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NOTE

Toxicology

Transgenerational effects of developmental neurotoxicity induced by exposure to a no-observed-adverse-effect level (NOAEL) of neonicotinoid pesticide clothianidin

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ABSTRACT. Neonicotinoid pesticides (NNs) transfer rapidly from mother to offspring, which exhibit neurobehavioral effects. However, no studies have investigated NNs' transgenerational effects. We exposed F0 generation mice (mothers) to a no-observed-adverse-effect level (NOAEL) of clothianidin (CLO) during gestation and lactation, and examined the adult neurobehavioral effects of three generations of offspring (F1, F2, F3). F1 had lower birth weight, decreased locomotor activity, and increased anxiety-like behavior. In F2, body weight was affected, and there was a decreasing trend in locomotor activity and an increasing trend in anxiety-like behavior. In F3, locomotor activity tended to increase. Thus, even when only the mothers were exposed, the effects of CLOs were still observed in F1, F2, and F3 but the effects became smaller.

KEYWORDS: behavioral test, developmental stage, fetal and lactational exposure, neonicotinoid, transgenerational toxicity

Many epidemiological studies have suggested that environmental factors are major contributors to disease [17, 39, 48]. In particular, environmental chemicals may alter the cellular epigenome during development, increasing disease susceptibility after birth; moreover, germ line disruption of epigenetic marks can transmit detrimental phenotypes across successive generations [49]. Common epigenetic gene targets have been identified in the brain, and more recent studies suggest that the gut microbiota may contribute to early brain development and that metabolic and immune processes may interact and ultimately affect neurodevelopment [1]. In addition, epigenetic signatures in germ cells shape offspring neurodevelopment and are mediated transgenerationally [34]. Epigenetic mutations caused by pesticides, a type of environmental chemicals, result in adverse health effects for humans, rodents, and other species [3]. Those effects have been shown to be inherited successively [28] and may even worsen across generations. For example, direct exposure to the organophosphorus herbicide glyphosate showed minimal adverse effects on F0 and F1 mice. However, significant effects were observed in F2 and F3 generations, including prostate disease, obesity, kidney disease, ovarian disease, and abnormal deliveries [2, 20]. Thus, there is an urgent need to elucidate the effects of pesticides on successive generations, not only for our own health but also for the health of future generations.

Neonicotinoids (NNs), which were registered and marketed in the 1990s, are agonists of insect nicotinic acetylcholine receptors

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(nAChRs) and exhibit insecticidal activity by continuously stimulating and disturbing neurons. Because the affinity of NNs for insect nAChRs is tens to hundreds of times higher than that for mammalian nAChRs [44], NNs were widely used as a safer pesticide for mammals compared to conventional pesticides. In 2012, however, Kimura-Kuroda reported that the NNs imidacloprid (IMI) and acetamiprid (ACE) induce nAChR-mediated neuronal excitation in the cerebellar neurons of newborn rats, and it has become clear that NNs may have adverse effects on human health, especially on the developing brain [18]. Recently it was reported that birds and mammals exposed to no-observed-adverse-effect levels (NOAELs) of NNs exhibit toxic effects on reproduction, the thymus, gut microbiota, and neurobehavior [4, 9–11, 13, 19, 24, 27, 29, 31, 36, 43, 50]. In addition, NNs were detected in the urine of adults, children, and newborns [15, 16, 32, 45]. Specifically, clothianidin (CLO), a type of NN, and its metabolites transfer rapidly from mother to fetus [30]. Moreover, CLO is metabolized and concentrated in the mother and then transfers rapidly to breast milk [35], indicating that pesticides are routinely transferred from mother to offspring. These findings suggest that the adverse health effects of NNs may extend to the next generation.

