No-observed-adverse-effect-level (NOAEL) clothianidin, a neonicotinoid pesticide, impairs hippocampal memory and motor learning associated with alteration of gene expression…

Hara, Yukako ; Shoda, Asuka ; Yonoichi, Sakura ; Ishida, Yuya ; Murata, Midori ; Kimura, Mako ; Ito, Makiko ; Nunobiki, Sarika ; Yoshimoto,…

(Citation)
Journal of Veterinary Medical Science,86(3):340-348

(Issue Date)
2024-03

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100488555

The Journal of Veterinary Medical Science

NOTE

Toxicology

No-observed-adverse-effect-level (NOAEL) clothianidin, a neonicotinoid pesticide, impairs hippocampal memory and motor learning associated with alteration of gene expression in cerebellum

Yukako HARA[1)], Asuka SHODA[1)], Sakura YONOICHI[1)], Yuya ISHIDA[1)], Midori MURATA[1)], Mako KIMURA[1)], Makiko ITO[1)], Sarika NUNOBIKI[1)], Ayano YOSHIMOTO[1)], Youhei MANTANI[2)], Toshifumi YOKOYAMA[1)], Tetsushi HIRANO[3)], Yoshinori IKENAKA[4-7)], Yoshiaki TABUCHI[3)], Nobuhiko HOSHI[1)]*

[1)]Laboratory of Animal Molecular Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, Hyogo, Japan
[2)]Laboratory of Histophysiology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, Hyogo, Japan
[3)]Faculty of Pharmaceutical Sciences, University of Toyama, Toyama, Japan
[4)]Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[5)]Translational Research Unit, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[6)]One Health Research Center, Hokkaido University, Hokkaido, Japan
[7)]Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

ABSTRACT. Neonicotinoid pesticides (NNs) have been associated with numerous neurobehavioral effects in rodents, raising concerns about their impact on cognitive function. Clothianidin (CLO), a type of NN, was orally administered to male mice (10 weeks old, C57BL/6N) at the no-observed-adverse-effect level (NOAEL) of 50 mg/kg/day as indicated in the pesticide risk assessment report. Behavioral tests (novel location recognition and rotarod tests) evaluated hippocampal memory and cerebellar motor learning. After each test, plasma monoamines (3-methoxytyramine, histamine, serotonin, tryptamine) were measured by LC-ESI/MS/MS (Liquid chromatography-electrospray ionization/tandem mass spectrometry), and cerebellar mRNA expression was quantified by microarray and qRT-PCR analyses. The NOAEL of CLO was found to impair hippocampal memory, leading to decreased spontaneous locomotor activity and motor function. We reported, for the first time, multiple alterations of gene expression in the cerebellum associated with motor dysfunction.

KEYWORDS: cerebellum, hippocampus, neonicotinoid, novel location recognition test, rotarod test

J Vet Med Sci
86(3): 340–348, 2024
doi: 10.1292/jvms.23-0516

Received: 22 December 2023
Accepted: 24 January 2024
Advanced Epub:
5 February 2024

Neonicotinoid pesticides (NNs) are nicotine analogues with agonist activity on the insect-type nicotinic acetylcholine receptor (nAChR). Their worldwide use has increased rapidly since their development as an alternative to organophosphorus pesticides. Seven NNs, characterized by their penetrating, residual, and selective toxicities, are currently in broad use: imidacloprid (IMI), nitenpyram, acetamiprid (ACE), thiamethoxam (TMX), thiacloprid, clothianidin (CLO), and dinotefuran (DIN) [3, 58]. These NNs exhibit insecticidal activity by continuously exciting and disturbing neurons as competitive modulators of nAChR.

However, from the 1990s through the 2000s, when the widespread use of NNs began, mass disappearances of honeybees were reported in some regions, including the EU and the United States. This phenomenon, called colony collapse disorder (CCD), is characterized by the disappearance of only the worker bees, with no trace of their carcasses, leaving behind the queen and larvae. While

*Correspondence to: Hoshi N: nobhoshi@kobe-u.ac.jp, Laboratory of Animal Molecular Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada, Kobe, Hyogo 657-8501, Japan
(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

the detailed mechanism remains unclear, overuse of NNs is considered a leading factor [38, 39]. During the 2000s, active research was conducted in Europe and the United States to investigate the relationship between CCD and NNs. It was reported that IMI, the first NN developed in Japan, inhibited the honeybee's homing instinct, spatial cognition, learning, and inter-individual communication [4, 11, 17]. This indicated that NNs impair the cognitive and learning functions of insects and that excessive NN use is dangerous.

Although NNs have been considered safe for mammals because their affinity for nAChRs is tens to hundreds of times higher in insects than in mammals, recent studies in mammals, primarily rodents, have indicated that nAChRs induce cognitive–emotional changes, such as anxiety-like behavior and decreased spontaneous locomotor activity [8, 9, 19–22, 24, 25, 37, 40, 44, 51–53, 55, 56, 62]. However, only a limited number of investigations on memory and learning in mammals have been reported, and the severity of effects at trace concentrations, along with the underlying mechanisms, remain unclear.

In this study, we specifically targeted the hippocampus, a region intricately involved in the formation of new memories, particularly spatial memories [41, 46], and the cerebellum, which plays a role in coordinated movement and motor learning [28]. We investigated the neurobehavioral effects of a trace amount of CLO on male mice by assessing hippocampal memory using the novel location recognition test (NLR) and evaluating cerebellar motor learning function through the rotarod test (RR).

C57BL/6N mice were purchased at 9 weeks of age and bred according to a previous report [21]. Forty male mice (10 mice per group) were used for the experiments, and each behavioral test was conducted at 10 weeks of age. The study was approved by the Institutional Animal Care and Use Committee (Permission #30-01-01) and managed in accordance with the Kobe University Laboratory Animal Regulations. The control groups (0 mg/kg/day) were designated as N0 (N for NLR) and R0 (R for RR). Following a previous study [21], the treatment groups were orally administered CLO (>95% purity) at 50 mg/kg/day based on the male no-observed-adverse-effect level (NOAEL) [14, 59] and are denoted as N50 and R50. Each behavioral test was conducted in the light phase using different sets of mice.

The NLR was conducted by modifying the previous protocol [36, 37, 43]. The experiment comprised habituation (DAY1), familiarization (DAY2), and test trials (DAY3), each separated by a 24-hr interval, spanning a 3-day period. After a single oral administration, mice were acclimated to the testing room conditions in their home cage for 1 hr prior to the trial's commencement (20–30 lux). Oral administration was omitted on DAY1. On DAY1, mice were placed in an open field (60 × 60 × 40 cm) with no objects, allowed to explore freely for 5 min, and then returned to the home cage. On DAY2, two Lego brick towers (5 × 5 × 8 cm) were positioned in a corner of the open field, and the mice were allowed to explore them for 5 min. On DAY3, one of the towers was relocated to another corner, and the mice were given 5 min for exploration. All behaviors of the mice were recorded by a video camera, and their trajectories were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Exploration was defined as the mouse touching an object with its nose. We calculated the percentage of explorations where objects were relocated to a new position out of the total number of explorations of all objects (frequency novel location exploration).

