



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

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ABSTRACT. Dinotefuran (DIN) belongs to the neonicotinoids (NNs), a class of globally applied pesticides originally developed to exhibit selective toxicity in insects. However, several reports have suggested that NNs also exert neurotoxic effects in mammals. We previously demonstrated neurobehavioral effects of DIN on mice under non-stressful conditions. For further toxicity assessments in the present study, we investigated the effects of DIN on mice exposed to stressful conditions. After subacutely administering a no-observed-effect-level (NOEL) dose of DIN and/or chronic unpredictable mild stress (CUMS) to mice, we conducted three behavioral tests (*i.e.*, open field test [OFT], tail suspension test [TST] and forced swimming test [FST]). In addition, serotonin (5-HT) and tryptophan hydroxylase 2 (TPH2) of the dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) and tyrosine hydroxylase (TH) of the ventral tegmental area and substantia nigra (SN) were evaluated immunohistochemically. A NOEL dose of DIN or CUMS alone increased of the total distance in OFT, decreased or increased the immobility time in TST or FST, respectively, and increased the positive intensity of 5-HT and TPH2 in the DRN/MRN, and TH in the SN. These changes were suppressed under the conditions of combined exposure to DIN and CUMS, though the blood corticosterone level was increased depending on the blood DIN values and the presence of CUMS. The present study suggests the multifaceted toxicity of the neurotoxin DIN.

KEY WORDS: behavioral test, chronic unpredictable mild stress, dinotefuran, monoamine, mouse

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Dinotefuran (DIN) belongs to the neonicotinoid (NN) family, a class of neuroactive pesticides chemically similar to nicotine. DIN was launched in 2002 as a third-generation NN, following the first-generation NNs imidacloprid (IMI), acetamiprid (ACE), nitenpyram (NTP) and thiacloprid (THI) and the second-generation NNs thiamethoxam (TMX) and clothianidin (CTD). The high water solubility of DIN facilitates its absorption into crops and accounts for its relatively rapid insecticidal effect compared to that of other NNs. Moreover, DIN has a broad insecticidal spectrum and selective agonistic action for the nicotinic acetylcholine receptors (nAChRs) of insects, which contributes to efficient pest control and a high degree of crop safety. For these reasons, DIN is the most widely used NN in Japan. On the other hand, NNs have been reported to have adverse effects on the nervous systems and behaviors of mammals in spite of the mentioned above. The metabolic fate of NNs in mice has been reported, demonstrating

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that NNs were delivered to the brain [13]. Kimura-Kuroda *et al.* revealed that ACE, IMI and nicotine exerted similar excitatory effects on mammalian nAChRs by using primary cultures of cerebellar neurons from neonatal rats [22]. Hirano *et al.* demonstrated that CTD induces anxiety-related behavior with human-audible vocalization in male mice [17, 18]. Moreover, clinical cases of depressive disorder caused by IMI ingestion have been reported [34].

Several reports have suggested the neurotoxicity of the first and second-generation NNs, whereas there are only a few reports of third-generation NN neurotoxicity in mammals. In our previous study, we examined the effects on mammalian behavior and neuroactivity of subacute exposure to an orally administered, no-observed-effect-level (NOEL) dose of DIN. Locomotor activity, anxiety-like behavior and behavioral despair are evaluated using a behavioral test such as the open field test (OFT), tail suspension test (TST) and forced swimming test (FST). Such behaviors in animals are closely related to the levels of the monoamine neurotransmitters serotonin (5-HT), dopamine (DA) and noradrenaline (NA) in the brain; for example, according to the monoamine hypothesis, depletion of these neurotransmitters can induce depression [9]. The biosynthesis of 5-HT and DA are rate-limited by tryptophan hydroxylase 2 (TPH2) and tyrosine hydroxylase (TH), respectively. DIN increased locomotor activity during the OFT and did not increase the likelihood of behavioral despair during the TST and FST. In mice under a condition without forced stress, DIN enhanced the intensity of TH positivity in the substantia nigra (SN) but did not decrease the number of 5-HT-positive cells in the dorsal raphe nuclei (DRN) [35, 41]. These results suggested that DIN induces an excited state by perturbing the monoamine system, unlike the first- and second-generation NNs.

Humans are exposed to various types of daily stress—including social and economic challenges; physical stressors such as noise, heat and cold; and chemical stressors such as drugs and environmental toxins—all of which can cause neurodevelopmental disorders with chronic exposure. It is thus necessary to conduct toxicity assessments that include daily life stressors among the experimental conditions [17]. Long-term exposure to various stressors is associated with behavioral changes in experimental animals. The chronic unpredictable mild stress (CUMS) model has been established as a depression model [38]. Here, we examined the effects of the combined exposure to DIN and CUMS using three behavioral tests (*i.e.*, OFT, TST and FST), immunohistochemical evaluations of 5-HT, TPH2 and TH, and analyses of the levels of DIN and corticosterone in blood samples.

MATERIALS AND METHODS

Experimental animals

Male C57BL/6NCrSlc mice (3 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained in 40.5 × 20.5 × 18.5 cm individually ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2°C) and humidity (50 ± 10%) conditions and on a 12-hr light/dark cycle in the Kobe University Life-Science Laboratory. Before DIN administration and CUMS exposure, a period of 1 week was provided to acclimate the mice to the breeding environment with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. This study was approved by the Institutional Animal Care and Use Committee (permission number: 26-05-07) and was carried out according to the Kobe University Animal Experimental Regulations.