Exposure to CLO during the fetal and neonatal periods has been shown to cause neurobehavioral abnormalities in the next generation of male offspring [24, 36], as well as toxicity to the immune system, gut microbiota [27], male reproductive organs [50], and female reproductive organs [19]. However, the neurobehavioral effects of CLO exposure on successive generations are not clear. In this study, we examined the neurobehavioral effects of F1, F2, and F3 offspring in adulthood exposed to the NOAEL of CLO during the fetal and neonatal periods.

Pregnant C57BL6/NCrSlc mice at 1.5 days gestation were purchased (n=12) from Japan SLC (Hamamatsu, Japan) and maintained as described elsewhere [9]. Fifty-eight dams (F0: n=12, F1: n=18, F2: n=16, F3: n=12) were used for CLO administration and mating, and 62 of their male offspring (F1: n=16, F2: n=24, F3: n=22) were used for behavioral tests. 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 Experimental Regulations.

The dams were divided into two groups, and CLO (95% purity: [10]) was administered as described in a previous study [24]. Briefly, CLO was administered to the dams at a volume of 0 or 65 mg/kg body weight from embryonic day (E) 1.5 to postnatal day (PND) 21 with reference to the NOAEL (ICR female mice: 65.1 mg/kg/day) [6, 46]. Rehydration gel (MediGel® Sucralose; ClearH_2O, Portland, ME, USA) with CLO or vehicle (1% dimethyl sulfoxide: DMSO) was used for administration. To standardize milk volume, a maximum of 6 offspring per litter were randomly selected on PND 3, and litters with fewer than 3 pups were eliminated from the experiment. To avoid litter bias, 1 or 2 offspring per litter were used. When F1 reached 10 weeks of age, F2 was created by mating males and females of F1 in the same group. Similarly, when F2 reached 10 weeks of age, males and females of F2 were mated to produce F3.

Male offspring were subjected to the open field test (OF) and the elevated plus maze test (EPM) at 10 weeks of age under conditions described previously [23]. In the OF, locomotor activity and anxiety-like behavior were assessed in a novel environment. Total distance traveled and moving speed (total distance [cm]/total moving time [sec]) were recorded as indices of locomotor activity in the novel environment, and time spent in the center zone (30 × 30 cm) was recorded as an index of anxiety-like behavior. A decrease in time spent in the center zone was defined as an increase in anxiety-like behavior. In the EPM, behavior under fear-inducing conditions without walls at a high place was evaluated. Total distance traveled and total number of arm entries were recorded as indices of locomotor activity. Time spent in the open arms and the percentage of open arm entries were recorded as indices of anxiety-like behavior. An increase in anxiety-like behavior was defined as a decrease in time spent in the open arms and in the percentage of open arm entries. The results of each behavioral test were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Infanticide and severe neglect were observed as maternal behaviors. Severe neglect was defined as death of the pups due to maternal negligence.

Statistical analyzes were performed with BellCurve for Excel (Version 3.23; SSRI, Tokyo, Japan). The behavioral data were analyzed by two-way ANOVA (CLO × generation) and Bonferroni *post hoc* tests. The gel intake data and differences in the number or weight of offspring were analyzed by Welch's *t*-test. The results were considered significant when the *P*-value was less than 0.05.

CLO had no significant effect on gel intake or litter size (Supplementary Tables 1 and 2). This suggests that CLO exposure in the fetal and neonatal periods has no effect on gel intake or litter size over successive generations.

