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FULL PAPER

Toxicology

Developmental stage-specific exposure and neurotoxicity evaluation of low-dose clothianidin during neuronal circuit formation

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ABSTRACT. Neonicotinoid pesticides (NN) were recently reported to exhibit adverse effects in higher vertebrates. Moreover, NNs are routinely transferred from mother to offspring, raising concerns about their effects on future generations. The fetal and neonatal periods are the most critical to the formation of neural circuits in the brain through neurogenesis and differentiation, neuronal migration, axon guidance, and synaptogenesis. NN exposure throughout the fetal and neonatal periods was found to affect the neurobehavior of the offspring, but the stage-specific neurobehavioral effects are unclear. We exposed fetal and neonatal mice to a no-observed-adverse-effect level (NOAEL) of clothianidin (CLO) for 4 days during each of four developmental stages: neurite proliferation and differentiation (fetal days 9–12, CLO-1), neurite outgrowth (fetal days 15–18, CLO-2), synapse formation and astrocyte differentiation (days 1–4 after birth, CLO-3), and synapse remodeling (days 11–14 after birth, CLO-4). CLO's neurobehavioral effects were evaluated in juveniles and adults, revealing that CLO-1 and CLO-2 caused behavioral abnormalities in adult mice. CLO-3 significantly increased locomotor activity and decreased juvenile neurons in the hippocampal dentate gyrus in adulthood. Comprehensive gene analysis of CLO-3 revealed high expression of genes related to neurite outgrowth and axonal branching in the hippocampus in juveniles and adults. These results revealed developmental stage-specific effects of a NOAEL of CLO in the fetal and neonatal periods, suggesting that the susceptibility of the fetus and neonate to CLO varies by developmental stage.

KEYWORDS: behavioral test, critical period, developmental stage, fetal and neonatal exposure, neonicotinoid

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It has been believed that both environmental and genetic factors have been implicated in developmental disorders, and exposure to environmental chemicals increases the risk of these disorders [19, 20]. In 2012, the American Academy of Pediatrics (AAP) warned that pesticides, environmental chemicals, are associated with developmental disorders, brain tumors, asthma, and low birth

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weight [7, 30]. In fact, it was shown that children exposed to organophosphorous pesticides have an increased risk of attention-deficit hyperactivity disorder (ADHD) [1] and that organophosphorous pesticides are dangerous for children's brain development [11]. In 2019, researchers in the United States showed that pyrethroids, organophosphorous, and organochlorine pesticides increase the risk of ADHD and autism [31], and the involvement of pesticides in developmental disorders is becoming clear. Neonicotinoid pesticides (NNs), which are used in large quantities along with organophosphorous pesticides, were identified by the European Food Safety Authority (EFSA) in 2013 as having the potential for developmental neurotoxicity [6]. There is an urgent need to clarify the effects of NNs on child development.

NNs are nicotinic analogues developed as alternatives to organophosphorous pesticides, and they exhibit insecticidal activity by continuously exciting and disturbing neurons as competitive modulators of insect-type nicotinic acetylcholine receptors (nAChRs). NNs were thought to be safe for mammals because their affinity for nAChRs is tens to hundreds of times higher than that of mammals [38]. Recently, however, studies exposing birds and mammals to no-observed-adverse-effect-levels (NOAELs) of NNs have revealed that NNs affect reproduction, the thymus, gut flora, and neurobehavior in higher vertebrates [4, 14, 21, 23, 26]. Moreover, it is clear that NNs are routinely transferred from mother to offspring, considering the rapid transfer of clothianidin (CLO), a type of NN, and its metabolites from mother to fetus via the placenta [27]; the metabolism and concentration of CLO in mothers and its rapid transfer into breast milk [34]; and the detection of NNs in the urine of Japanese adults, children, and newborns [17, 18, 28, 39]. The fetal and neonatal periods correspond to the most important stages in the development of brain functions. Although the timing of development varies depending on the brain region, neurogenesis, neuronal migration, neurite and axon elongation, synapse formation and pruning, etc., occur during the prenatal and early postnatal periods [5, 8, 36]. These periods correspond to critical stages of high plasticity of neural circuits [3], when the blood–brain barrier is still underdeveloped and greatly affected by chemical substances and the surrounding environment [32]. The neurodevelopmental toxicity of exposure to NNs during this period has been reported in experiments using mice [16, 23, 25, 33], and there is no doubt that NNs will affect the brain nervous system of the next generation.

The environment, such as exposure to pesticides and other chemicals, during the fetal and neonatal periods influences future health and may lead to diseases in adulthood. In fact, it has been shown that exposure to CLO throughout the fetal and neonatal periods inhibits neurogenesis and causes different behavioral abnormalities in juveniles and adults [23]. On the other hand, the developmental stage-specific effects of NN exposure during these periods are not clear. In this study, we aimed to clarify the critical windows for neural circuit formation in the brain during the fetal and neonatal periods, and we exposed mice to a NOAEL dose of CLO at four different stages during these periods. We then focused on the behavior and nervous system of the exposed juveniles and adults, investigated the time points at which NNs have the greatest influence on brain development in the next generation of mice, and discussed the details of this influence.

MATERIALS AND METHODS

Animals

C57BL6/N mice (Japan SLC, Hamamatsu, Japan) at 1.5 days (n=11) and 14.5 days (n=25) gestation were purchased and bred according to a previous report [12]. Eleven mothers and 26 male offspring were used for the experiments during the fetal period, and 25 mothers and 78 male offspring were used for the experiments during the neonatal period. Of the 78 offspring, 55 were used for behavioral analysis and 23 for gene expression analysis. This study was approved by the Institutional Animal Care and Use Committee (Permission #30-01-01) and carried out according to the Kobe University Animal Experiment Regulations.

Administration of CLO

To examine the effects of fetal exposure, CLO (95% purity: [13]) was administered to the mothers at fetal days 9–12 during the neurite proliferation and differentiation period or at fetal days 15–18 during the neurite outgrowth period. As in a previous study, rehydration gel was used to avoid physical stress that could affect parturition [23]. The offspring of the mothers administered CLO on fetal days 9–12 were designated CLO-1, and those of the mothers administered CLO on fetal days 15–18 were designated CLO-2. Next, to examine the effects of neonatal exposure, CLO or solvent (0.5% carboxymethylcellulose, 10 mL/kg) was administered to female offspring at days 1–4 after birth during the synaptogenesis and astrocyte differentiation period or at days 11–14 after birth during the synaptic remodeling period. Maternal mice were orally administered with reference to the NOAEL dose at a volume of 0 or 65 mg/kg body weight on each of 4 consecutive days. Mouse pups treated on days 1–4 after birth were designated CLO-3, and those treated on days 11–14 after birth were designated CLO-4. Two or three pups per litter were used in order to avoid litter bias.

