomotor activity in the CAP-160 group. RyR1 is mainly expressed in the sarcoplasmic reticulum of mammalian skeletal muscle and plays a role in excitation–contraction coupling [53, 63]. Humans with specific genetic mutations in RyR1 are known to develop malignant hyperthermia (MH), a condition in which exposure to volatile anesthetics or other triggering agents enhances Ca^{2+} release and causes generalized muscle contraction. In studies using malignant hyperthermia susceptibility (MHS) mice transgenic with RyR1 mutations, the core body temperature of mice under heat stress conditions is measured to evaluate the heat stress response. It has been reported that CAP administration does not affect the change in core temperature during heat stress in wild-type and MHS mice [57]. This suggests that CAP does not cause motor dysfunction in skeletal muscle via enhanced Ca^{2+} release from RyR1.

It has been reported that RyR3 knockout mice exhibit increased locomotor activity and decreased anxiety-like behavior [24, 51]. However, in a report in which RyR3 knockout mice were subjected to several behavioral tests, there were no significant differences in indicators of anxiety-like behavior [35]. Those authors suggested that RyR3 is not involved in the regulation of anxiety-like behavior. In the present study, the absence of change in locomotor activity in the CAP-160 group suggests that CAP has no effect on, or is insensitive to, RyR3, which is highly expressed in the brain. Therefore, Ca^{2+} signaling via RyR1 or RyR2 may be the primary cause of anxiety-like behavior observed in this study. The CAP binding site in rabbit RyR1 has been identified and shown to open the channel [32]. Atrioventricular block and bradycardia have been reported in humans who had ingested large doses of CAP products [4, 36]. Therefore, we speculate that CAP acts on RyR1 and RyR2.

The hippocampus is vital for cognitive and emotional functions as well as stress responses. RyR1 and RyR2 are highly expressed