CLO-65 in F1 showed significantly lower birth weight compared to CLO-0 [t (29.84)=3.18, $P<0.01$] (Supplementary Table 2). Body weight at 3 weeks of age in F1 was significantly lower in CLO-65 compared to CLO-0 [t (39.84)=2.86, $P<0.01$], and that at 10 weeks of age in F2 was significantly higher in CLO-65 compared to CLO-0 [t (35.39)=2.399, $P<0.05$]. No significant effects of CLO were observed in F3 (Supplementary Table 3). Previous studies have shown that CLO transfers rapidly from mother to offspring via both the placenta during pregnancy and breast milk during lactation [30, 35], that CLO affects the immune system and gut microbiota of the next generation [27], and that CLO affects the gut, causing loose stool and suppressing body weight gain in rats [31]. Peristalsis is controlled by the enteric nervous system and autonomic innervation, and ACh, the ligand of nAChR, plays an important role as a neurotransmitter [7, 25]. It has been reported that the enteric nervous system, which begins to develop during the fetal period, regulates various gastrointestinal tract functions including intestinal motility [22], and that structural and functional changes in the mucosal immune system of the gut that occur before and after birth are controlled by developmental and environmental factors [33]. In other words, CLO exposure in F1 during the fetal and neonatal periods, which including some stages of intestinal development, may have some effect on the intestinal immune system or may activate peristalsis, resulting in inadequate nutrient and water absorption at feeding. Postnatal weight gain due to fetal CLO exposure [41, 42], the relationship between NNs and obesity [37, 38], and changes in metabolic function in F2 [14, 21] suggest that CLO may affect F2 metabolic function and may be associated with weight gain in adulthood.

In the F1 generation, infanticide (n=1) and severe neglect (n=3) were observed in CLO-0, and severe neglect (n=4) was observed in CLO-65. In the F2 generation, infanticide (n=3) and severe neglect (n=3) were observed only in CLO-65. No effects were observed

in the F0 and F3 generations (Supplementary Table 4). In a previous study in which CLO exposure occurred during the same period [19], CLO-65 of the F1 generation showed infanticide and severe neglect, and oxytocin and prolactin levels were low. The rates of infanticide and severe neglect increased with each generation from F0 to F1 to F2 (data not shown). The infanticide and severe neglect may have been caused by direct exposure to CLO or by inadequate care from mothers with low oxytocin and prolactin levels, and the same effects may have been involved in the F1 generation in this study. In addition, infanticide and severe neglect were observed in the F1 and F2 generations in this study, suggesting that the same effects as in the F1 generation were inherited across generations. However, no CLO effects were observed in the F3 generation, which indicated that CLO may not affect maternal behavior in the third and subsequent generations, but the details remain to be clarified. Further studies on the effects of CLO on maternal behavior are needed.

To assess locomotor activity and anxiety-like behavior in the novel environment, all mice were subjected to the OF. The representative trajectory maps show no difference in CLO-65 compared to CLO-0 in each generation (Fig. 1A). There was no significant effect on the total distance traveled in the OF, but generation showed a significant main effect on moving speed [$F(2, 56)=3.827$, $P<0.05$] (Figs. 1B and 1C). There was no significant effect on the time spent in the center zone in the OF (Fig. 1D). EPM was performed to evaluate behavior under fear-inducing conditions without walls at a high place. The representative trajectory map showed that CLO-65 mice in F1 rarely entered the open arms (Fig. 2A). A significant interaction between CLO and generation was found for the total distance traveled in the EPM [$F(2, 55)=3.502$, $P<0.05$]. *Post hoc* tests showed an increasing trend for CLO-65 in F3 compared to CLO-0, indicating an increasing trend in locomotor activity ($P=0.054$) (Fig. 2B). CLO and generation had significant effects on the total number of arm entries in the EPM, with significant interaction between CLO and generation [$F(1, 55)=7.143$, $P<0.01$; $F(2, 55)=7.901$, $P<0.001$; $F(2, 55)=4.563$, $P<0.05$]. *Post hoc* tests showed a significant decrease in CLO-65 in F1 compared to CLO-0 and a decreasing trend in CLO-65 in F2 compared to CLO-0 ($P<0.01$; $P=0.086$). In other words, F1 showed a significant decrease in locomotor activity in CLO-65, while F2 showed a decreasing trend in locomotor activity in CLO-65 (Fig. 2C). CLO and generation had no significant effect on time spent in open arms in the EPM. *Post hoc* tests showed a decreasing trend for CLO-65 in F2 compared to CLO-0, indicating an increasing trend for anxiety-like behavior ($P=0.058$) (Fig. 2D). Significant main effects of CLO and generation, along with a significant CLO-by-generation interaction, were found on the percentage of open arm entries in the EPM [$F(1, 55)=4.197$, $P<0.05$; $F(2, 55)=9.014$, $P<0.001$; $F(2, 55)=3.555$, $P<0.05$]. *Post hoc* tests showed that CLO-65 in F1 was significantly decreased compared to CLO-0, indicating a significant increase in anxiety-like behavior ($P<0.01$) (Fig. 2E). No human-audible vocalizations of mice were observed in either OF or EPM. In other words, CLO exposure during gestation and lactation in F0 mothers decreased