Behavioral tests

The male pups were subjected to the open field test (OF) and the elevated plus maze test (EPM) at 3 and 10 weeks of age under conditions described in a previous study [22]. The OF was performed to assess locomotor activity and anxiety-like behavior in a novel environment. Total distance traveled and moving speed (total distance [cm]/total moving duration [s]) were recorded as indices of locomotor activity in the novel environment, and the time spent in the center zone (30 × 30 cm) was recorded as an index of anxiety-like behavior. EPM was conducted to assess behavior under fearful conditions at high altitudes without walls. Total distance traveled and total number of arm entries were recorded as measures of locomotor activity, and both the percentage of open arm entries and the time spent in the open arm were recorded as measures of anxiety-like behavior. An increase in anxiety-like behavior was defined as a decrease in the percentage of open arm entries or in time spent in the open arm. The results of each behavioral test were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical analysis

After completion of the behavioral tests, all mice were euthanized under isoflurane deep anesthesia using an inhalation anesthesia apparatus (BS-400T; Brain Science Idea, Osaka, Japan) by whole blood collection. The brains were excised, immersed in 4% paraformaldehyde solution for 20 hr at 4°C, dehydrated in ethanol and xylene, and embedded in paraffin. Coronal sections (−1.58 mm to −2.06 mm and 0.38 mm to −0.10 mm from the bregma) were thinly sliced at 3 µm thickness and mounted on a glass slide precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

Rabbit polyclonal anti-doublecortin antibody (DCX, 1:2,000; ab18723; Abcam, Cambridge, UK) was used in the hippocampal dentate gyrus (DG). The HRP-labeled, highly sensitive polymer method (EnVision + System HRP-Labeled Polymer; Dako, Glostrup, Denmark) was used as the detection system. Immunostaining of all sections was performed as previously described [12]. DCX in the subgranular zone (SGZ), the inner layer of the DG, was counted and the number of immunopositive cells per area was calculated using ImageJ software.

RNA extraction and microarray analysis

Total RNA from the hippocampus of 3- and 10-week-old mice was extracted using the NucleoSpin® RNA isolation kit (Macherey–Nagel GmbH & Co., Düren, Germany). The RNA samples with RIN (RNA integrity number) values of 8.0 or higher were used for microarray analysis (3 weeks [wk], Control group n=2, CLO group n=2; 10 wk, Control group n=2, CLO group n=2). The microarray data (.CEL files) were deposited in a public database (Gene Expression Omnibus) under accession number GSE218520.

cDNA synthesis and quantitative reverse transcription PCR

The RNA samples (1 µg) were reverse transcribed to cDNA using the Prime Script RT reagent kit with gDNA Eraser (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. The gene expression was quantitatively analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using TB Green Premix Ex Taq II (Takara Bio Inc.) with specific primers (Supplementary Table 1) on a Thermal Cycler Dice Real Time System (Takara Bio Inc.). Thermal cycling was performed for *β-actin* (*Actb*) and 18S ribosomal RNA (*Rn18s*) by initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, and extension at 60°C for 30 sec for 40 cycles; for brain-derived neurotrophic factor (*Bdnf*) by initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, and extension at 60°C for 30 sec for 50 cycles; for the other genes, initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, and extension at 56°C for 30 sec for 40 cycles. The gene copy number was calculated using a standard curve and normalized by the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), *Actb*, and *Rn18s* according to The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [2, 40]. All samples were assayed in duplicate, and the specificity of PCR products was confirmed by melting curves.

Statistical analyzes

Statistical analyzes were performed using BellCurve for Excel (Version 3.23; SSRI, Tokyo, Japan). The behavioral data from the fetal and neonatal periods were analyzed by two-way ANOVA (CLO × age) and Bonferroni *post hoc* tests. The gel intake data, quantitative immunohistochemical data, brain weight, and differences in the number or weight of offspring were analyzed by one-way ANOVA or the Kruskal–Wallis test in the fetal period. Welch's *t*-test was used to detect differences in the quantitative immunohistochemistry data, qRT-PCR data, litter size or weight, and brain weight in the neonatal period. Abnormal values were excluded from the immunohistochemical analysis based on the standard deviation (SD) of twice or half the mean. The results were considered significant when the *P*-value was less than 0.05.

RESULTS

Effects of CLO on general health status

Gel intake and brain weight did not differ significantly between the control and treatment groups (Supplementary Tables 2, 3). Litter size was significantly higher in CLO-4 than in Ctrl-4 [$t(7.571)=2.778$, $P<0.05$], but there were no significant effects in the other groups (Supplementary Table 2). In adulthood, CLO-2 had significantly lower body weight than CLO-1 [$F(2, 23)=4.481$, $P<0.05$], but none of the groups showed a significant effect on body weight in juveniles and adults compared to the control group (Supplementary Table 3).

Behavioral test results for groups exposed to CLO in utero

The behavioral test results showed that age had a significant main effect on the total distance traveled and on the moving speed in the OF [$F(2, 46)=44.11$, $P<0.001$; $F(2, 46)=23.71$, $P<0.001$] (Fig. 1A and 1B). Age had a significant main effect on the time spent in the center zone in the OF, with a significant interaction [$F(2, 46)=24.15$, $P<0.001$; $F(2, 46)=3.973$, $P<0.05$]. The *post hoc* test showed a significant decrease in adult CLO-1 and CLO-2 compared to Ctrl-1–2 ($P<0.05$) (Fig. 1C). The EPM results showed age had significant main effects on the total distance traveled and the time spent in the open arm [$F(2, 46)=16.38$, $P<0.001$; $F(2, 46)=25.66$, $P<0.001$] (Fig. 1D and 1F). As for the percentage of open arm entries, CLO had an almost significant main effect and age had a significant main effect [$F(2, 46)=2.762$, $P=0.0736$; $F(2, 46)=25.27$, $P<0.001$]. The *post hoc* test showed an increasing tendency for adult CLO-1 and CLO-2 compared to Ctrl-1–2 ($P=0.055$; $P=0.078$) (Fig. 1G). There was no significant effect on the total number of arm entries for either juveniles or adults (Fig. 1E).