The RR was conducted using a modified protocol [50]. A single-lane rotarod (MK-630B, Muromachi-Kikai, Tokyo, Japan) fitted with a rat rotor (90 mm diameter) was utilized. The day before the RR started, all mice were placed on the rotarod for 5 min every 3 hr (3 times/day) to acclimate them to the machine. After a single oral administration, mice were acclimated to the testing room conditions in their home cage for 1 hr prior to the trial's commencement. On DAY1, mice were trained by walking at 4 rpm for 1 min, followed by walking at 10 rpm for up to 5 min. On DAY2–DAY4, after acclimation on a nonrotating rotor for 1 min, mice were walked at 10 rpm for up to 5 min. The time spent on the rod was measured 5 times a day.

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. Plasma obtained by centrifuging whole blood at 4°C and 3,000 rpm was utilized for LC-ESI/MS/MS analysis [19, 45] to measure the levels of plasma corticosterone, 3-methoxytyramine (3MT), histamine, serotonin, and tryptamine and to assess the impact of CLO after behavioral tests. In accordance with a previous report [40], the brains were excised and sliced at a thickness of 3 µm (−1.58 mm to −2.06 mm), and immunohistochemical analysis was performed in the hippocampal dentate gyrus (DG) using rabbit monoclonal anti-c-fos (1:10,000; #2353; Cell Signaling Technology, Beverly, MA, USA). c-Fos expression in the DG was quantified by counting the immunopositive cells per area. This analysis was performed using ImageJ software.

After the RR study, the cerebellum of some mice was excised and frozen in liquid nitrogen. RNA extraction, quality analysis, and microarray analysis were performed as previously reported [44], with n=2 in the R0 group and n=2 in the R50 group. The microarray data (.CEL files) were deposited in a public database (Gene Expression Omnibus, accession number: GSE251773).

The qRT-PCR analysis was conducted by modifying the previous protocol [51]. Thermal cycling was performed by initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec, and extension at 72°C for 20 sec for 50 cycles. The gene copy number was calculated using a standard curve and normalized by the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and *β-actin* (*Actb*) according to The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [6, 60]. All samples were assayed in duplicate, and the specificity of PCR products was confirmed by melting curves (n=6).

Statistical analyses were performed with BellCurve for Excel (Version 4.04; SSRI, Tokyo, Japan). The behavioral data were analyzed by two-way ANOVA and Bonferroni's *post hoc* tests. Welch's *t*-test was used to detect differences in the quantitative immunohistochemistry data and qRT-PCR data. The Smirnov–Grubbs two-tailed test was used to exclude outliers. The results were considered significant when the *P*-value was less than 0.05.

The NLR results showed significant main effects of CLO administration on total travel distance, moving speed, and the total number of explorations during DAY2 and DAY3 ($F(1, 17)=11.96$, $P<0.01$; $F(1, 17)=5.44$, $P<0.05$; $F(1, 17)=5.04$, $P<0.05$). These main effects were lower than those in the N0 group (Fig. 1A-C, Supplementary Fig. 1A). There was a significant interaction between the days and CLO administration ($F(1, 12)=5.78$, $P<0.05$), and *post hoc* tests revealed a significantly lower frequency of exploration on DAY3

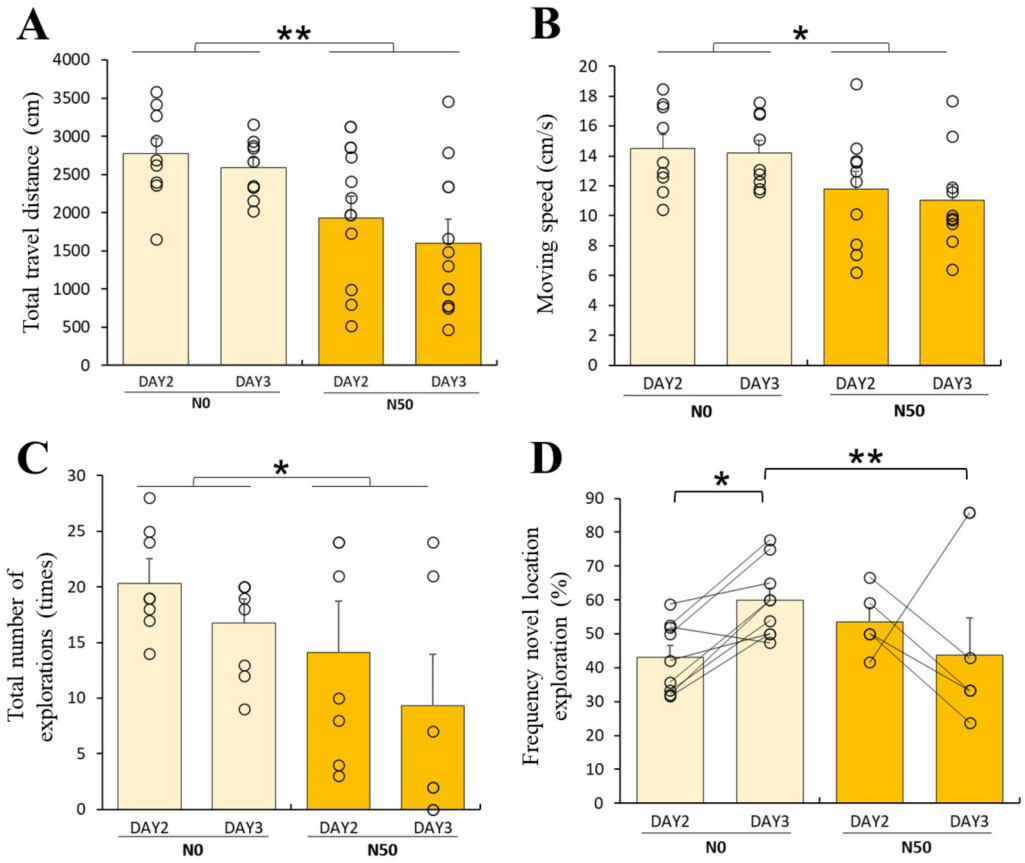

Fig. 1. Effects of clothianidin (CLO) exposure on total travel distance (**A**), moving speed (**B**), explorations (**C**), and frequency of novel location exploration (**D**) in 10-week-old male mice during the novel location recognition test (NLR). **A**, **B**, **C**: CLO showed significant main effects on total travel distance, moving speed, and total number of explorations; **D**: significant interaction was found between CLO and days for frequency novel location exploration, with a significant simple main effect of days for the N0 group. The N50 group exhibited significantly lower values for DAY3 compared to N0. Data represent mean + SE for each group (n=5–9), and each result is plotted. **P*<0.05, ***P*<0.01 vs. control group (two-way ANOVA followed by Bonferroni's *post hoc* test).

compared to the N0 group ($P<0.01$). The simple main effect was significant ($P<0.05$) only in the control group (Fig. 1D). Plasma 3MT and tryptamine levels tended to be lower or significantly lower in the N50 group ($P=0.067$, $P<0.05$) (Fig. 2). Human-audible vocalizations, which characterize anxiety-like behavior [21], were noted in all individual mice in the N50 group during the behavioral test (Supplementary Fig. 1B).