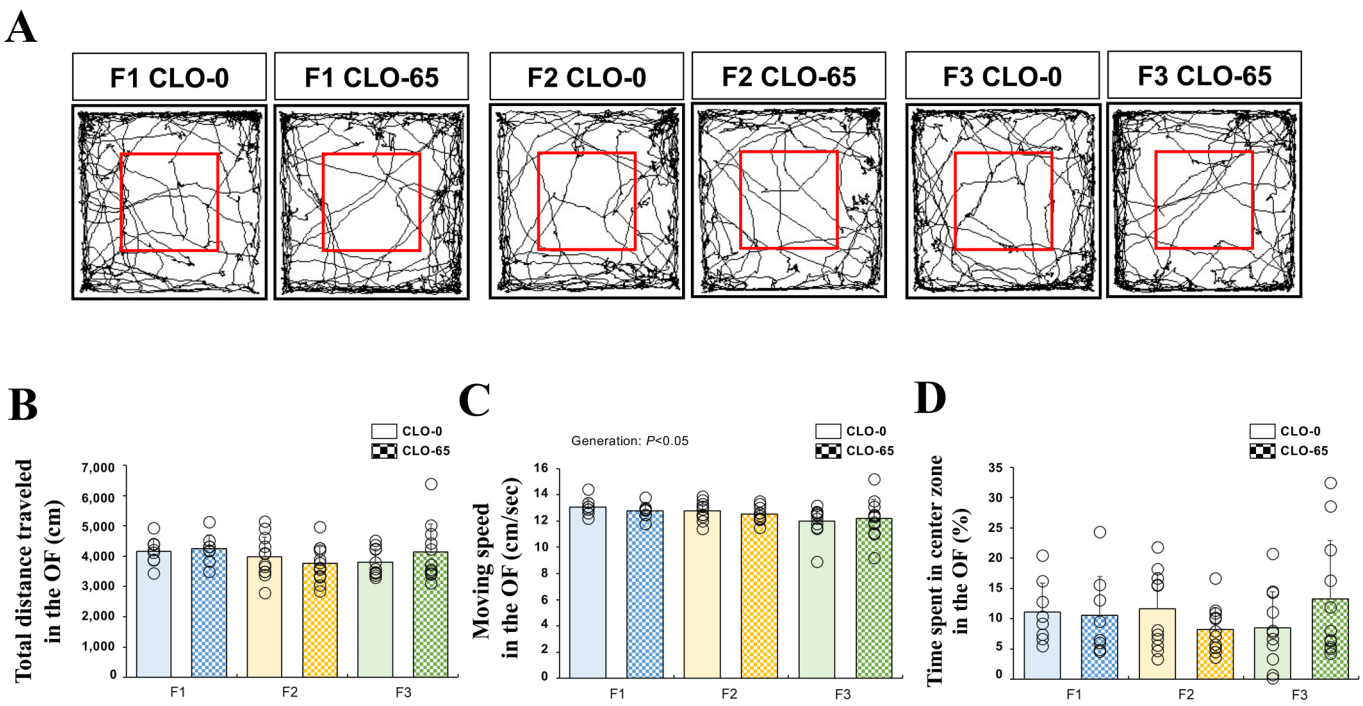

Fig. 1. Neurobehavioral effects of exposure of F0 dams to clothianidin (CLO) during gestation and lactation on F1, F2, and F3 in the open field test (OF) at 10 weeks of age. **A**: Representative trajectory maps show no difference between CLO-65 and CLO-0 in each generation. The red square indicates the center zone (30 × 30 cm). **B**: There was no significant effect on the total distance traveled. **C**: Generation had a significant main effect on moving speed. **D**: There was no significant effect on time spent in the center zone. Data are reported in the form of mean + SD, and each result is plotted. The numbers of mice per group are as follows: F1, CLO-0 (n=8); F1, CLO-65 (n=8); F2, CLO-0 (n=12); F2, CLO-65 (n=12); F3, CLO-0 (n=11); F3, CLO-65 (n=11). No significant differences were found between the groups when comparing the *P*-values with other groups using a two-way ANOVA followed by a Bonferroni *post hoc* test.