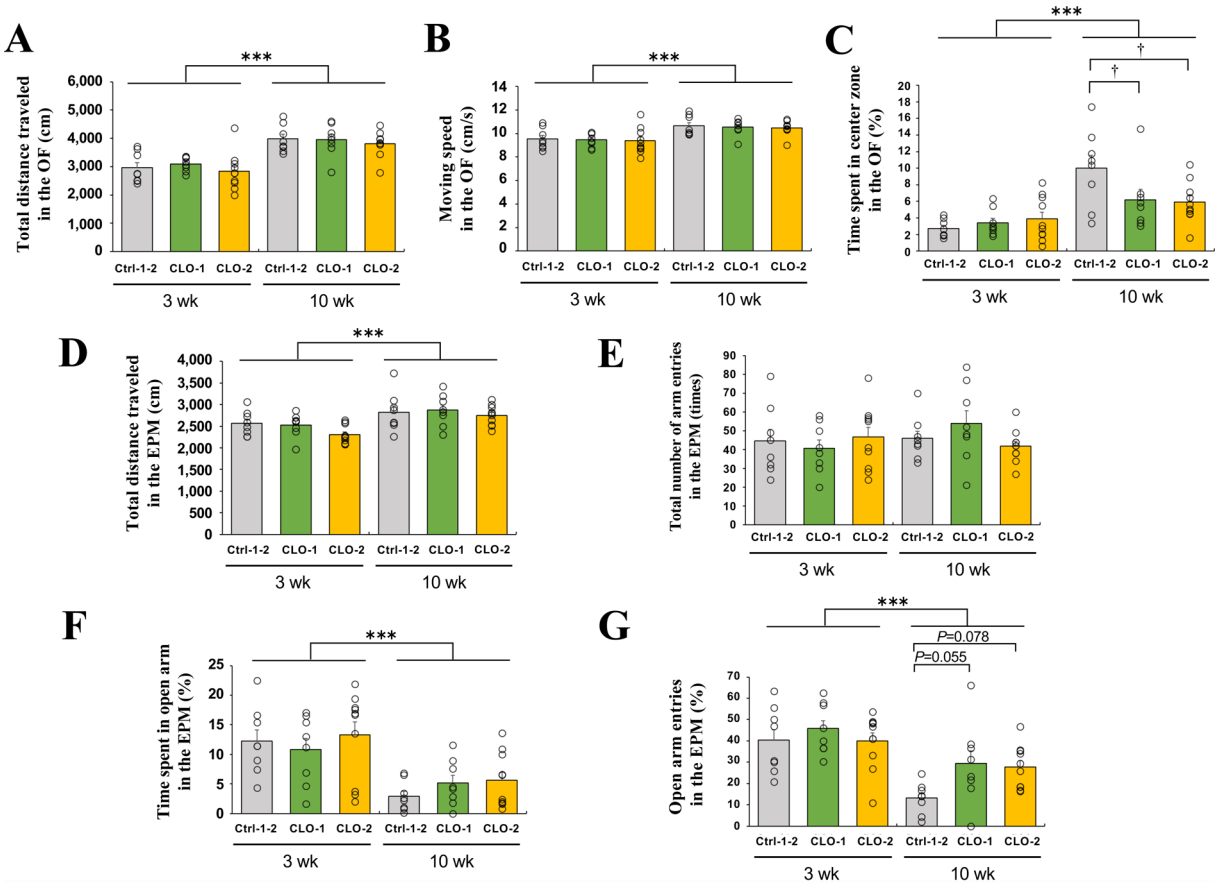

Fig. 1. The effects of clothianidin (CLO) exposure at fetal days 9–12 or 15–18 on total distance traveled (**A**), moving speed (**B**), time spent in the center zone (**C**) in the open field test (OF), total distance traveled (**D**), total number of arm entries (**E**), time spent in the open arm (**F**), and the percentage of open arm entries (**G**) in the elevated plus maze test (EPM) at 3 and 10 weeks of age. Data are reported in the form of mean + SD, and each result is plotted. The numbers of mice in the groups are as follows: 3 wk Ctrl-1–2 (n=8); 3 wk CLO-1 (n=8); 3 wk CLO-2 (n=10); 10 wk Ctrl-1–2 (n=8); 10 wk CLO-1; (n=8); 10 wk CLO-2; (n=10). A, B: Significant main effects of age were found on total distance traveled and moving speed in the OF. C: There was a significant main effect of age and a significant interaction between CLO and age on time spent in the center zone in the OF. CLO-1 and CLO-2 at 10 weeks of age spent significantly less time in the center zone in the OF compared to Ctrl-1–2. D, F: Age had significant main effects on total distance traveled and time spent in the open arm in the EPM. G: CLO had a close to significant main effect and age had a significant main effect on the percentage of open arm entries in the EPM. CLO-1 and CLO-2 at 10 weeks of age showed an increasing tendency compared to Ctrl-1–2. E: There was no significant effect for either juveniles or adults. †P<0.05, ***P<0.001, *vs.* other groups (two-way ANOVA followed by Bonferroni's *post hoc* test), †: significant interaction between CLO and age, *: significant main effect of age.

Behavioral test results for groups exposed to CLO during lactation

The mice exposed during the neonatal period showed a close to significant main effect of CLO [F(1, 68)=2.931, P=0.091] and a significant main effect of age in the total distance traveled in the OF for CLO-3 [F(1, 68)=24.14, P<0.001] (Fig. 2A). Age showed a significant main effect on the time spent in the center zone for CLO-3 [F(1, 68)=10.84, P<0.01] (Fig. 2C). Moving speed showed no significant effect in either juveniles or adults (Fig. 2B). Although age showed significant main effects on the total distance traveled and moving speed in the OF for CLO-4 [F(1, 34)=10.379, P<0.01; F(1, 34)=7.761, P<0.01] (Fig. 2D and 2E), it showed no significant effect on the time spent in the center zone for CLO-4 for either juveniles or adults (Fig. 2F).