The RR results showed significant main effects of days and CLO administration on the time spent on the rod per day ($F(3, 13)=27.31$, $P<0.001$; $F(3, 13)=11.99$, $P<0.001$; $F(1, 13)=11.21$, $P<0.01$). A significant interaction between the days and CLO administration was observed, with *post hoc* tests showing significantly lower values for DAY2, DAY3, and DAY4 compared to the R0 group ($P<0.05$, $P<0.001$, $P<0.001$). The simple main effect of the days was significant ($F(3, 39)=39.95$, $P<0.001$) only in the R0 group (Fig. 3). As in the NLR, human-audible vocalizations were observed in all mice in the R50 group during the RR. Plasma 3MT and histamine levels were significantly higher ($P<0.01$, $P<0.001$), and tryptamine levels were significantly lower ($P<0.001$), in the R50 group (Fig. 4).

The results of these behavioral tests revealed that exposure to NOAEL doses of CLO induced behavioral abnormalities and hindered memory formation in 10-week-old mice. The NLR results suggested that exposure to CLO in 10-week-old mice may decrease spontaneous locomotion and exploratory behavior, making it challenging to remember the relative positions of objects. Additionally, the RR results indicated that CLO exposure in 10-week-old mice may significantly impair coordination and hinder the mice's ability to learn walking locomotion.

The LC-ESI/MS/MS results revealed that plasma 3MT and tryptamine varied between the N50 and R50 groups. The discrepancy in the direction of changes may stem from the differing time intervals between the end of the behavioral test and sampling for the NLR and RR groups. Monoamines may not readily cross the blood–brain barrier, making it difficult to directly interpret the relationship between CLO and behavioral effects based on plasma monoamine alone. However, IMI altered several monoamine secretions in the striatum, cerebellum, and plasma of mice, alongside behavioral abnormalities, suggesting an association between central nervous system disorder and changes in brain and plasma monoamine levels [19]. Furthermore, CLO has been reported to induce dopamine release from the rat striatum via nAChR [13], and the dopamine metabolite 3MT [42] in plasma is considered a biological marker of brain dopamine activity.

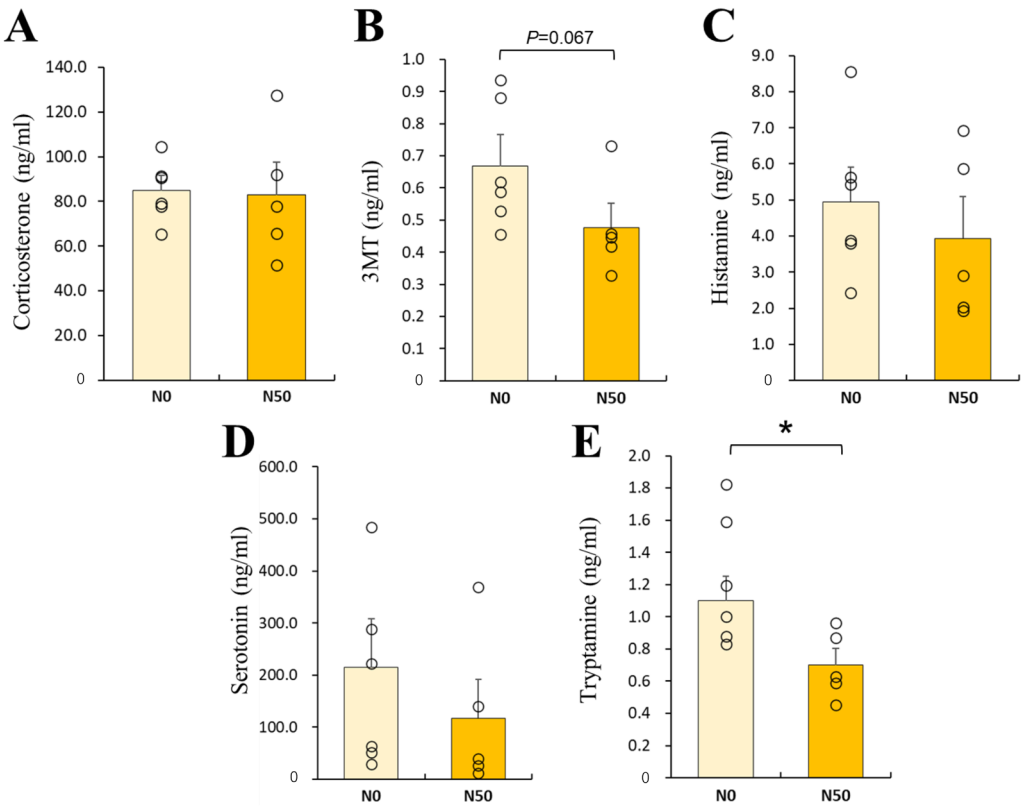

Fig. 2. Effects of clothianidin (CLO) exposure on plasma concentrations of corticosterone (**A**), 3-methoxytyramine (3MT) (**B**), histamine (**C**), serotonin (**D**), and tryptamine (**E**) in 10-week-old male mice, 2 hr after the novel location recognition test (NLR). **A**, **C**, **D**: The N50 group showed no significant difference compared to N0; **B**: N50 tended to decrease in 3MT compared to N0; **E**: N50 significantly decreased in tryptamine compared to N0. Data represent mean + SE for each group (n=5–6), and each result is plotted. **P*<0.05, ***P*<0.01 vs. control group (Welch's *t*-test).

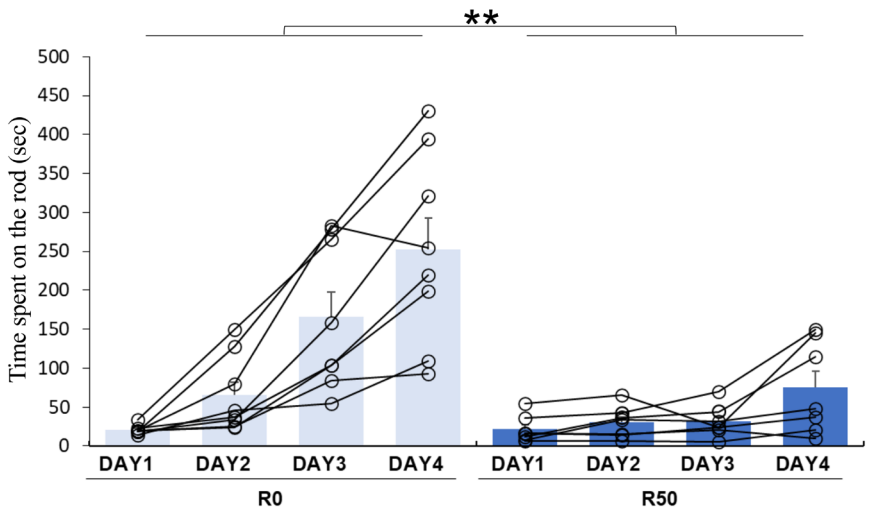

Fig. 3. Time spent on the rod in the rotarod test (RR). Significant main effects were found for days and clothianidin (CLO), with a significant interaction between days and CLO. A simple main effect of days was found for only the R0 group. Data represent mean + SE for each group (n=7–8), and each result is plotted. ***P*<0.01 vs. control group (two-way ANOVA followed by Bonferroni's *post hoc* test).