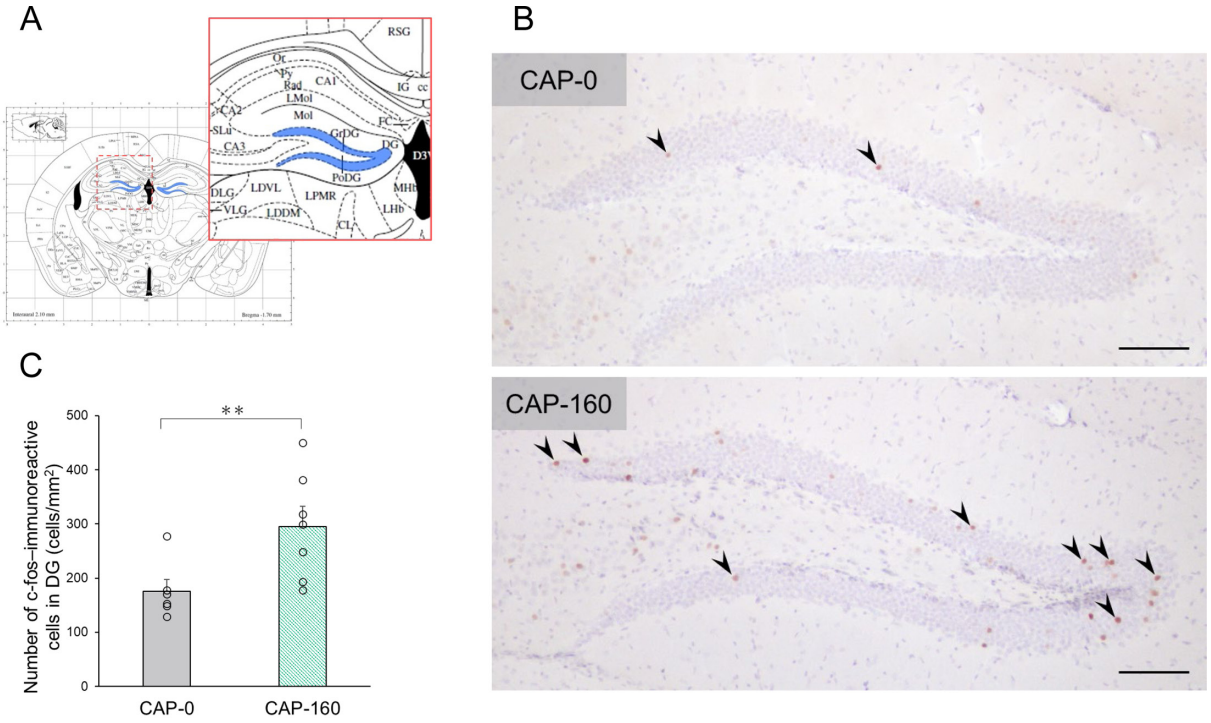

Fig. 4. Evaluation of neuronal activity by immunohistochemistry. (**A**) The granule cell layer of the hippocampal dentate gyrus (DG) is based on the brain atlas [42] (blue area). (**B**) Representative images of immunoreactivity of c-fos in the DG in the CAP-administered group. The arrowheads show representative c-fos–immunoreactive cells. Scale bars=100 µm. (**C**) Bar graph showing the number of c-fos–immunoreactive cells per area in the DG. The number of c-fos–immunoreactive cells was significantly increased in the CAP-160 group. Circles show individual values (CAP-0: n=6, CAP-160: n=7). Data represent the mean + SD, **: $P<0.01$ vs. each control group (Welch's *t*-test).

in DG in mice, and it has been shown that the leakage of Ca^{2+} through RyR2 is involved in stress-induced cognitive dysfunction [12, 31]. In DG granule cells in the CAP-160 group, c-fos–immunoreactive cells were significantly increased after the elevated plus-maze test, indicating that CAP enhances neuronal activity. It has been reported that activation of voltage-gated L-type Ca^{2+} channel promotes c-fos expression and that intracellular Ca^{2+} regulation of RyR is largely involved in this pathway [5]. Thus, hippocampal c-fos expression by CAP administration may be due to RyR activation.

Mice administered imidacloprid (one of the neonicotinoid insecticides) showed decreased plasma and brain histamine levels [16]. In the present study, plasma histamine increased in the CLO-50 group after the elevated plus-maze test. These findings suggest that neonicotinoids affect histamine synthesis, but even insecticides with similar mechanisms of action on insects may have different effects depending on the specific compound. Plasma 3-Methoxytyramine and histamine were decreased at the time to maximum concentration of CAP, which differed from the results for plasma collected 2 hr after the behavioral test. This discrepancy could be because the CAP-0 group was also affected by the stress induced by the behavioral test, resulting in monoamine fluctuation, causing no significant difference to be observed, or it could be due to differences in the duration since administration of the compound at the time of blood collection. Histamine functions as a neurotransmitter in addition to contributing to inflammation and allergic reactions. Decreased histamine in the brain has been reported to decrease locomotor activity, increase anxiety-like behavior, and impair cognitive function [1, 46, 60]. These findings suggest that histamine levels in the brain modulate behavioral effects. Depletion of histamine by more than half resulted in decreased locomotor activity and increased anxiety-like behavior, while depletion by one-third resulted in only increased anxiety-like behavior [46, 60]. Taken together, CAP inhibited histamine synthesis through some mechanism and induced anxiety-like behavior.

In summary, these findings demonstrate that diamide insecticides affect neural activity and induce neurotransmitter disturbances in mice. As mentioned above, the structure of RyR is thought to be highly related to the binding affinity of diamide insecticides, and it is unclear whether the same phenomenon is induced in humans as it was in this study. Considering that humans are chronically exposed to various chemicals including diamide insecticides in their daily lives, further studies are needed.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest associated with this manuscript.