The EPM results showed that CLO and age had significant main effects on the total distance traveled for CLO-3, with a significant interaction [F(1, 68)=5.746, P<0.05; F(1, 68)=37.52, P<0.001; F(1, 68)=4.738, P<0.05]. The *post hoc* tests showed a significant increase in total distance traveled by CLO-3 compared to Ctrl-3 (P<0.01) (Fig. 3A). CLO and age had significant main effects on the total number of arm entries in the EPM for CLO-3, with a significant interaction [F(1, 68)=4.534, P<0.05; F(1, 68)=4.498, P<0.05; F(1, 68)=5.439, P<0.05]. The *post hoc* tests showed a significant increase in adult CLO-3 compared to Ctrl-3 (P<0.01) (Fig. 3B). Age had a significant main effect on time spent in the open arm in the EPM for CLO-3 [F(1, 68)=19.39, P<0.001] (Fig. 3C). Age also had a significant main effect on the percentage of open arm entries in the EPM for CLO-3, with a significant interaction [F(1, 68)=57.06, P<0.001; F(1, 68)=4.427, P<0.05] (Fig. 3D). Age had a significant main effect on the total distance traveled, total number of arm entries, and percentage of open arm entries in the EPM for CLO-4 [F(1, 33)=24.79, P<0.001; F(1, 33)=7.424, P<0.05; F(1, 33)=26.94, P<0.001] (Fig. 3E, 3F, 3H). CLO and age also had significant main effects on time spent in the open arm [F(1, 33)=4.382, P<0.05; F(1, 33)=5.332, P<0.05]. The *post hoc* tests showed a decreasing tendency in CLO-4 compared to Ctrl-4 in the juvenile period (P=0.067) (Fig. 3G).

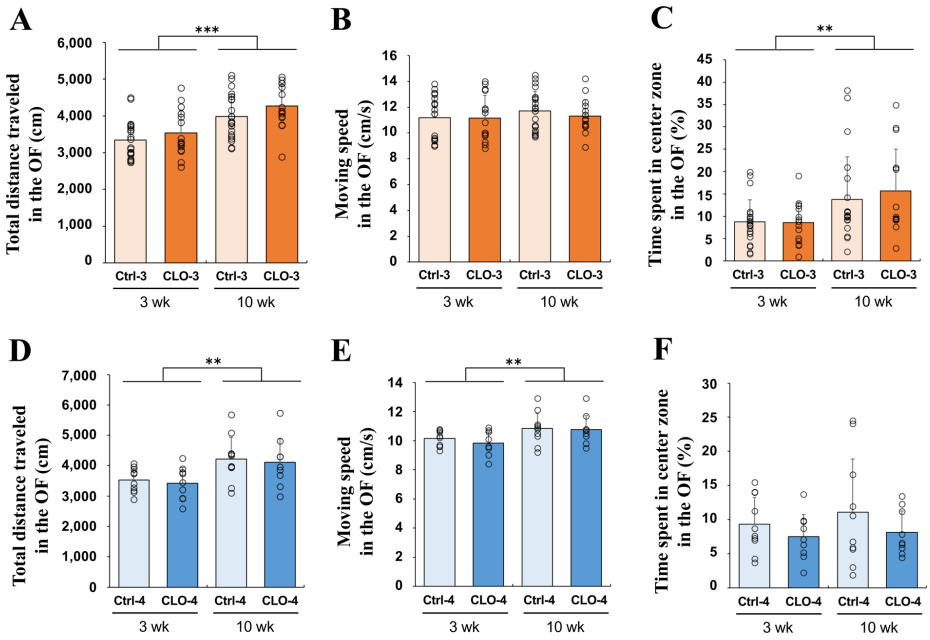

Fig. 2. The effects of clothianidin (CLO) exposure at 1–4 days after birth or 11–14 days after birth on total distance traveled (**A, D**), moving speed (**B, E**), and time spent in the center zone (**C, F**) in the open field test (OF) at 3 and 10 weeks of age. Data are reported in the form of mean + SD, and each result is plotted. The numbers of mice in the groups are as follows: 3 wk Ctrl-3 (n=21); 3 wk CLO-3 (n=15); 3 wk Ctrl-4 (n=10); 3 wk CLO-4 (n=9); 10 wk Ctrl-3 (n=21); 10 wk CLO-3 (n=15); 10 wk Ctrl-4 (n=10); 10 wk CLO-4 (n=9). **A**: CLO-3 had a close to significant main effect of CLO and a significant main effect of age on total distance traveled in the OF. **B**: There was no significant effect on moving speed in the OF in CLO-3. **C**: Age had a significant main effect on time spent in the center zone in the OF in CLO-3. **D, E**: Age had significant main effects on total distance traveled and moving speed in the OF in CLO-4. **F**: There was no significant effect for either juveniles or adults. $^{**}P<0.01$, $^{***}P<0.001$, *vs.* other groups (two-way ANOVA followed by Bonferroni's *post hoc* test), *: significant main effect of age.

Effects of CLO exposure on DCX-positive cells

The immunohistochemistry of DCX showed positive reactions mainly in the inner layers of DG cell bodies and dendrites in all groups (Fig. 4A). No significant effect was observed on the number of DCX-positive cells per unit area in the SGZ of DG in CLO-1, CLO-2, or CLO-4 (Fig. 4B and 4D), but a significant decrease was observed in CLO-3 [$t(10.86)=2.8302$, $P<0.05$] (Fig. 4C).

Effects of CLO exposure on global gene expression in the CLO-3 group hippocampus

The behavioral tests and the immunohistochemical analysis of DCX revealed that exposure to CLO at days 1–4 after birth had statistically significant neurobehavioral effects, and that the effects were greater than those in groups exposed to CLO *in utero* (CLO-1 and CLO-2). Therefore, we analyzed gene expression in CLO-3, and the results suggested that this period is crucial during the formation of brain neural circuits. We examined the effects of CLO on gene expression in the mouse hippocampus and found that the expression of 1,023 genes fluctuated more than 1.5-fold (upregulated: 502 genes, downregulated: 521 genes) in the treatment group during the juvenile period, and that 892 genes fluctuated more than 1.5-fold (upregulated: 365 genes, downregulated: 527 genes) in the treatment group during the adult period. The biological functions, canonical pathways, and gene networks of upregulated genes were identified in both the juvenile and adult periods, based on gene data from ingenuity pathway analysis (IPA) software. The biological functions with a z-score of 2.0 or higher during the juvenile period are summarized in Table 1. CLO exposure increased the functions of "Development of neural cells", "Neuritogenesis", "Branching of neurites", "Axonogenesis", and "Branching of axons" in the juvenile hippocampus. The results suggest that the hippocampus in the early juvenile period shows increased differentiation of neurons as well as increased levels of neurite outgrowth, branching of axons, memory function, and microglial activation. A gene network was created, and the expression of many genes related to "Branching of neurites" and "Neuritogenesis" was found to be upregulated (Fig. 5A). Activated or repressed pathways are shown in Fig. 5B. Genes related to oxidative phosphorylation and mitochondrial dysfunction were highly detected. Table 2 summarizes the biological functions related to neurodevelopment with z-scores in the adult period. The biological function with a z-score of 2.0 or higher was "Outgrowth of neurites", and the other biological functions were "Neuritogenesis", "Growth of axons", "Dendritic growth/branching", and "Formation of hippocampus". These results suggest that the hippocampus in adulthood undergoes accelerated neuronal differentiation, neurite/axon elongation and branching, increased neurogenesis, and accelerated development of the architecture of the hippocampus. The results of the gene network analysis indicated that "Outgrowth of neurites", "Growth of axons", and "Dendritic growth/branching" were upregulated (Fig. 5C).