The N0 and N50 groups, sampled 2 hr after the NLR, exhibited lower 3MT levels. Flexible synaptic changes are crucial for memory and learning, and a transient decrease in dopamine (DA dip) released in the striatum is thought to trigger synaptic plasticity [26]. Additionally, dopamine release is said to enhance memory in spatial learning involving the hippocampus, and normal dopamine regulation is crucial for memory formation [30]. In the N50 group, the temporary dopamine release induced by CLO may have

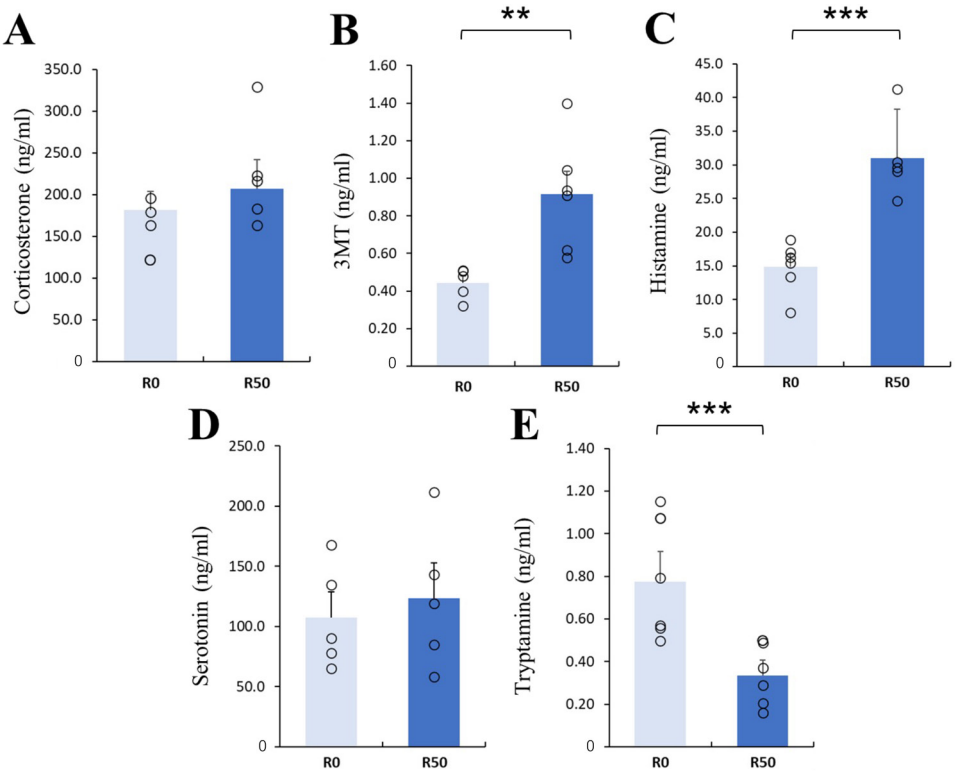

Fig. 4. Effects of clothianidin (CLO) exposure on plasma concentrations of corticosterone (**A**), 3-methoxytyramine (3MT) (**B**), histamine (**C**), serotonin (**D**), and tryptamine (**E**) in 10-week-old male mice immediately after the rotarod test (RR). **A**, **D**: The R50 group showed no significant difference compared to R0; **B**: R50 significantly increased in 3MT compared to R0; **C**: R50 significantly increased in histamine compared to R0; **E**: R50 significantly decreased in tryptamine compared to R0. Data represent mean + SE for each group (n=5–6), and each result is plotted. **P*<0.05, ***P*<0.01 vs. control group (Welch's *t*-test).

prevented a dip in DA levels and the elicitation of synaptic plasticity, possibly hindering the dopamine release necessary for memory formation. However, the R0 and R50 groups, sampled immediately after the RR, displayed a direct effect of CLO, with elevated 3MT in the R50 group. 3MT is implicated in motor function, and its association with neurological disorders characterized by impaired dopaminergic signaling, such as Parkinson's disease and schizophrenia, has been suggested [54]. The increase in plasma 3MT in the R50 group is believed to result from the metabolization of dopamine released by CLO and flowing into the plasma. In other words, excessive dopamine or 3MT release may contribute to behavioral abnormalities.

In addition, tryptamine, a metabolite of tryptophan that can cross the blood–brain barrier [2], is believed to regulate dopaminergic, glutaminergic, and serotonergic neurotransmission by activating trace amine-related receptors expressed in the mammalian brain [15, 16]. This suggests that the decrease in tryptamine observed in this study may have influenced behavior through disturbances in neurotransmitter regulation.

To further investigate the influence of CLO on neurons in each behavioral test, we conducted immunohistochemical staining to visualize c-fos expression in the DG, which is crucial for memory and learning [61]. c-Fos is commonly used as a marker for neural activity, since it is expressed when neurons fire and generate action potentials [23]. Immunohistochemistry for c-fos showed a positive reaction in all groups that underwent the NLR. The number of c-fos–positive cells per unit area in the DG was significantly (P<0.05) reduced in the N50 group compared to the N0 group (Fig. 5). However, there was no significant difference in the number of c-fos–positive cells per unit area after the RR test (results not shown).

In the NLR, both behavioral assessments and immunohistochemical analysis of the hippocampus using c-fos revealed significant neurobehavioral effects, confirming previous findings that NN adversely affects memory and learning functions associated with the hippocampus [29, 37]. In addition, the LC-ESI/MS/MS results newly suggested that excessive dopamine release induced by CLO may have hindered the DA dip associated with synaptic plasticity [26], and caused dopamine dysregulation, thereby inhibiting normal memory formation. These results are at odds with a previous study [37], which reported an increase in the number of c-fos–positive cells in the DG of male mice subjected to the Barnes maze after exposure to a NOAEL dose of CLO. However, this inconsistency is likely attributable to differences in the learning process and stress load between the NLR and the Barnes maze. The DG, a brain region specifically activated in the NLR, was not activated during memory formation and recall, aligning with the outcomes of the current behavioral study. An experiment in which IMI was administered to adult rats revealed impaired cognitive function in a spatial learning task, along with an increase in the mRNA expression of muscarinic receptors (M1) associated with memory and learning in the hippocampal DG [29]. Similarly, alterations in gene expression related to learning are suggested to have contributed to the decline