DIN administration and CUMS exposure

Water-soluble Arubarin[®] containing 20% DIN (Mitsui Chemical, Tokyo, Japan) was administered to mice via their drinking water for 4 weeks from the time they reached 4 weeks of age. With reference to the NOEL dose of 550 mg/kg/day in ICR mice [12], we divided the mice into six groups (n=6 mice in each group) as follows: DIN-0 (vehicle as control), DIN-500 (500 mg/kg/day) and DIN-2500 (2,500 mg/kg/day) in the presence or absence of CUMS exposure. In the CUMS groups, mice were exposed to 2 of the following 6 stressors each day: 24-hr food deprivation, 24-hr wet bedding, 1-hr restraint stress, lights on overnight, 1-hr cage tilting (45 degrees) and 30-min horizontal cage shaking (80 rpm). Twice a week, we measured the body weight of each mouse.

Extraction and analysis of DIN in blood samples

A 100- μ l aliquot from a whole blood sample was fortified with 100 μ l of a deuterium-labeled DIN standard (dinotefuran-d₃, 100 ppb). Three ml of 1% formic acid in acetonitrile was added to the sample for protein precipitation. The sample was vortexed for 3 min and then centrifuged at 10,000G for 10 min. The supernatant was collected and supplemented with 3-ml methanol for the second extraction. The mixture was then vortexed for 3 min and centrifuged at 10,000G for 10 min, and the methanol extract (supernatant) was collected. The two extracts (supernatants) were combined and subjected to the solid-phase extraction (SPE) procedure. Specifically, the extracts were passed through a serially connected phospholipid remover (InertSep[™], 100 mg/3 ml; GL Science, Tokyo, Japan) and a primary-secondary amine (PSA) cartridge (InertSep[™], 500 mg/6 ml; GL Science) which had been preconditioned with 5 ml of acetonitrile. The analytes were subsequently eluted from the cartridges using 5 ml of acetone. The eluates were concentrated to dryness using a centrifugal concentrator (CVE-200D with UT-2000; EYELA, Tokyo, Japan) and reconstituted with 100- μ l cotinine-d₃ solution (100 ppb). Finally, the extracts were transferred into vials for the LC-MS/MS analysis.

The LC-ESI/MS/MS instrument (Shimadzu 20 A series with LCMS8040; Shimadzu, Kyoto, Japan) was equipped with a Cadenza column CD-C18 (150 × 3 mm) (Imtakt, Kyoto, Japan) for sample analyses. The HPLC solvents A and B consisted of 0.1% formic acid and 10-mM acetic acid in water, and 0.1% formic acid and 10-mM acetic acid in methanol, respectively, with an initial solvent B concentration of 20%, and were applied with the following gradient: t=0 to 2 min, keep 20% solvent B; t=2 to 11

Table 1. Multiple reaction monitoring (MRM) used for mass spectrometry

Target neonicotinoids	MRM	Polarity for ESI
Dinotefuran	203.0>129.1	+
Dinotefuran-d3	206.0>132.3	+

ESI: electrospray ionization.

min, gradient from 20% to 95% solvent B; $t=11$ to 13 min, keep 95% solvent B. The column oven temperature and flow rate were 45°C and 0.5 ml/min, respectively. Multiple reaction monitoring for mass spectrometry was programmed as described in Table 1, and the recovery of dinotefuran-d3 standard was confirmed to be >70%. Analytical reproducibility was confirmed with a relative standard deviation of <10% for all detected compounds. The analytes were quantitated using external standard methods, and calibration curves were generated for each analyte at five calibration points (0.05, 0.125, 0.25, 0.375 and 0.5 mg/l). Linearity of the calibration curves was found above $R^2=0.998$.

Measurement of corticosterone in plasma

Plasma corticosterone levels were determined using commercially available ELISA kits (501320; Cayman Chemical, Ann Arbor, MI, USA) according to the procedure recommended by the manufacturer. The antibody in the kit specifically reacts with corticosterone and has less than 1% cross-reactivity with other adrenal hormones (e.g., aldosterone and cortisol). The absorbance was read at 405 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The levels of corticosterone were determined by comparing samples to the standard curve generated with the kit.

Open field test (OFT)

OFT was conducted during the light phase 33 days after the beginning of DIN administration and CUMS exposure. The mouse was placed on the corner of the open field (60 × 60 × 40 cm) (Tom Products, Tokyo, Japan) with LED illumination. Animals with higher levels of locomotor activity show longer total distance of locomotion, and the more anxious animals showed a shorter duration spent in the center square. All of the mouse's activities were recorded by a video camera for the subsequent 10 min. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the total distance traveled and the time spent in the center square (30 × 30 cm), which are considered to represent locomotor activity and anxiety-like behavior, respectively.

Tail suspension test (TST)

TST was performed 34 days after the beginning of DIN administration and CUMS exposure; the protocol was a modified version of that described elsewhere [31]. Inside of a white box, each mouse was suspended 60 cm above the surface of a table by a piece of plastic tape attached to the tail about 1 cm from the tip; the other end of the piece of tape was pierced and attached to a hook at the top of the box. The presence of immobility behaviors during TST is considered to reflect behavioral despair. The mouse was considered "immobile" once it had become completely motionless. After a 2-min acclimatization period, the time from onset of immobility was recorded from a side-view video camera for 4 min. The percentage of time spent immobile during this 4-min period was calculated.