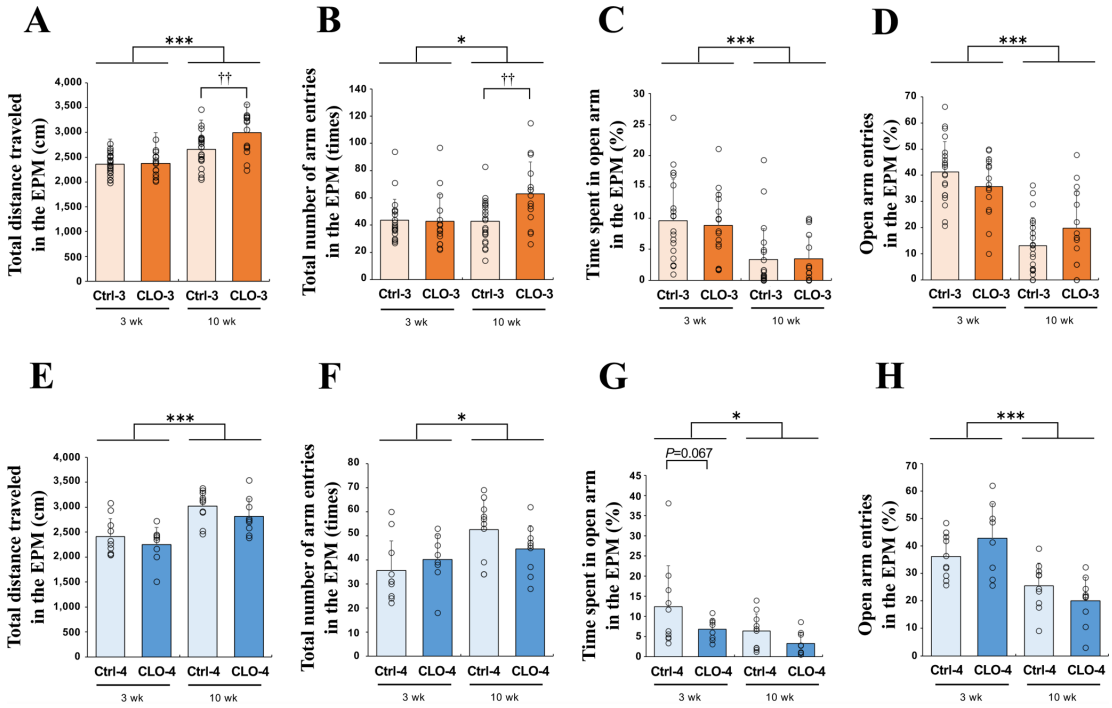

Fig. 3. The effects of clothianidin (CLO) exposure at days 1–4 or 11–14 after birth on total distance traveled (**A, E**), total number of arm entries (**B, F**), time spent in the open arm (**C, G**), and the percentage of open arm entries (**D, H**) in the elevated plus maze test (EPM) at 3 and 10 weeks of age. Data are reported in the form of mean + SD, and each result is plotted. The numbers of mice in the groups are as follows: 3 wk Ctrl-3 (n=21); 3 wk CLO-3 (n=15); 3 wk Ctrl-4 (n=10); 3 wk CLO-4 (n=8); 10 wk Ctrl-3 (n=21); 10 wk CLO-3 (n=15); 10 wk Ctrl-4 (n=10); 10 wk CLO-4 (n=9). **A**: CLO and age had significant main effects on total distance traveled in the EPM in CLO-3, with a significant interaction between CLO and age. CLO-3 at 10 weeks of age was significantly higher than Ctrl-3. **B**: CLO and age had significant main effects on the total number of arm entries in the EPM in CLO-3, with a significant interaction between CLO and age. CLO-3 at 10 weeks of age was significantly higher than Ctrl-3. **C**: Age had a significant main effect on time spent in the open arm in the EPM in CLO-3. **D**: Age had a significant main effect on the percentage of open arm entries in the EPM in CLO-3, with a significant interaction between CLO and age. **E, F, H**: Age had significant main effects on total distance traveled, total number of arm entries, and the percentage of open arm entries in the EPM in CLO-4. **G**: CLO and age had significant main effects on time spent in the open arm in the EPM in CLO-4. CLO-4 tended to be lower than Ctrl-4 at 3 weeks of age. ††P<0.01, *P<0.05, ***P<0.001, *vs.* other groups (two-way ANOVA followed by Bonferroni's *post hoc* test), †: significant interaction between CLO and age, *: significant main effect of age.

Validation of microarray results by quantitative reverse transcription PCR

To confirm the microarray results, the expression levels of upregulated genes were analyzed using qRT-PCR. Ten genes important for biological functions, canonical pathways, and gene networks were selected: fibroblast growth factor receptor 1 (*Fgfr1*), dicer 1, ribonuclease type III (*Dicer1*), Ndufa4, mitochondrial complex associated (*Ndufa4*), ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9) (*Atp5g1*), mitochondrial calcium uniporter (*Mcu*), MAP/microtubule affinity regulating kinase 4 (*Mark4*), MAP/microtubule affinity regulating kinase 2 (*Mark2*), ras homolog family member B (*Rhob*), PTK2 protein tyrosine kinase 2 beta (*Ptk2b*), and brain-derived neurotrophic factor (*Bdnf*). The quantitative results shown in Fig. 5 indicate that exposure to a NOAEL dose of CLO from days 1–4 after birth resulted in significantly higher gene expression of *Fgfr1* in the juveniles [t(8.187)=2.621, P<0.05] (Fig. 5D). The expression levels of other genes were also increased in the CLO group, although not significantly; the increases were highest in the juvenile group (Fig. 5D and 5E).