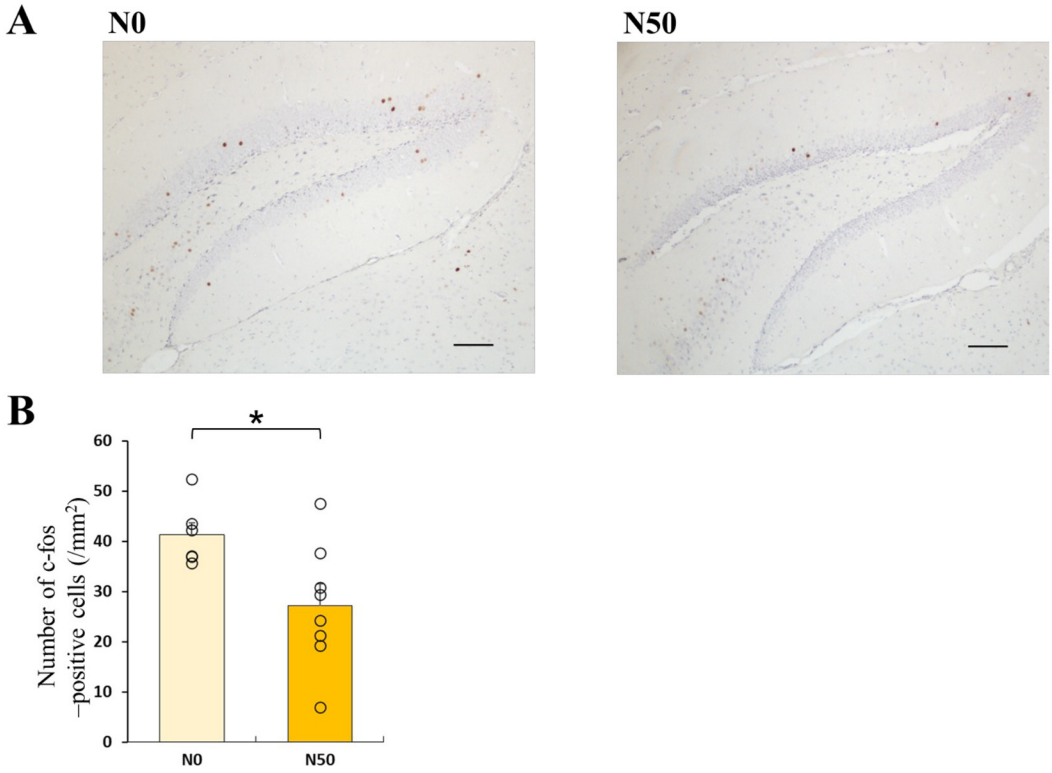

Fig. 5. Representative histology and immunohistochemistry of c-fos in the hippocampal dentate gyrus (DG) at 10 weeks (**A**) and the number of c-fos–positive cells in the DG (**B**). **B**: The N50 group was significantly reduced compared to N0. Data represent mean + SE for each group (n=6–8), and each result is plotted. Bar is 100 µm. *P<0.05, vs. control group (Welch's t-test).

in hippocampal memory.

To support the notable findings of the RR, we conducted gene expression analysis of the cerebellum, a crucial region for motor function. Microarray analysis of cerebellar mRNA revealed that 510 genes exhibited upregulated expression, while 430 genes showed downregulated expression. Based on Ingenuity Pathway Analysis (IPA), we elucidated the biological functions and canonical pathways associated with the altered genes. CLO exposure resulted in diminished functions related to "Motor dysfunction or movement disorder", "Behavioral deficit", "Movement disorder", and "Ataxia", while functions associated with "Development neurons", "Neuritogenesis", "Formation of dendrites", "Dendritic growth/branching", and "Branching of neurites" were enhanced (Supplementary Tables 2, 3). In essence, exposure to a low dose of CLO indicated a potential to induce motor impairments, mirroring the results of the behavioral tests. Furthermore, the findings suggested an acceleration in neuronal differentiation and promotion of the differentiation, elongation, and branching of neurites and axons. The gene networks for "Neuritogenesis" and "Motor dysfunction or movement disorder" are depicted in Fig. 6A. The two annotations are closely related, as several genes are connected to either function via another gene. Additionally, pathways related to mitochondrial function and calcium signaling were either suppressed or stimulated in the R50 group (Fig. 6B).

To confirm the microarray results, the expression levels of upregulated genes were confirmed using qRT-PCR. We selected 9 crucial genes known for their significance in biological functions, canonical pathways, and gene networks: *Cacna1g* (calcium voltage-gated channel subunit alpha 1g), *Cacna1d* (calcium voltage-gated channel subunit alpha 1d), *Lrrk2* (leucine-rich repeat kinase 2), *Lrp1b* (low-density lipoprotein receptor-related protein 1b), *Syne1* (spectrin repeat-containing nuclear envelope protein 1), *Mtcl1* (microtubule crosslinking factor 1), *Rora* (retinoic acid receptor-related orphan receptor alpha), *Mark4* (MAP/microtubule affinity-regulating kinase 4), and *Fgfr1* (fibroblast growth factor receptor 1). The quantitative results revealed significantly lower values in *Cacna1g* (P<0.001), while higher values or a tendency toward higher values were observed in *Lrp1b*, *Syne1*, and *Rora* (P=0.0654, P<0.05, P<0.05) (Fig. 6C).

In the R0 group, the significantly reduced expression of *Cacna1g*, a gene encoding a T-type calcium channel involved in calcium signaling, is noteworthy. *Cacna1g* is highly expressed in various brain regions, including the cerebellar Purkinje cell layer, brainstem, and spinal cord [57]. Ca^{2+}, serving as a second messenger, plays a crucial role in diverse cellular functions and activities, regulating physiological processes such as neurotransmitter transmission and synaptic plasticity [47]. One hypothesis suggests that dysregulation of Ca^{2+} may contribute to the onset of neurodegenerative diseases [1, 31]. In both humans and mice, *Cacna1g* expression decreases with age at both the mRNA and protein levels, and further reduction in expression has been reported in Alzheimer's disease [48]. In a study using a rat cerebellar cell model, variations in the expression of two types of calcium channels have been observed, suggesting that NNs may disrupt calcium regulation in cerebellar neurons *in vitro* [35]. The decrease in *Cacna1g* expression does not directly correlate with the microarray results. This discrepancy may be attributed to the limited number of samples in the microarray study, the potential occurrence of transcript mutations or mRNA degradation, and differences in target positions of primers and probes.