Forced swimming test (FST)

FST was performed 35 days after the beginning of DIN administration and CUMS exposure; the protocol was a modified version of that described elsewhere [28]. The mouse was placed in an acrylic cylinder (40 cm in height, 20 cm in diameter; Tom Products) containing 20-cm-deep water kept at 23–25°C. The presence of immobility behaviors during FST is considered to reflect behavioral despair. The mouse was considered "immobile" when it remained floating in the water, except for movements needed to keep its head above the water to breathe. After a 2-min acclimatization period, the time spent immobile was recorded from a side-view video camera for 4 min. The percentage of time spent immobile during this 4-min period was calculated.

Tissue preparation and immunohistochemical analysis

Mice were euthanized 36 days after the beginning of DIN administration and CUMS exposure, and brains were excised, weighed and embedded in paraffin in the same manner as previously reported [35]. Serial sections of each brain were then cut at 10- μ m thickness on a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Germany) and mounted on slide glasses (521611; Muto Pure Chemicals, Tokyo, Japan) precoated with 2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan) after being washed with 1% Tween-20. All sections were stored at -30°C until use in the following steps. To detect 5-HT and TPH2 in the DRN and median raphe nuclei (MRN), and TH in the SN and ventral tegmental area (VTA), we performed immunohistochemistry according to the protocol described elsewhere [35]. The combinations of blocking reagents and antibodies used for the detection of each protein by immunohistochemistry are listed in Table 2. The data were evaluated using following criteria: -, 50–80%; \pm , 100%; +, 120–150%; ++, 150–180% compared to DIN-0 group.

Table 2. Combination of blocking reagents and antibodies used for immunohistochemistry

Detection	Blocking reagent	Primary antibody	Secondary antibody
5-HT	Blocking One Histo (Nacalai Tesque, Kyoto, Japan)	Rabbit polyclonal antibody against 5-HT (20080, 1:80,000) (ImmunoStar, Hudson, NJ, USA)	EnVision+ System-HRP Labeled Polymer Anti-Rabbit (Dako, Glostrup, Denmark)
TPH2	Blocking One Histo (Nacalai Tesque, Kyoto, Japan)	Rabbit polyclonal antibody against TPH2 (PA1-778, 1:4,000) (Thermo Fisher, Waltham, MA, USA)	EnVision+ System- HRP Labeled Polymer Anti-Rabbit (Dako, Glostrup, Denmark)
TH	Blocking reagent A and B (Nichirei Bioscience, Tokyo, Japan)	Mouse monoclonal antibody against TH (MAB318; 1:500) (Merck Millipore, Darmstadt, Germany)	Histofine MAX-PO (M) (Histofine Simple Stain system) (Nichirei Bioscience, Tokyo, Japan)

5-HT: serotonin, TPH2: tryptphan hydroxylase 2, TH: tyrosine hydroxylase.

Statistical analysis

Statistical analyses were performed with the software package Excel Statistics 2012 (version 1.00; SSRI, Tokyo, Japan). All data were analyzed by two-way ANOVA (DIN and CUMS) followed by Tukey-Kramer's *post hoc* test. The results were considered significant when the *P*-value was <0.05.

RESULTS

Body and brain weight

The body and brain weights of the mice at 8 weeks of age are shown in Table 3. The values in the DIN-500, DIN-2500, DIN-0+CUMS, DIN-500+CUMS and DIN-2500+CUMS groups had not changed significantly compared to those in DIN-0 group.

Table 3. Body weight and brain weight

	Weight (g)	Non-CUMS	CUMS
DIN-0	Body	22.24 ± 1.07	21.20 ± 0.80
	Brain	0.449 ± 0.004	0.437 ± 0.007
DIN-500	Body	23.92 ± 0.28	21.53 ± 0.46
	Brain	0.454 ± 0.006	0.437 ± 0.004
DIN-2500	Body	21.53 ± 0.47	19.34 ± 0.35
	Brain	0.442 ± 0.006	0.434 ± 0.005

CUMS: chronic unpredictable mild stress. Mean ± SEM, n=5 or 6.

DIN and corticosterone levels in blood

In the blood of DIN-0 and DIN-0+CUMS groups, no DIN was detected. In the groups administered DIN either with or without CUMS, the blood concentrations of DIN were increased as the administered concentrations of DIN were increased. Moreover, higher values were observed in the DIN-2500+CUMS group than the DIN-2500 group (Fig. 1A).

In the groups with and without CUMS, the level of corticosterone was significantly high, in a DIN-concentration-dependent manner [F (2, 34)=12.82, *P*<0.01]. The levels of corticosterone in the DIN-0+CUMS, DIN-500+CUMS and DIN-2500+CUMS groups were significantly higher than the DIN-0, DIN-500 and DIN-2500 groups, respectively [F (1, 34)=9.742, *P*<0.01] (Fig. 1B).

OFT

The trajectory maps revealed that the DIN-0+CUMS group exhibited a longer trajectory than the DIN-0 group, although the other groups showed very similar trajectories to that of DIN-0 group (Fig. 2A). The locomotor activity and the anxiety-like behavior were evaluated by the total travel distance and the time spent in the center square, respectively (Fig. 2B and 2C). The mean total distance traveled was shorter in the DIN-2500 group and the mean time spent in the center square was higher in the DIN-2500 group, as compared to those in the DIN-0 group (Fig. 2B and 2C). In the DIN-0+CUMS group, the total distance traveled was significantly longer than that in the DIN-0 group (*P*<0.01), and the mean time in the center square was also longer than that in the DIN-0 group (Fig. 2B and 2C). Moreover, in the DIN-500+CUMS and DIN-2500+CUMS groups, the mean total distance traveled and the mean time spent in the center square were both shorter than those in the DIN-0+CUMS groups (Fig. 2B and 2C).