DISCUSSION

CLO had no effect on gel intake, brain weight, or body weight compared to controls, indicating that CLO exposure during the four periods in this study had no effect on gel intake or general health status. CLO-4 significantly increased litter size, but this may be a physiological result and not an effect of CLO. This is because we evaluated litter size to see whether there was a decrease due to CLO administration, and the results did not indicate a decrease. It may also be related to the fact that maternal body weight was higher in the CLO group than in the control group from the time of purchase at 14.5 days of gestation.

Behavioral studies of prenatal exposure to CLO found that exposure at specific times during the fetal period led to behavioral abnormalities in adulthood. The OF results showed that exposure to CLO at fetal days 9–12 and 15–18 increased anxiety-like behaviors in adulthood. On the other hand, the EPM results showed that exposure to CLO at fetal days 9–12 and 15–18 tended to suppress anxiety

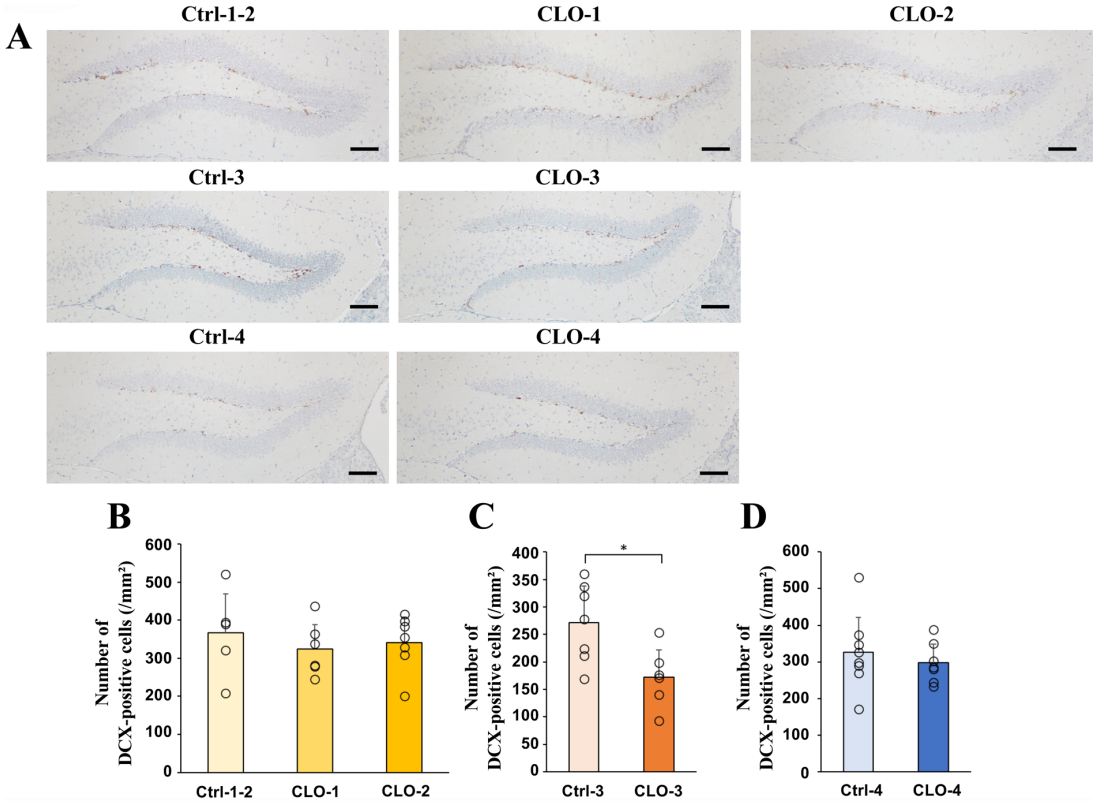

Fig. 4. Representative histology and immunohistochemistry of doublecortin (DCX) in the hippocampal dentate gyrus (DG) at 10 weeks of age (**A**) and the number of DCX-positive cells in the subgranular zone (SGZ) (**B, C, D**). Bar=100 µm. Data are reported in the form of mean + SD, and each result is plotted. **A, C**: clothianidin (CLO)-3 significantly decreased the number of DCX-positive cells in the SGZ compared to Ctrl-3. **A, B, D**: CLO-1, CLO-2, and CLO-4 were not significantly affected compared to the control group. The numbers of mice in the groups are as follows: Ctrl-1–2 (n=5); CLO-1; (n=6); CLO-2; (n=7); Ctrl-3 (n=7); CLO-3 (n=6); Ctrl-4 (n=8); CLO-4 (n=7). One-way ANOVA followed by Bonferroni's *post hoc* test was used for B, and Welch's *t*-test for C and D. **P*<0.05, *vs*. control group.

Table 1. Biological functions related to neurodevelopment of upregulated genes in 3-wk-old offspring

Diseases or functions annotation	*P*-value	Predicted activation state	Activation z-score	Number of molecules
Development of neural cells	7.27.E-04	Increased	3.650	41
Neuritogenesis	4.27.E-03	Increased	3.384	29
Branching of neurites	5.54.E-03	Increased	3.241	17
Development of neurons	9.26.E-04	Increased	3.144	39
Axonogenesis	1.37.E-02	Increased	2.768	12
Branching of axons	1.12.E-02	Increased	2.383	6

Activation z-score >2.0 is significantly predictive.

levels in adulthood. Although the results of these two behavioral tests differ, it is clear that the effects of CLO exposure persist into adulthood and cause abnormal behavior in male offspring. Although the OF and EPM were used in the present study, several behavioral tests detect anxiety-like behaviors, and anxiety-related profiles of animals may differ among behavioral tests [9]. In a previous report [42], male pups that had been exposed to an organophosphorus fire retardant (OPFR) mixture from gestation day 7 to postnatal day 14 went on to exhibit increased locomotor activity and decreased time spent in the center zone in the OF in adulthood. Those male pups also exhibited an anti-anxiety effect by increasing the percentage of open arm entries in the EPM in adulthood. These conflicting results are similar to those reported in the present study, suggesting that the effects of CLO on anxiety levels may differ depending on the behavioral test. In other words, the CLO exposure during the two prenatal periods in this study had a negative effect on anxiety levels in adult males. These results suggest that CLO exposure on fetal days 9–12 and 15–18 may affect brain development and lead to behavioral deficits later in life.