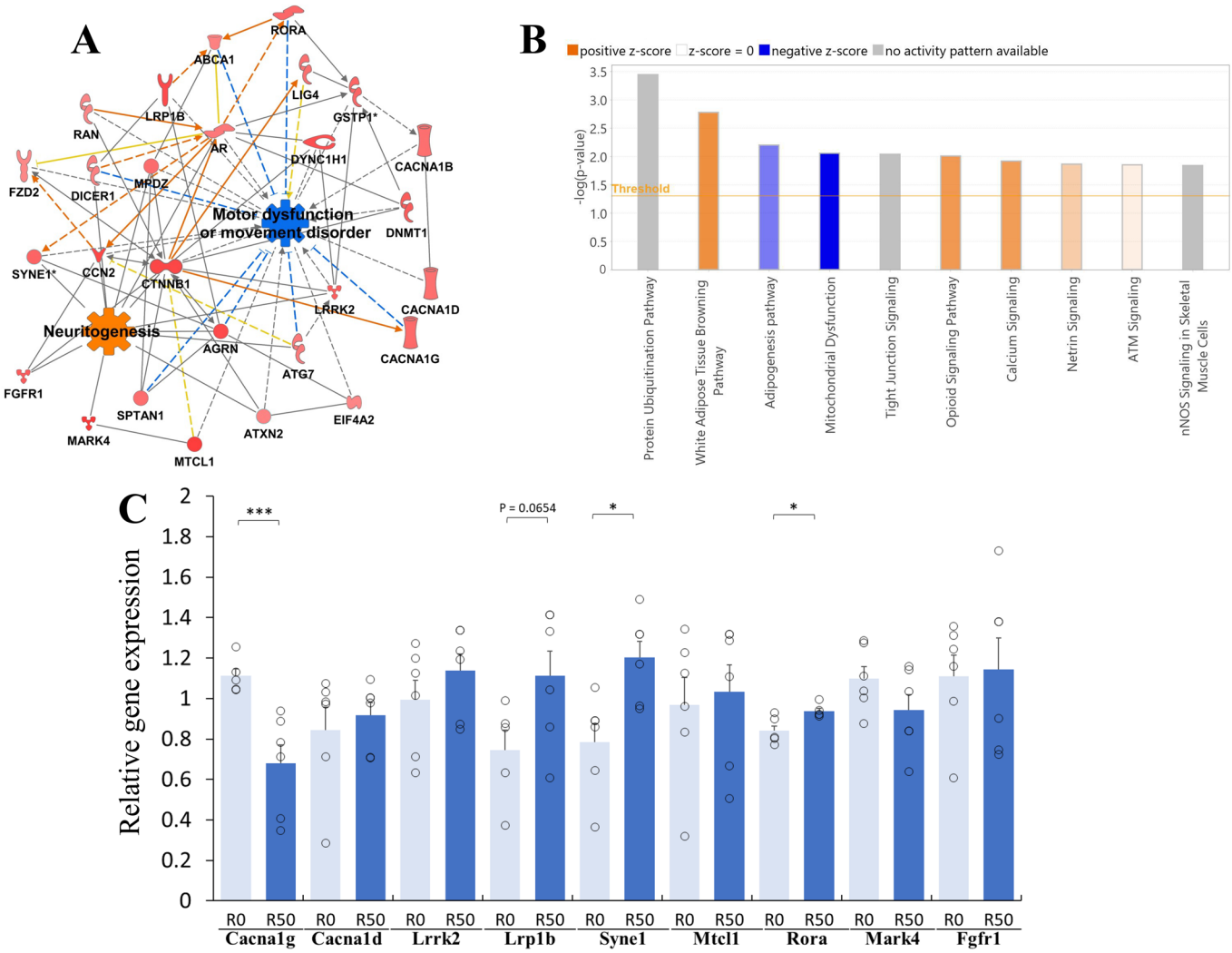

Fig. 6. Microarray analysis (**A**, **B**: n=2) and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis (**C**: n=6) in 10-week-old mice exposed to clothianidin (CLO). **A**: Gene network map showing the interaction between the upregulated genes and other molecules at 10 weeks of age, focusing on neural functions and motor function. **B**: Signaling pathways activated in the cerebellum at 10 weeks of age. **C**: Gene expressions in the cerebellum of 10-week-old mice. Data represent mean + SE for each group. **P*<0.05, ***P*<0.01, vs. control group.

Syne1, which exhibits high levels of expression in the Purkinje cell layer, encodes multiple variants and is implicated in processes such as the binding of the cytoskeleton to the nuclear membrane [7]. *Syne1* is associated with cerebellar ataxia [18, 27], aligning with the results of the present behavioral study and microarray results, where motor deficits were observed. *Rora* is implicated in dendritic differentiation in Purkinje cells, with overexpression shown to promote differentiation, highlighting its importance in dendritic remodeling regulation [5].

In summary, our findings from the RR suggest that, in addition to cognitive impairment linked to calcium dysregulation, the altered expression of genes involved in cerebellar function maintenance, including cytoskeletal and dendritic differentiation, contributes significantly to motor disorder.

In the present study, we revealed that exposure of 10-week-old male mice to NOAEL doses of CLO impairs hippocampal memory and cerebellar motor learning function. In recent years, NN and other pesticides have been considered factors in the initiation of developmental disorders [12, 32–34, 49], and in 2013, the European Food Safety Authority (EFSA) officially announced that NNs can affect the neurodevelopment not only of honeybees but also of other organisms, including humans [10]. In light of these findings, it is necessary to clarify the neurodevelopmental effects of exposure to NOAEL doses of CLO during the developmental period on subsequent memory and learning functions and to evaluate the future risks of pesticide exposure.

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

ACKNOWLEDGMENTS. This work was supported in part by Grants-in-Aid for Scientific Research A (JP18H04132 & JP23H00512: YI) and B (JP22H03750 & JP19H04277: NH), as well as by Grants-in-Aid for Challenging Research (Exploratory JP21K19846: NH;