TST

The mean percentage of immobility time was shorter in the DIN-2500 group than in the DIN-0 group (Fig. 3A). The mean percentage of immobility time was shorter in the DIN-0+CUMS group than in the DIN-0 group; this decrease was counteracted in the DIN-500+CUMS group, but not in the DIN-2500+CUMS group (Fig. 3A). The percentage of immobility time was significantly short in a DIN-concentration-dependent manner [F (2, 34)=4.23, *P*<0.05]

FST

The mean percentage of immobility time was longer in the DIN-500 and DIN-2500 groups than in the DIN-0 group (Fig. 3B). The mean percentage of immobility time was higher in the DIN-0+CUMS group than in the DIN-0 group (Fig. 3B), and this increase was counteracted in the DIN-500+CUMS group (Fig. 3B).

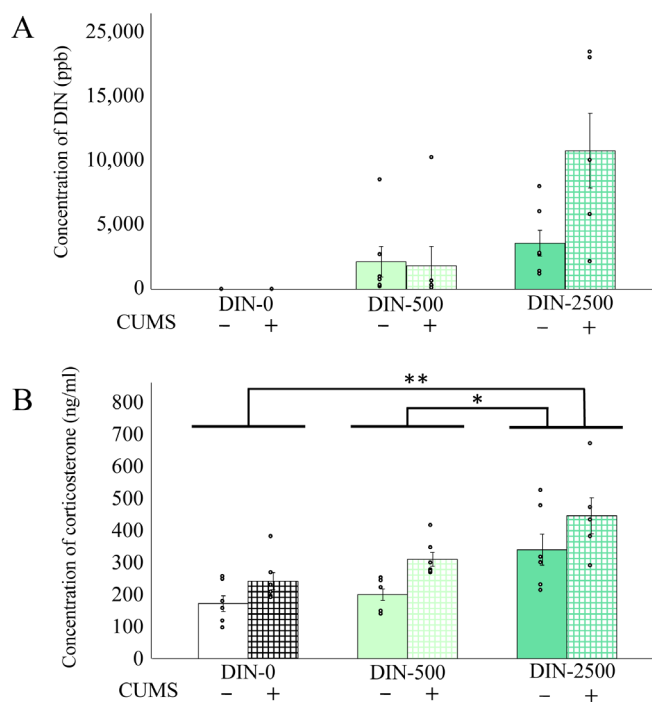


Fig. 1. The level of (A) dinotefuran (DIN) and (B) corticosterone in blood samples in each DIN exposed group with or without chronic unpredictable mild stress (CUMS) (mean \pm SEM). (A) DIN was not detected in the DIN-0 and DIN-0+CUMS groups. The level of DIN in the DIN-2500+CUMS group was higher than the DIN-2500 group. (B) The levels of corticosterone in the DIN-0+CUMS, DIN-500+CUMS and DIN-2500+CUMS groups were higher than the DIN-0, DIN-500 and DIN-2500 groups, respectively (Tukey-Kramer's *post hoc* test, * P <0.05 and ** P <0.01). The level of corticosterone showed high in a DIN-level-dependent manner.

OFT

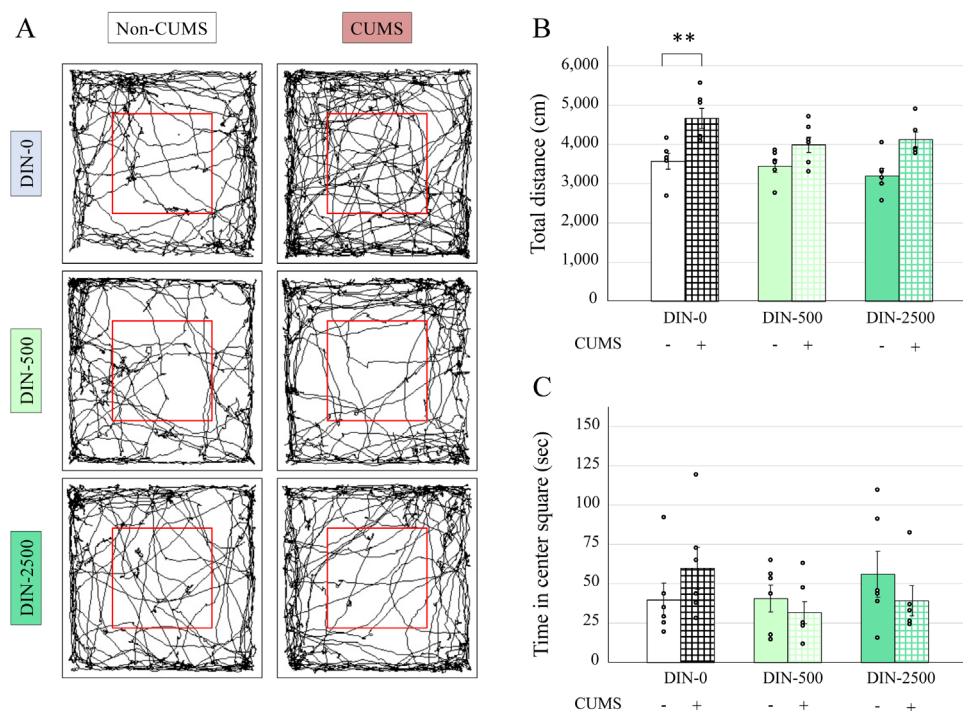


Fig. 2. Behavioral effects of combined exposure to dinotefuran (DIN) and chronic unpredictable mild stress (CUMS) in the open field test (OFT). (A) The representative trajectory maps of each group are shown. (B) The total travel distances of each group are shown (mean \pm SEM); these distances are considered to reflect locomotor activities (Tukey-Kramer's *post hoc* test, ** P <0.01). (C) The times spent in the center square of each group are shown (mean \pm SEM); these times are considered to reflect anxiety-like behavior.