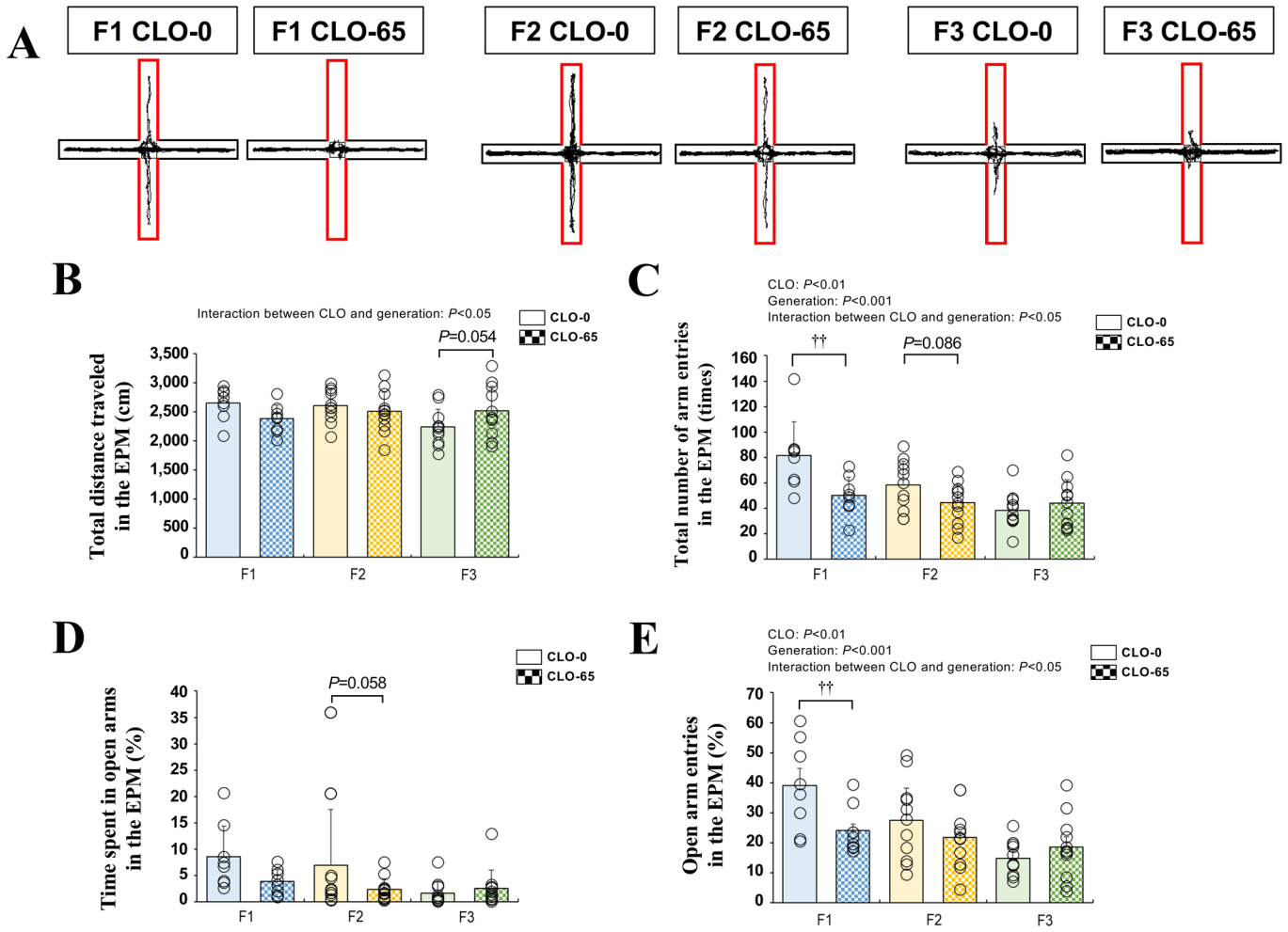

Fig. 2. Neurobehavioral effects of exposure of F0 dams to clothianidin (CLO) during gestation and lactation on F1, F2, and F3 in the elevated plus maze test (EPM) at 10 weeks of age. **A**: Representative trajectory map shows that CLO-65 mice in F1 rarely entered the open arms. The upper and lower red lines indicate open arms, and the left and right black lines indicate enclosed arms. **B**: There was significant interaction between CLO and generation on the total distance traveled. *Post hoc* tests showed an increasing trend for CLO-65 in F3 compared to CLO-0. **C**: CLO and generation had significant main effects on the total number of arm entries, with significant interaction. *Post hoc* tests showed a significant decreasing trend for CLO-65 in F1 compared to CLO-0 and a decreasing trend for CLO-65 in F2 compared to CLO-0. **D**: Time spent in the open arms showed no significant main effect of CLO and generation. *Post hoc* tests showed a decreasing trend for CLO-65 in F2 compared with CLO-0. **E**: CLO and generation had a significant main effect, with a significant interaction effect, on the percentage of open arm entries. *Post hoc* tests showed a significant decreasing trend in CLO-65 in F1 compared to CLO-0. Data are reported in the form of mean + SD, and each result is plotted. The numbers of mice per group are as follows: F1, CLO-0 (n=8); F1, CLO-65 (n=8); F2, CLO-0 (n=11); F2, CLO-65 (n=12); F3, CLO-0 (n=11); F3, CLO-65 (n=11). ††: $P<0.01$ *vs.* other groups (two-way ANOVA followed by Bonferroni *post hoc* test).