Pioneering JP22K18425: YI) and by Grants-in-Aid for Early-Career Scientists (JP19K19406 & 22K17342: TH) from the Japan Society for the Promotion of Science. It was also supported in part by "Act Beyond Trust" (GIA) civil grants in 2020–2023 (NH). In addition, we acknowledge financial support from the Nakajima Foundation, the Sumitomo Foundation, the Nihon Seimei Foundation, and the Triodos Foundation (to YI). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Alzheimer's Association Calcium Hypothesis Workgroup. 2017. Calcium Hypothesis of Alzheimer's disease and brain aging: A framework for integrating new evidence into a comprehensive theory of pathogenesis. *Alzheimers Dement* **13**: 178–182.e17. [Medline] [CrossRef]
2. Anderson GM. 2021. The quantitative determination of indolic microbial tryptophan metabolites in human and rodent samples: A systematic review. *J Chromatogr B Analyt Technol Biomed Life Sci* **1186**: 123008. [Medline] [CrossRef]
3. Bass C, Denholm I, Williamson MS, Nauen R. 2015. The global status of insect resistance to neonicotinoid insecticides. *Pestic Biochem Physiol* **121**: 78–87. [Medline] [CrossRef]
4. Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S, Porrini C. 2003. Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. *Bull Insectol* **56**: 63–67.
5. Boukhtouche F, Janmaat S, Vodjdani G, Gautheron V, Mallet J, Dusart I, Mariani J. 2006. Retinoid-related orphan receptor alpha controls the early steps of Purkinje cell dendritic differentiation. *J Neurosci* **26**: 1531–1538. [Medline] [CrossRef]
6. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**: 611–622. [Medline] [CrossRef]
7. Cartwright S, Karakesisoglou I. 2014. Nesprins in health and disease. *Semin Cell Dev Biol* **29**: 169–179. [Medline] [CrossRef]
8. Costas-Ferreira C, Faro LRF. 2021. Neurotoxic effects of neonicotinoids on mammals: what is there beyond the activation of nicotinic acetylcholine receptors? A systematic review. *Int J Mol Sci* **22**: 8413. [Medline] [CrossRef]
9. Dhouib IB, Annabi A, Doghri R, Rejeb I, Dallagi Y, Bdiri Y, Lasram MM, Elgaaied A, Marrakchi R, Fazaa S, Gati A. 2017. Neuroprotective effects of curcumin against acetamiprid-induced neurotoxicity and oxidative stress in the developing male rat cerebellum: biochemical, histological, and behavioral changes. *Environ Sci Pollut Res Int* **24**: 27515–27524. [Medline] [CrossRef]
10. EFSA Panel on Plant Protection Products and their Residues. 2013. Scientific opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid. *EFSA* Journal **11**: 3471.
11. Eiri DM, Nieh JC. 2012. A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *J Exp Biol* **215**: 2022–2029. [Medline] [CrossRef]
12. Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcín C, Montiel-Nava C, Patel V, Paula CS, Wang C, Yasamy MT, Fombonne E. 2012. Global prevalence of autism and other pervasive developmental disorders. *Autism Res* **5**: 160–179. [Medline] [CrossRef]
13. Faro LRF, Tak-Kim H, Alfonso M, Durán R. 2019. Clothianidin, a neonicotinoid insecticide, activates α4β2, α7 and muscarinic receptors to induce *in vivo* dopamine release from rat striatum. *Toxicology* **426**: 152285. [Medline] [CrossRef]
14. Food and Agriculture Organization of the United Nations. 2020. FAO Specifications and Evaluations for Agricultural Pesticides Clothianidin. http://www.fao.org/3/ca7726en/ca7726en.pdf [accessed on November 30, 2023].
15. Freyberg Z, Saavedra JM. 2020. Trace amines and trace amine-associated receptors: A new frontier in cell signaling. *Cell Mol Neurobiol* **40**: 189–190. [Medline] [CrossRef]
16. Gainetdinov RR, Hoener MC, Berry MD. 2018. Trace amines and their receptors. *Pharmacol Rev* **70**: 549–620. [Medline] [CrossRef]
17. Goñalons C, Farina WM. 2015. Effects of sublethal doses of imidacloprid on young adult honeybee behaviour. *PLoS One* **10**: e0140814. [Medline] [CrossRef]
18. Gros-Louis F, Dupré N, Dion P, Fox MA, Laurent S, Verreault S, Sanes JR, Bouchard JP, Rouleau GA. 2007. Mutations in SYNE1 lead to a newly discovered form of autosomal recessive cerebellar ataxia. *Nat Genet* **39**: 80–85. [Medline] [CrossRef]
19. Hirai A, Yamazaki R, Kobayashi A, Kimura T, Nomiyama K, Shimma S, Nakayama SMM, Ishizuka M, Ikenaka Y. 2022. Detection of changes in monoamine neurotransmitters by the neonicotinoid pesticide imidacloprid using mass spectrometry. *Toxics* **10**: 696. [Medline] [CrossRef]
20. Hirano T, Miyata Y, Kubo S, Ohno S, Onaru K, Maeda M, Kitauchi S, Nishi M, Tabuchi Y, Ikenaka Y, Ichise T, Nakayama SMM, Ishizuka M, Arizono K, Takahashi K, Kato K, Mantani Y, Yokoyama T, Hoshi N. 2021. Aging-related changes in the sensitivity of behavioral effects of the neonicotinoid pesticide clothianidin in male mice. *Toxicol Lett* **342**: 95–103. [Medline] [CrossRef]
21. Hirano T, Yanai S, Takada T, Yoneda N, Omotehara T, Kubota N, Minami K, Yamamoto A, Mantani Y, Yokoyama T, Kitagawa H, Hoshi N. 2018. NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. *Toxicol Lett* **282**: 57–63. [Medline] [CrossRef]
22. Hirano T, Yanai S, Omotehara T, Hashimoto R, Umemura Y, Kubota N, Minami K, Nagahara D, Matsuo E, Aihara Y, Shinohara R, Furuyashiki T, Mantani Y, Yokoyama T, Kitagawa H, Hoshi N. 2015. The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice. *J Vet Med Sci* **77**: 1207–1215. [Medline] [CrossRef]
23. Hoffman GE, Smith MS, Verbalis JG. 1993. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrinol* **14**: 173–213. [Medline] [CrossRef]
24. Hoshi N. 2021. Adverse effects of pesticides on regional biodiversity and their mechanisms. pp. 235–247. In: Risks and Regulation of New Technologies (Matsuda T, Wolff J, Yanagawa T eds.), Springer, Singapore.
25. Hoshi N, Hirano T, Omotehara T, Tokumoto J, Umemura Y, Mantani Y, Tanida T, Warita K, Tabuchi Y, Yokoyama T, Kitagawa H. 2014. Insight into the mechanism of reproductive dysfunction caused by neonicotinoid pesticides. *Biol Pharm Bull* **37**: 1439–1443. [Medline] [CrossRef]
26. Iino Y, Sawada T, Yamaguchi K, Tajiri M, Ishii S, Kasai H, Yagishita S. 2020. Dopamine D2 receptors in discrimination learning and spine enlargement. *Nature* **579**: 555–560. [Medline] [CrossRef]
27. Indelicato E, Nachbauer W, Fauth C, Krabichler B, Schossig A, Eigentler A, Dichtl W, Wenning G, Wagner M, Fanciulli A, Janecke A, Boesch S. 2019. SYNE1-ataxia: novel genotypic and phenotypic findings. *Parkinsonism Relat Disord* **62**: 210–214. [Medline] [CrossRef]
28. Ito M. 2000. Mechanisms of motor learning in the cerebellum. *Brain Res* **886**: 237–245. [Medline] [CrossRef]
29. Kara M, Yumrutas O, Demir CF, Ozdemir HH, Bozgeyik I, Coskun S, Eraslan E, Bal R. 2015. Insecticide imidacloprid influences cognitive functions and alters learning performance and related gene expression in a rat model. *Int J Exp Pathol* **96**: 332–337. [Medline] [CrossRef]