Immunohistochemical findings

Immunohistochemical analyses were conducted of the DRN and MRN visualization of 5-HT (Fig. 4) and TPH2 (Fig. 5), and of the SN and VTA visualization of TH (Fig. 6). The positive intensity of 5-HT immunostaining in the DRN and MRN was higher in the

DIN-500, DIN-2500 and DIN-0+CUMS groups than the DIN-0 group (Fig. 4). These increases in the positive intensity of 5-HT immunostaining in the DRN and MRN were counteracted in both the DIN-500+CUMS and DIN-2500+CUMS groups (Fig. 4).

The positive intensity of TPH2 immunostaining in the DRN and MRN was higher in the DIN-500, DIN-2500 and DIN-0+CUMS groups than the DIN-0 group (Fig. 5). These increases in the positive intensity of TPH2 immunostaining in the DRN and MRN were counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups (Fig. 5).

The positive intensity of TH was lower in the VTA in the DIN-500, DIN-2500, DIN-0+CUMS, DIN-500+CUMS and DIN-2500+CUMS groups than the DIN-0 group (Fig. 6). The positive intensity of TH was higher in the SN in the DIN-500, DIN-2500 and DIN-0+CUMS groups than the DIN-0 group (Fig. 6). The increase in the positive intensity of TH in the SN was counteracted in the DIN-500+CUMS group (Fig. 6).

DISCUSSION

Levels of exposure concentrations to DIN in the human body have risen steadily over the past 20 years in Japan [37]. Quite recently, reports from scientific organizations such as the World Health Organization/the United Nations Environment Programme and the American Academy of Pediatrics have suggested a causal relationship between pesticide exposure and developmental disorders [10, 40]. Moreover, humans are chronically exposed to social, physical and non-pesticidal chemical stressors. These exposures to stress are thought to be related to psychiatric disorders such as posttraumatic stress disorder, major depressive disorder and anxiety disorder. It is possible that humans suffer the consequences of developmental disorders after unknowingly

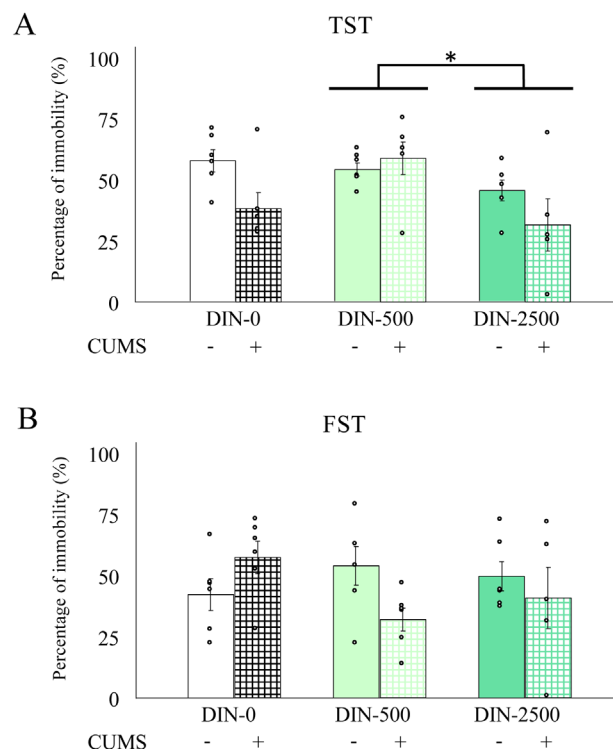


Fig. 3. Behavioral effects of combined exposure to dinotefuran (DIN) and chronic unpredictable mild stress (CUMS) in the (A) tail suspension test (TST) (Tukey-Kramer's *post hoc* test, $*P<0.05$) and (B) forced swimming test (FST) (mean \pm SEM). Immobility behaviors during the two tests are considered as reflective behavioral despair.