locomotor activity and increased anxiety-like behavior in F1. Moreover, a decreasing trend in locomotor activity and an increasing trend in anxiety-like behavior were observed in F2, and an increasing trend in locomotor activity was observed in F3. Anxiety-like behavior was increased and locomotor activity was decreased in F1 in the present study. However, in a previous study, locomotor activity was increased in F1 [24]. The reason for this difference may be due to individual differences involving the epigenome [26, 40] and differences in susceptibility [12], but the fact that significant behavioral abnormalities were induced by CLO in the F1 generation in the present study is consistent with previous findings. We found that the NOAEL of CLO exposure during gestation and lactation in F0 mothers has neurobehavioral successional effects across generations, albeit in a decreasing manner. F0 mothers exposed to CLO have direct CLO effects on their F1 offspring via the placenta and breast milk. Epigenetic changes in the germ cells of F1 offspring further influence F2 offspring, in a sense directly. F2 offspring also maintain their epigenetic changes, thereby exerting CLO effects on F3, which is not directly exposed to CLO [5]. 'Intergenerational' and 'transgenerational' inheritance is occurring. Likewise, CLO may have other developmental impacts. This is because CLO affects germ cells and both male and female reproductive organs [10, 13, 19, 43, 50] and, like other NNs, it may affect DNA methylation and epigenetic mechanisms [8, 47]. The process of reprogramming the epigenome in response to environmental challenges, such as maternal stress, makes the organism more or less adaptive to future challenges. Hence, the epigenetic marks received in the germ line are shaping future generations even before conception [1]. However,