30. Kempadoo KA, Mosharov EV, Choi SJ, Sulzer D, Kandel ER. 2016. Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory. *Proc Natl Acad Sci USA* **113**: 14835–14840. [Medline] [CrossRef]
31. Khachaturian ZS. 1994. Calcium hypothesis of Alzheimer's disease and brain aging. *Ann N Y Acad Sci* **747**: 1–11. [Medline] [CrossRef]
32. Kimura E, Endo T, Yoshioka W, Ding Y, Ujita W, Kakeyama M, Tohyama C. 2016. *In utero* and lactational dioxin exposure induces Sema3b and Sema3g gene expression in the developing mouse brain. *Biochem Biophys Res Commun* **476**: 108–113. [Medline] [CrossRef]
33. Kimura-Kuroda J, Komuta Y, Kuroda Y, Hayashi M, Kawano H. 2012. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. *PLoS One* **7**: e32432. [Medline] [CrossRef]
34. Kimura-Kuroda J, Nagata I, Kuroda Y. 2007. Disrupting effects of hydroxy-polychlorinated biphenyl (PCB) congeners on neuronal development of cerebellar Purkinje cells: a possible causal factor for developmental brain disorders? *Chemosphere* **67**: S412–S420. [Medline] [CrossRef]
35. Kimura-Kuroda J, Nishito Y, Yanagisawa H, Kuroda Y, Komuta Y, Kawano H, Hayashi M. 2016. Neonicotinoid insecticides alter the gene expression profile of neuron-enriched cultures from neonatal rat cerebellum. *Int J Environ Res Public Health* **13**: 987. [Medline] [CrossRef]
36. Kitamura Y, Kanemoto E, Sugimoto M, Machida A, Nakamura Y, Naito N, Kanzaki H, Miyazaki I, Asanuma M, Sendo T. 2017. Influence of nicotine on doxorubicin and cyclophosphamide combination treatment-induced spatial cognitive impairment and anxiety-like behavior in rats. *Naunyn Schmiedebergs Arch Pharmacol* **390**: 369–378. [Medline] [CrossRef]
37. Kubo S, Hirano T, Miyata Y, Ohno S, Onaru K, Ikenaka Y, Nakayama SMM, Ishizuka M, Mantani Y, Yokoyama T, Hoshi N. 2022. Sex-specific behavioral effects of acute exposure to the neonicotinoid clothianidin in mice. *Toxicol Appl Pharmacol* **456**: 116283. [Medline] [CrossRef]
38. Lu C, Chang CH, Lemos B, Zhang Q, Maclntosh D. 2020. Mitochondrial Dysfunction: a plausible pathway for honeybee colony collapse Disorder (CCD). *Environ Sci Technol Lett* **7**: 254–258. [CrossRef]
39. Lu C, Warchol KM, Callahan RA. 2012. *In situ* replication of honey bee colony collapse disorder. *Bull Insectol* **65**: 99–106.
40. Maeda M, Kitauchi S, Hirano T, Ikenaka Y, Nishi M, Shoda A, Murata M, Mantani Y, Tabuchi Y, Yokoyama T, Hoshi N. 2021. Fetal and lactational exposure to the no-observed-adverse-effect level (NOAEL) dose of the neonicotinoid pesticide clothianidin inhibits neurogenesis and induces different behavioral abnormalities at the developmental stages in male mice. *J Vet Med Sci* **83**: 542–548. [Medline] [CrossRef]
41. McNaughton BL, Barnes CA, Gerrard JL, Gothard K, Jung MW, Knierim JJ, Kudrimoti H, Qin Y, Skaggs WE, Suster M, Weaver KL. 1996. Deciphering the hippocampal polyglot: the hippocampus as a path integration system. *J Exp Biol* **199**: 173–185. [Medline] [CrossRef]
42. Molinoff PB, Axelrod J. 1971. Biochemistry of catecholamines. *Annu Rev Biochem* **40**: 465–500. [Medline] [CrossRef]
43. Murai T, Okuda S, Tanaka T, Ohta H. 2007. Characteristics of object location memory in mice: behavioral and pharmacological studies. *Physiol Behav* **90**: 116–124. [Medline] [CrossRef]
44. Nishi M, Sugio S, Hirano T, Kato D, Wake H, Shoda A, Murata M, Ikenaka Y, Tabuchi Y, Mantani Y, Yokoyama T, Hoshi N. 2022. Elucidation of the neurological effects of clothianidin exposure at the no-observed-adverse-effect level (NOAEL) using two-photon microscopy in vivo imaging. *J Vet Med Sci* **84**: 585–592. [Medline] [CrossRef]
45. Ohno S, Ikenaka Y, Onaru K, Kubo S, Sakata N, Hirano T, Mantani Y, Yokoyama T, Takahashi K, Kato K, Arizono K, Ichise T, Nakayama SMM, Ishizuka M, Hoshi N. 2020. Quantitative elucidation of maternal-to-fetal transfer of neonicotinoid pesticide clothianidin and its metabolites in mice. *Toxicol Lett* **322**: 32–38. [Medline] [CrossRef]
46. O'Keefe J, Dostrovsky J. 1971. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res* **34**: 171–175. [Medline] [CrossRef]
47. Pozzan T, Rizzuto R, Volpe P, Meldolesi J. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiol Rev* **74**: 595–636. [Medline] [CrossRef]
48. Rice RA, Berchtold NC, Cotman CW, Green KN. 2014. Age-related downregulation of the CaV3.1 T-type calcium channel as a mediator of amyloid beta production. *Neurobiol Aging* **35**: 1002–1011. [Medline] [CrossRef]
49. Roberts JR, Karr CJ, Council on Environmental Health. 2012. Pesticide exposure in children. *Pediatrics* **130**: e1765–e1788. [Medline] [CrossRef]
50. Shiotsuki H, Yoshimi K, Shimo Y, Funayama M, Takamatsu Y, Ikeda K, Takahashi R, Kitazawa S, Hattori N. 2010. A rotarod test for evaluation of motor skill learning. *J Neurosci Methods* **189**: 180–185. [Medline] [CrossRef]
51. Shoda A, Murata M, Kimura M, Hara Y, Yonoichi S, Ishida Y, Mantani Y, Yokoyama T, Hirano T, Ikenaka Y, Tabuchi Y, Hoshi N. 2023. Developmental stage-specific exposure and neurotoxicity evaluation of low-dose clothianidin during neuronal circuit formation. *J Vet Med Sci* **85**: 486–496. [Medline] [CrossRef]
52. Shoda A, Murata M, Kimura M, Hara Y, Yonoichi S, Ishida Y, Mantani Y, Yokoyama T, Hirano T, Ikenaka Y, Hoshi N. 2023. Transgenerational effects of developmental neurotoxicity induced by exposure to a no-observed-adverse-effect level (NOAEL) of neonicotinoid pesticide clothianidin. *J Vet Med Sci* **85**: 1023–1029. [Medline] [CrossRef]
53. Shoda A, Nishi M, Murata M, Mantani Y, Yokoyama T, Hirano T, Ikenaka Y, Hoshi N. 2023. Quantitative elucidation of the transfer of the neonicotinoid pesticide clothianidin to the breast milk in mice. *Toxicol Lett* **373**: 33–40. [Medline] [CrossRef]
54. Sotnikova TD, Beaulieu JM, Espinoza S, Masri B, Zhang X, Salahpour A, Barak LS, Caron MG, Gainetdinov RR. 2010. The dopamine metabolite 3-methoxytyramine is a neuromodulator. *PLoS One* **5**: e13452. [Medline] [CrossRef]
55. Takada T, Yoneda N, Hirano T, Onaru K, Mantani Y, Yokoyama T, Kitagawa H, Tabuchi Y, Nimako C, Ishizuka M, Ikenaka Y, Hoshi N. 2020. Combined exposure to dinotefuran and chronic mild stress counteracts the change of the emotional and monoaminergic neuronal activity induced by either exposure singly despite corticosterone elevation in mice. *J Vet Med Sci* **82**: 350–359. [Medline] [CrossRef]
56. Takada T, Yoneda N, Hirano T, Yanai S, Yamamoto A, Mantani Y, Yokoyama T, Kitagawa H, Tabuchi Y, Hoshi N. 2018. Verification of the causal relationship between subchronic exposures to dinotefuran and depression-related phenotype in juvenile mice. *J Vet Med Sci* **80**: 720–724. [Medline] [CrossRef]
57. Talley EM, Cribbs LL, Lee JH, Daud A, Perez-Reyes E, Bayliss DA. 1999. Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. *J Neurosci* **19**: 1895–1911. [Medline] [CrossRef]
58. Tomizawa M, Casida JE. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol* **48**: 339–364. [Medline] [CrossRef]
59. Uneme H, Konobe M, Akayama A, Yokota T, Mizuta K. 2006. Discovery and development of a novel insecticide "clothianidin", Sumitomo Kagaku 2. pp. 1–14. https://www.sumitomo-chem.co.jp/english/rd/report/files/docs/20060202_h6t.pdf [accessed on November 30, 2023].
60. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: RESEARCH0034. [Medline] [CrossRef]
61. Vivar C, van Praag H. 2013. Functional circuits of new neurons in the dentate gyrus. *Front Neural Circuits* **7**: 15. [Medline] [CrossRef]
62. Yoneda N, Takada T, Hirano T, Yanai S, Yamamoto A, Mantani Y, Yokoyama T, Kitagawa H, Tabuchi Y, Hoshi N. 2018. Peripubertal exposure to the neonicotinoid pesticide dinotefuran affects dopaminergic neurons and causes hyperactivity in male mice. *J Vet Med Sci* **80**: 634–637. [Medline] [CrossRef]