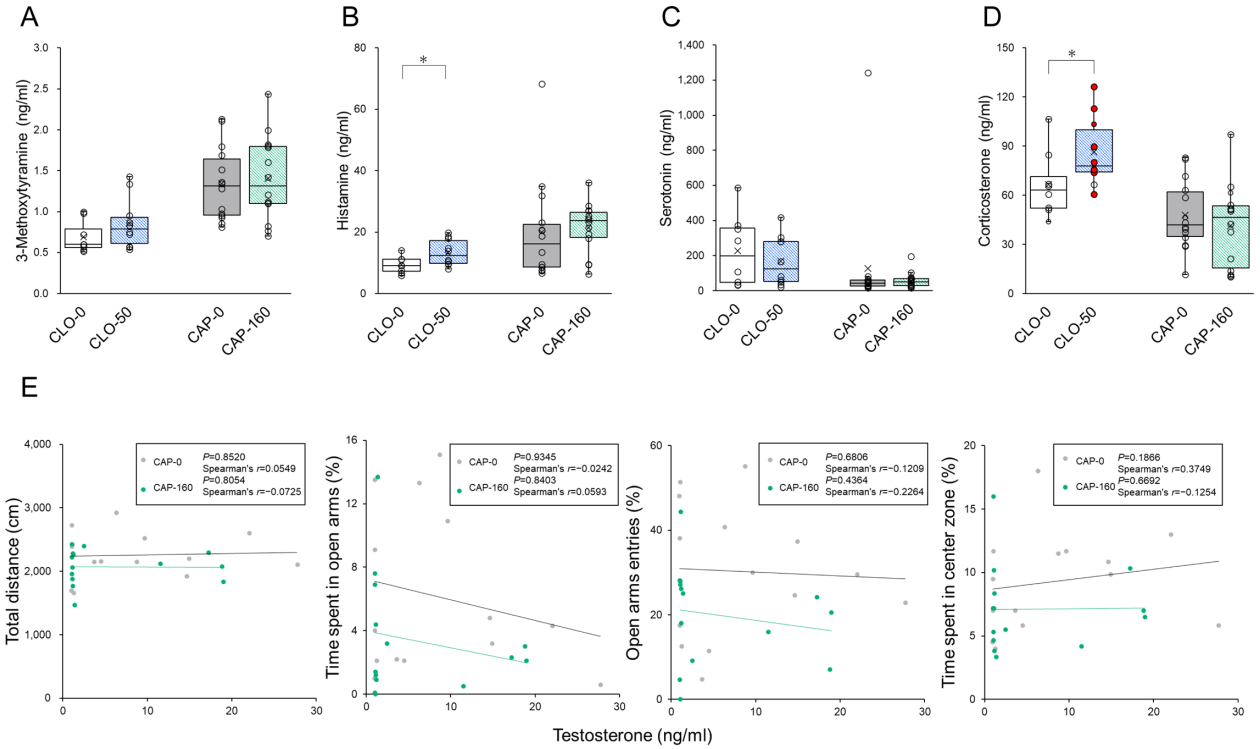

Fig. 5. Plasma endocrine substances in CLO- or CAP-administered mice after the elevated plus-maze test. (**A**) 3-Methoxytyramine, (**B**) histamine, (**C**) serotonin, and (**D**) corticosterone are shown in box-and-whisker plots. Plasma histamine and corticosterone were significantly increased in the CLO-50 group, and there was no difference in the CAP-administered group. Black circles and red circles show individual values and abnormal vocalizations in mice (CLO-0: n=8, CLO-50: n=10, CAP-0: n=14, CAP-160: n=14), *: $P<0.05$ vs. each control group (Welch's *t*-test). ×: the mean value. (**E**) Correlations between behavioral measures and plasma testosterone in the CAP-0 and the CAP-160 groups are shown. There was little correlation between the behavior and testosterone levels of the mice in either group (CAP-0: n=14, CAP-160: n=14, Spearman's rank correlation coefficient and test of no correlation).

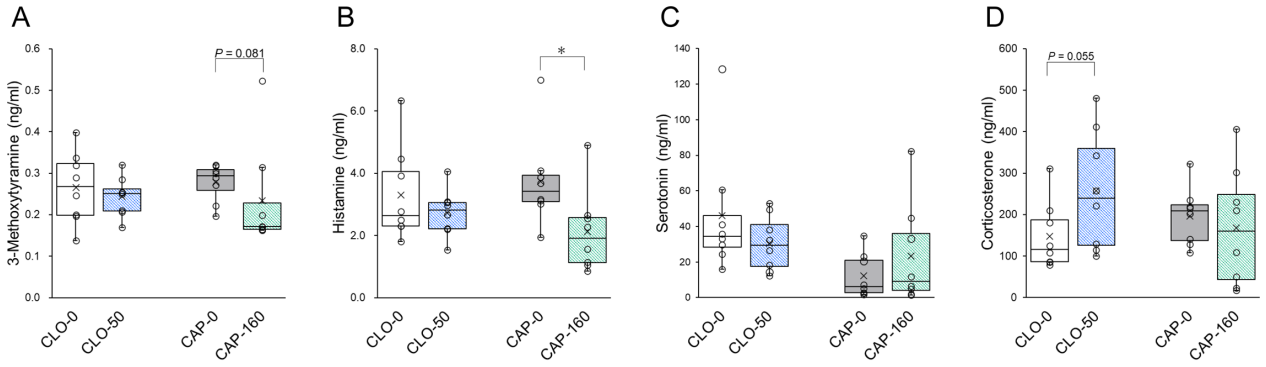

Fig. 6. Plasma endocrine substances in CLO- or CAP-administered mice at the time to maximum concentration. (**A**) 3-Methoxytyramine, (**B**) histamine, (**C**) serotonin, and (**D**) corticosterone are shown in box-and-whisker plots. Corticosterone showed an increasing trend in the CLO-50 group, 3-Methoxytyramine showed a decreasing trend in the CAP-160 group, and histamine significantly decreased in the CAP-160 group. Black circles show individual values (CLO-0: n=8, CLO-50: n=8, CAP-0: n=8, CAP-160: n=8), *: $P<0.05$ vs. each control group (Welch's *t*-test). ×: the mean value.

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