The results of the neonatal OF and EPM revealed that exposure to CLO during days 1–4 after birth significantly increased locomotor activity in adults and that exposure to CLO during days 11–14 after birth increased anxiety-like behavior in juveniles. A previous

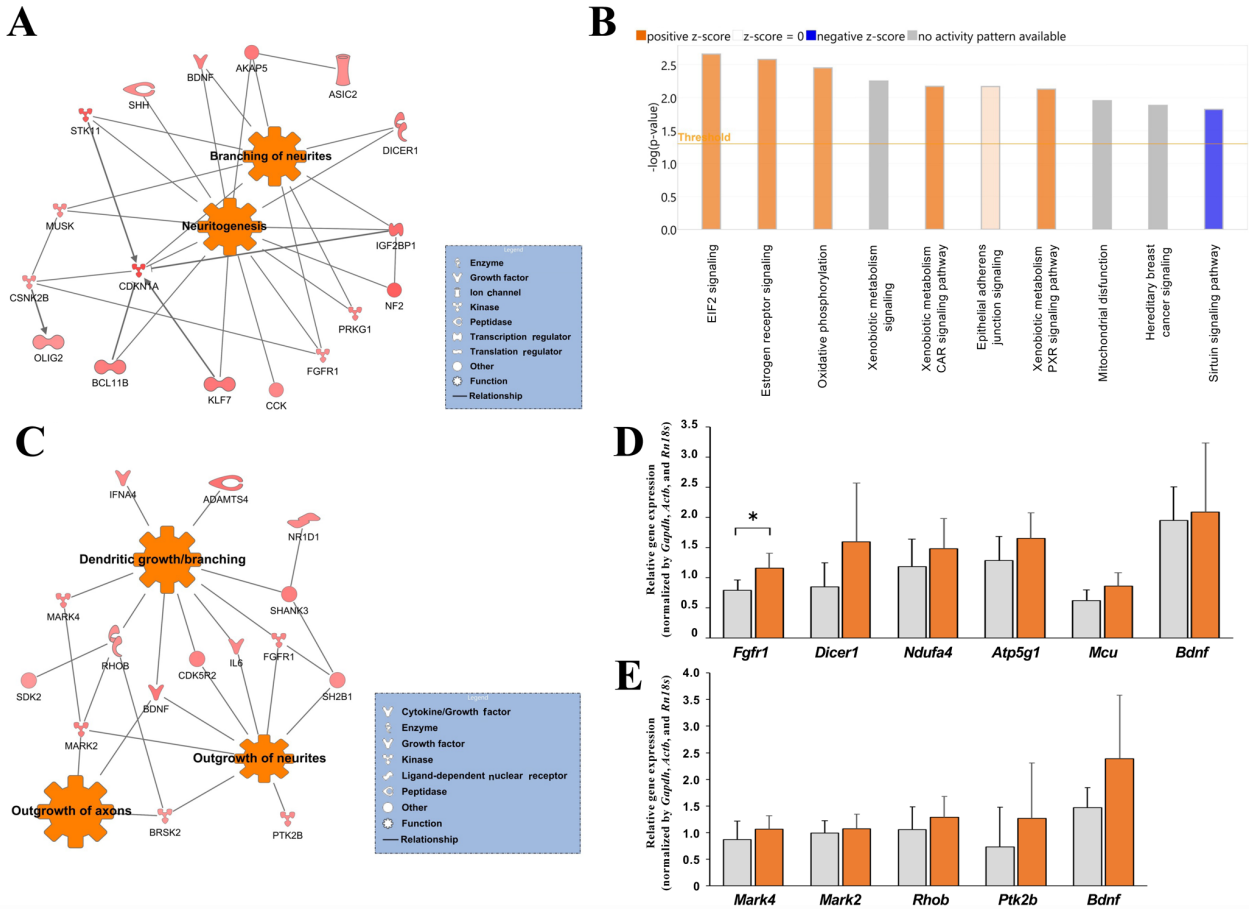

Fig. 5. The canonical pathways and network maps of upregulated genes at 3 and 10 weeks of age altered by clothianidin (CLO) exposure at days 1–4 after birth were identified by ingenuity pathway analysis (IPA) software (n=2 for each). **A**: The gene network map showing the interaction between the upregulated genes and other molecules at 3 weeks of age, focusing on neural functions. **B**: The signaling pathways activated in the hippocampus at 3 weeks of age. **C**: The gene network map showing the interaction between the upregulated genes and other molecules at 10 weeks of age, focusing on neural functions. **D, E**: The validation of microarray results using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 3-week-old (**D**) and 10-week-old (**E**) mouse hippocampus. Fibroblast growth factor receptor 1 (*Fgfr1*), dicer 1, ribonuclease type III (*Dicer1*), ndufa4, mitochondrial complex associated (*Ndufa4*), ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9) (*Atp5g1*), mitochondrial calcium uniporter (*Mcu*), MAP/microtubule affinity regulating kinase 4 (*Mark4*), MAP/microtubule affinity regulating kinase 2 (*Mark2*), ras homolog family member B (*Rhob*), PTK2 protein tyrosine kinase 2 beta (*Ptk2b*), and brain-derived neurotrophic factor (*Bdnf*) were normalized by the geometric mean of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), *β-actin* (*Actb*), and 18S ribosomal RNA (*Rn18s*), and were compared with the control group. Data represent mean + SD for each group (n=4–7 for each). **P*<0.05, *vs*. control group

Table 2. Biological functions related to neurodevelopment of upregulated genes in 10-wk-old offspring

Diseases or functions annotation	*P*-value	Predicted activation state	Activation z-score	Number of molecules
Outgrowth of neurites	1.23.E-02	Increased	2.136	14
Neuritogenesis	8.41.E-03		1.996	22
Growth of neurites	3.97.E-03		1.964	18
Development of neurons	6.32.E-03		1.918	28
Proliferation of neuronal cells	1.86.E-04		1.835	24
Branching of neurites	4.35.E-03		1.111	14
Excitation of neurons	9.38.E-03		1.069	5
Formation of hippocampus	2.88.E-03		1	7
Development of cerebral cortex	1.05.E-02		1	8
Dendritic growth/branching	4.04.E-03		0.773	12
Growth of axons	1.55.E-03		0.423	10
Regeneration of neurons	1.03.E-02		0.186	6