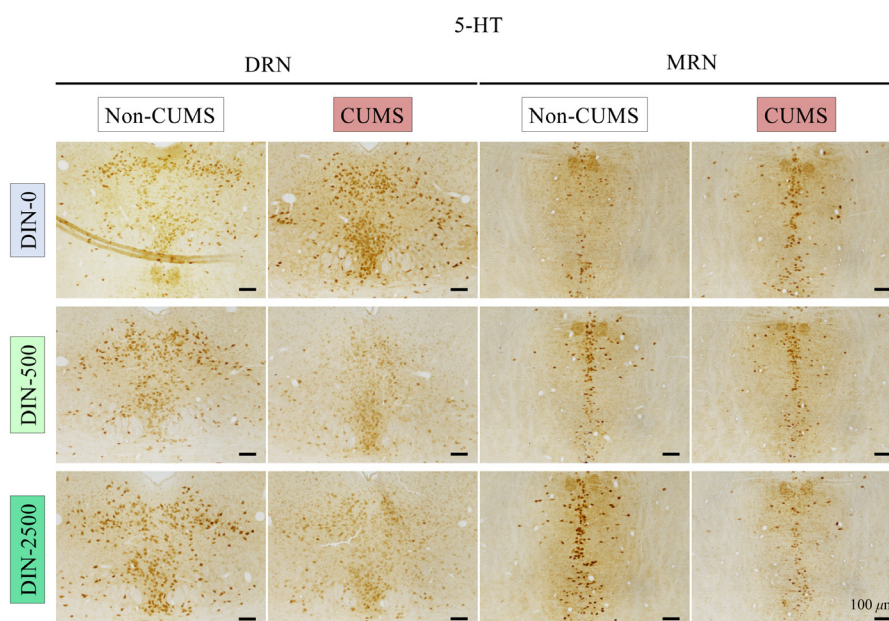


Fig. 4. Representative immunohistochemistry for serotonin (5-HT) in the dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) in all groups. The positive intensities of 5-HT in the DRN and MRN were increased in the DIN-500, DIN-2500 and DIN-0+CUMS groups, as compared to those in the DIN-0 group. The increase in the DIN-0+CUMS group was counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups. CUMS, chronic unpredictable mild stress.

having been exposed to DIN in concert with other stressors in daily life.

The present study employed non-invasive administration methods to examine the combined effects of subacute exposure to NOEL doses of DIN and CUMS on behaviors and monoaminergic systems in developing male mice. The results in this study are summarized in Fig. 7.

The detection of DIN in the blood confirms that only the DIN-administrated group was exposed to DIN in a dose-dependent manner. Also, the level of DIN in blood was higher in the DIN-2500+CUMS group than in the DIN-2500 group. A previous

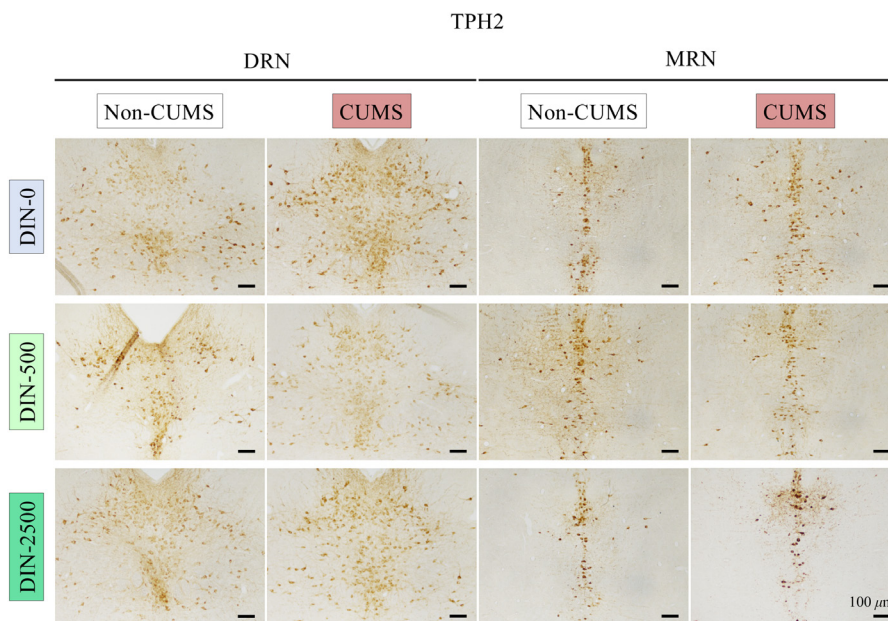


Fig. 5. Representative immunohistochemistry for tryptophan hydroxylase 2 (TPH2) in the dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) in all groups. The positive intensities of TPH2 in the DRN and MRN were increased in the DIN-500 and DIN-0+CUMS groups, as compared to those in the DIN-0 group. The increase in the DIN-0+CUMS group was counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups. CUMS, chronic unpredictable mild stress.