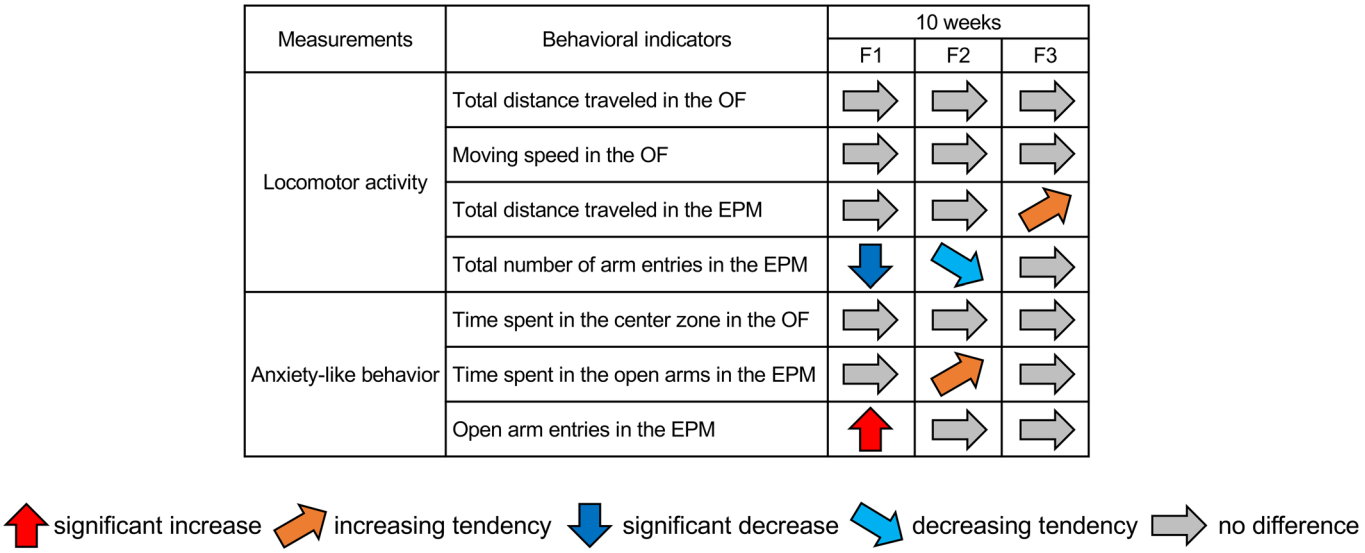

Fig. 3. Summary of the transgenerational effects of clothianidin (CLO) exposure on locomotor activity and anxiety-like behavior as analyzed by the open field test (OF) and elevated plus maze test (EPM). F1 showed a significant decrease in locomotor activity and a significant increase in anxiety-like behavior. F2 showed a decreasing trend in locomotor activity and an increasing trend in anxiety-like behavior, while F3 showed an increasing trend in locomotor activity.

the details of the epigenetic effects of CLO are not clear. Further investigation is needed.

While F1, which received direct maternal CLO exposure, caused statistically significant behavioral abnormalities, F2, which was exposed to CLO via the F1 germline, was less affected than F1. Moreover, F3, which inherited its epigenetic changes, was little affected compared to F1 and F2 (Fig. 3). In other words, it is clear that the NOAEL of CLO affects the neurobehavior of F1 and F2 generations but has no epigenetic neurobehavioral effects on the F3 generation. However, this is at least a neurobehavioral result, and the transgenerational effects of CLO exposure during fetal and neonatal periods on the immune system, learning and memory functions, etc., remain unclear. Therefore, further research is needed, and the obtained results should lead to a prompt review of the use of NNs.

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

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