Activation z-score >2.0 is significantly predictive.

study found that exposure to CLO throughout the fetal and neonatal periods increased anxiety-like behavior in juveniles and locomotor activity in adults [23]. This report is consistent with the results of the two groups exposed to CLO during the neonatal period in the present study. Newborns are nourished by breast milk, and previous reports have shown that CLO is metabolized in the mother's body and is rapidly transferred to and concentrated in the breast milk [34]. Moreover, CLO has been reported to have adverse neurodevelopmental and neurobehavioral effects [12–14, 23]. Together these findings suggest that newborns, which are vulnerable to chemical substances, are exposed to high concentrations of CLO, which may impair their neurodevelopment and lead to behavioral abnormalities in adulthood. Nicotine, like CLO, targets nicotinic acetylcholine receptors. It has been reported that rats exposed to nicotine at 4–9 days after birth show hyperactivity at 18–19 days after birth [37], and that animals exposed to nicotine from 8–14 days of age show hypoactivity at 19–21 days of age [10]. According to these reports, nicotine exposure is thought to be associated with developmental abnormalities in the cholinergic system, perturbations in the development of the hippocampus, and induction of apoptosis. It is possible that similar disturbances occurred in the present study.

In all groups, several parameters showed large individual differences in the results of the behavioral tests. Although the mice used in the experiments were reared and tested under identical conditions, maternal care and the timing of eating and sleeping were not always identical. In fact, in addition to synapse formation in the hippocampus, the expression of genes that regulate behavioral and endocrine responses to stress naturally changes with maternal care, and has been shown to be the basis for the formation of individual differences [24]. It is also believed that the movement and handling of animals prior to a test can change their sensitivity to the test [15], and in the present study, individual differences in gene expression and sensitivity may have caused variations in the test results.

To examine the effects of CLO on neurodevelopment at different time points, DCX in DG was visualized by immunohistochemical staining. DCX is expressed in neural progenitor cells and immature neurons. No significant effect was observed on the number of DCX-positive cells per unit area in the SGZ of DG in CLO-1, CLO-2, or CLO-4, but a significant decrease was observed in CLO-3. These results indicate that exposure to CLO from 1–4 days after birth decreases the number of juvenile neurons in the adult DG. This result is consistent with a previous finding that CLO exposure in the fetal and neonatal periods decreased the number of DCX-positive cells [23]. Therefore, our results indicate that CLO exposure during 1–4 days after birth produces similar results as exposure throughout the fetal and neonatal periods.

The gene expression analyzes suggested that CLO exposure during days 1–4 after birth excessively promotes neurite outgrowth and the branching of neurites and axons in the hippocampus of juveniles. In short, we found that the decreased number of juvenile neurons in the adult hippocampus may be the result of impaired development during the proper developmental period due to the excessive promotion of neurodevelopment, and that these effects may persist into adulthood and influence emotional behavior. Similar results to the present study have been reported in experiments modeling neurodevelopmental deficits due to prenatal nicotine exposure in human-induced pluripotent stem cell (hiPSC)-derived brain organoids, showing that nicotine exposure leads to early neuronal differentiation and neurite outgrowth [41]. Mouse offspring with maternal nicotine exposure was also reported to induce cognitive and behavioral abnormalities and increased neurite length in the molecular layer and hippocampal CA1 [43], suggesting that CLO exposure at days 1–4 after birth may similarly affect neurite outgrowth in the brain and lead to behavioral abnormalities in adulthood.

The developmental processes of the brain nervous system are common between humans and rodents. However, the temporal scales are different; for example, the early postnatal period in mice corresponds to the third trimester of human pregnancy [29]. After birth, the exposure pathway changes from the placenta to breast milk, so the effects of CLO exposure via the breast milk pathway during the early postnatal period in mice may not be similar to those of CLO exposure via the placenta during the third trimester of pregnancy in humans. Nevertheless, most drugs administered during pregnancy in humans are thought to enter the fetal circulation to some extent by passive diffusion [35]. Thus, it is highly possible that CLO enters the fetal circulation via the placenta in humans and affects the proper development of the nervous system.

This is the first report of developmental stage-specific effects of CLO exposure with a NOAEL dose in the fetal and neonatal periods. All results of the behavioral tests are summarized in Fig. 6. We found that CLO exposure at fetal days 9–12 or 15–18 resulted in

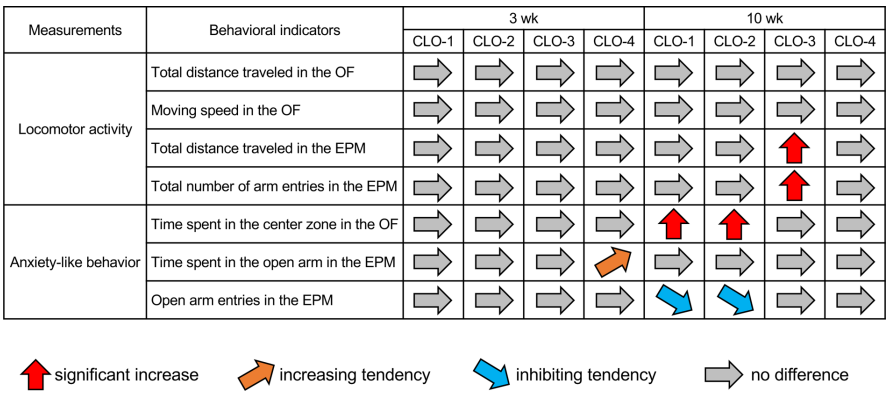

Fig. 6. Summary of the effects of clothianidin (CLO) exposure on locomotor activity and anxiety-like behavior at four developmental stages in the fetal and neonatal periods, as analyzed by the open field test (OF) and elevated plus maze test (EPM). In the juvenile period, a trend toward increased anxiety-like behavior was observed in CLO-4. In the adult period, anxiety-like behaviors were affected in CLO-1 and CLO-2, and locomotor activity increased in CLO-3, *vs*. the control group.

behavioral abnormalities in adulthood. In addition, CLO exposure at days 1–4 after birth was associated with an increase in locomotor activity in adulthood by promoting excessive neurite outgrowth and axonal outgrowth in the brain. The present results suggest a relationship between developmental stage-specific exposure to NNs and developmental disorders during brain function formation, which may lead to changes in what is considered the appropriate use of NNs in the future.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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