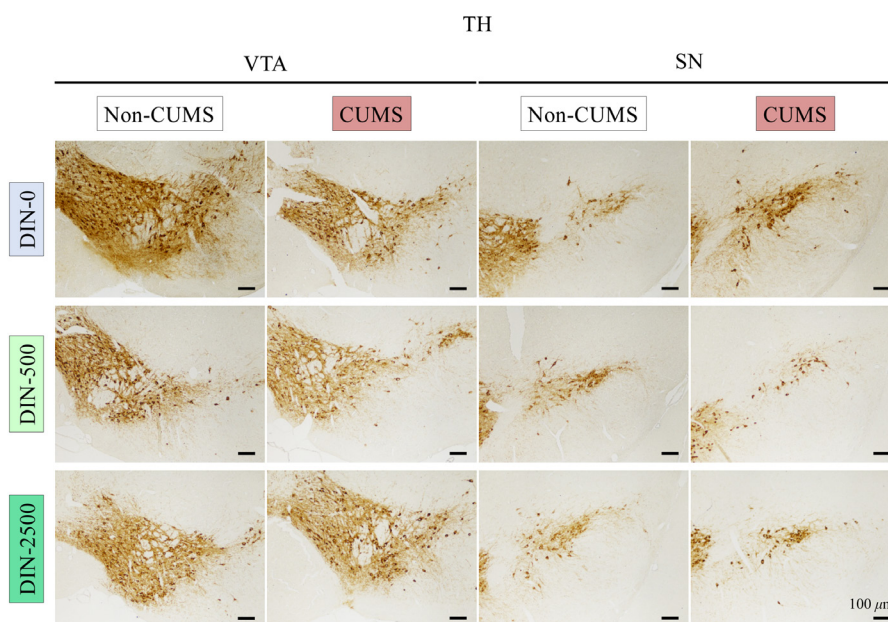


Fig. 6. Representative immunohistochemistry for tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and substantia nigra (SN) in all groups. The positive intensities of TH were decreased in the VTA and were increased in the SN in the DIN-500, DIN-2500 and DIN-0+CUMS groups, as compared to the corresponding values in the DIN-0 group. The increase in the positive intensity of TH in the SN in the DIN-0+CUMS group was counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups. CUMS, chronic unpredictable mild stress.

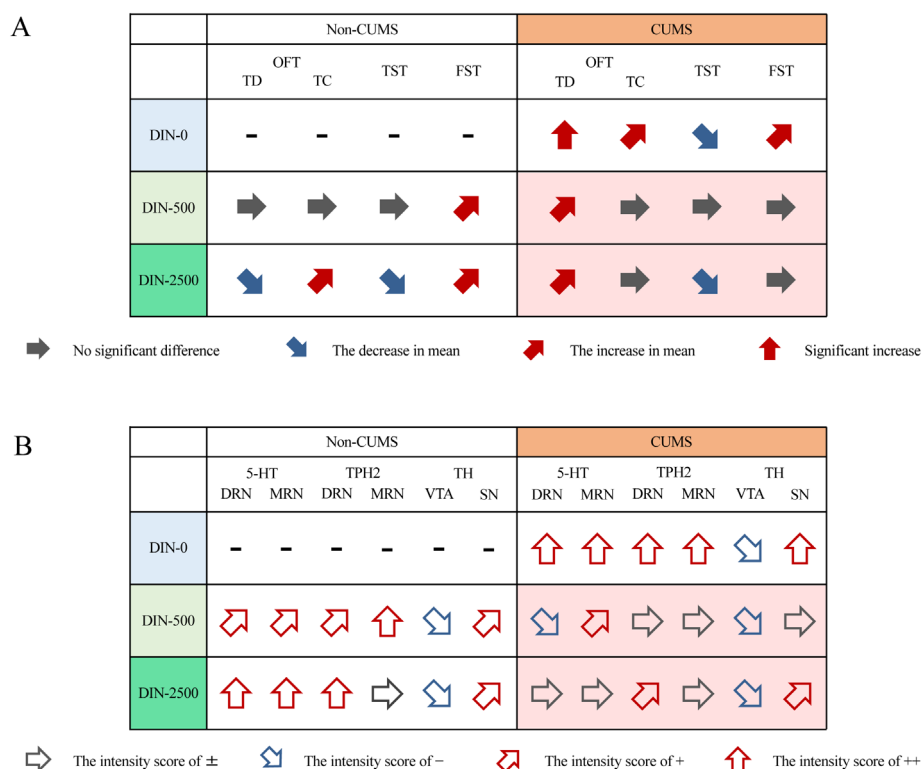


Fig. 7. The summary of the results of (A) behavioral tests and (B) immunohistochemical analyses. (A) The arrows in this figure represent the differences compared to DIN-0 group. (B) The positive intensities are indicated as follows: -, 50–80%; ±, 100%; +, 120–150%; ++, 150–180% compared to DIN-0 group. The abbreviations used in this figure: CUMS, chronic unpredictable mild stress; OFT, open field test; TD, total travel distances; TC, times spent in the center; TST, tail suspension test; FST, forced swimming test; 5-HT, serotonin; TPH2, tryptophan hydroxylase 2; TH, tyrosine hydroxylase; DRN, dorsal raphe nuclei; MRN, median raphe nuclei; VTA, ventral tegmental area; SN, substantia nigra.

study reported that stress reduced circulating nicotine levels [39], but in the present study, DIN—which is chemically similar to nicotine—was increased in the animals exposed to CUMS. Further research is needed to determine what caused the difference in the effects of stress on circulating levels of nicotine and DIN. Moreover, the levels of corticosterone increased with DIN in dose dependent manner and/or CUMS. This result suggests that DIN chronically acted in mice as a chemical stressor.

The locomotor activity was significantly increased and the mean anxiety behavior was decreased by CUMS. The increase of locomotor activity and the decrease of anxiety behavior were suppressed in both the DIN-500+CUMS and DIN-2500+CUMS groups. The mean locomotor activity of all groups exposed to CUMS was higher than that of the corresponding groups lacking CUMS exposure. These results are supported by previous studies in which chronic mild stress induced hyperactivity [16] and early life stress induced attention-deficit hyperactivity disorder like behaviors [5] in a rodent model.

The mean percentage of immobility was decreased in the TST and increased in the FST by CUMS. The decrease in TST was suppressed in the DIN-500+CUMS group, but not in the DIN-2500+CUMS group. The increase in FST was suppressed in both the DIN-500+CUMS and DIN-2500+CUMS groups. A previous study reported that nicotine administration suppressed depression-like behaviors in the FST induced by chronic unpredictable stress [4]. Since DIN and nicotine share similar mechanisms of action, similar results may have been observed in the present study. Although both the TST and FST are used in studies examining the effects of antidepressants, the effects of DIN on the percentage of immobility time of mice differed between the two tests. The cause for this discrepancy might be differences between the neuronal mechanisms involved in the TST and FST. For instance, sensitivity to the antidepressant effects of 5-HT uptake inhibitors is greater in the TST than in the FST [32]. Monoamine metabolism changes are known to follow the FST, but not the TST [29]. Several 5-HT_{1A} agonists generally decrease the duration of immobility in the FST, whereas they increase the duration of immobility in the TST [7].

The positive intensities of 5-HT and TPH2 immunostaining were increased in both the DIN-500 and DIN-2500 groups, as compared with those in the DIN-0 group. The excitability of 5-HT neurons in the DRN is known to be increased by presynaptic α 4 β 2 nAChR on glutamatergic neuronal endings [14], and nAChR partial agonists have been shown to augment the antidepressant effects of 5-HT medications [24]. DIN might act on presynaptic α 4 β 2 nAChR on glutamatergic neuronal endings, which induces the increase of the excitability of 5-HT neurons. Moreover, the positive intensities of 5-HT and TPH2 immunostaining in the DRN and MRN were higher in the DIN-0+CUMS group than the DIN-0 group. These results are supported by the previous report which chronic stress increased 5-HT in the rat brain [2]. The increases of the positive intensities of 5-HT and TPH2 immunostaining in the DIN-500, DIN-2500 and DIN-0+CUMS groups were suppressed in the DIN-500+CUMS and DIN-2500+CUMS groups.

We hypothesized that there were two possible causes for these results: the reduction of nAChR on presynaptic glutamatergic neurons and negative feedback of gamma-aminobutyric acid (GABA) neurons to the 5-HT neurons. Chronic immobilization stress is known to reduce the expression of nAChRs [20]. Also, CHRNA7 coding the $\alpha 7$ subunit of nAChRs contains a stress hormone response element [23]. The activation of 5-HT neurons by DIN might be decreased following the change of the response of nAChR by CUMS. The 5-HT nerve activity in the DRN is suppressed by many intervening GABA neurons from various parts of the brain, such as the medial prefrontal cortex, nucleus accumbens and hypothalamus [3, 8]. The exposure to both DIN and CUMS strongly activated the 5-HT neurons, which may have caused negative feedback in 5-HT neurons by activating the GABA neurons.

The positive intensities of 5-HT and TPH2 immunostaining in the DIN-2500+CUMS group were higher than those in the DIN-500+CUMS group. This finding can be attributed to the desensitization of nAChRs, which generally accounts for a loss of response after prolonged or repeated application of stimulus [26]. Exposure to nicotine for a long period of time also causes desensitization of nAChRs [27]. Here, reduced effects of DIN were observed, which in turn would be expected to cause weaker negative feedback to the 5-HT neurons in the DRN in the DIN-2500+CUMS group than those in the DIN-500+CUMS group.

The positive intensity of TH immunostaining was decreased in the VTA in the DIN-500 and DIN-2500 groups and increased in the SN in the DIN-500 and DIN-2500 groups, as compared with those in the DIN-0 group. The following facts were reported that nicotine increases the expression of the TH gene [19] and CTD directly injected into the brain evokes a striatal DA surge via nAChRs [11]. DIN might activate DA neurons in the SN like nicotine or CTD. The positive intensities of TH immunostaining were higher in the SN and were lower in the VTA in the DIN-0+CUMS group than the DIN-0 group. These results are supported by those of previous reports; chronic stress induced loss of dopaminergic neurons in the VTA [33] and chronic corticosterone enhancement aggravated SN neurodegeneration in mice [6].

The activity of midbrain DA neurons is thought to be strongly regulated by 5-HT_{2A} receptors [21]. The high positive intensity of TH immunostaining was seen in the SN but not in the VTA in the group with high positive intensity of 5-HT immunostaining in the DRN. These results may be explained by the fact that DRN stimulation differentially modulates DA neurons in the SN and VTA [15]. Moreover, nicotine exposure can increase sensitivity to stress and promote long-lasting activity in DA neurons [25].

DIN and CUMS may have canceled the effects of each exposure by combining different mechanisms of action: Tanida *et al.* demonstrated that mixed exposure to environmental toxins with different mechanisms of action (bisphenol A, di-(2-ethylhexyl)-phthalate and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) counteracts the effects of single exposure on mouse midbrain DA nuclei. They hypothesized that the results were caused by thyroid hormones and/or aryl hydrocarbon receptor-related mechanisms [36].

The present study showed that DIN changed the corticosterone levels, the behaviors and neurotransmitter levels of mice, even at the NOEL dose. On the other hand, some reports of NNs show the different results from the present study. TMX has been shown to significantly inhibit locomotor activity [30], CTD was shown to significantly increase anxiety-like behavior [17] and the levels of 5-HT, GABA and DA have been shown to be significantly reduced by IMI administration [1]. The effects on locomotor activities, the anxiety-like behaviors and the neurotransmitters of mice were observed to vary according to the type of NNs.

To summarize the above, the changes in behavior and monoaminergic neuronal activity observed with a NOEL dose of DIN or CUMS were suppressed by combined exposure to DIN and CUMS. On the other hand, the blood corticosterone level was increased depending on the DIN dose level. The present study suggests that DIN exhibits multifaceted toxicity, disrupting both neurotransmission and stress hormone secretion.

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