Molecular morphological study of the environmental factors and neurobehavioral change

Takada, Tadashi

(Degree)
博士（農学）
(Date of Degree)
2020-03-25
(Date of Publication)
2021-03-01
(Resource Type)
doctoral thesis
(Report Number)
甲第7793号
(URL)
https://hdl.handle.net/20.500.14094/D1007793

Doctoral Dissertation

Molecular morphological study of the environmental factors and neurobehavioral change

January, 2020

Department of Animal Science,

Graduate School of Agricultural Science, Kobe University

Tadashi TAKADA

博士論文

環境因子と神経行動変化に関する分子形態学的研究

2020 年 1 月

神戸大学大学院農学研究科

資源生命科学専攻

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CONTENTS

PREFACE

The number of patients receiving medical treatment due to mental illness, and the number of pupils receiving special needs education have been increasing in Japan [Ministry of Education, Culture, Sports, Science and Technology, 2017; Ministry of Health, Labour and Welfare, 2017]. In particular, diseases with disturbances of neurotransmission in the brain are prominent, such as depression, attention-deficit hyperactivity disorder (ADHD), learning disorder, autism and emotional disorder. There is great concern regarding the potential role of pesticides in the induction of these diseases; for example, high numbers of mood disorder hospitalization in an area with intensive use of pesticides [Meyer *et al.*, 2010], contribution of organophosphate pesticides to ADHD prevalence [Bouchard *et al.*, 2010] and an association between pesticides and the development of autism spectrum disorders [Sealey *et al.*, 2016] have all been described. Especially in recent years, reports from the World Health Organization (WHO)/ United Nations Environment Programme (UNEP) and the American Academy of Pediatrics have suggested a causal relationship between developmental disorders and pesticides [Elsabbagh *et al.*, 2012; WHO and UNEP, 2012].

Neonicotinoids (NNs), which were developed in the 1980s as a novel type of pesticides, induce persistent neural excitement by selective agonistic action on insect nicotinic

acetylcholine receptors (nAChRs). The use of NNs increased rapidly because of their excellent water solubility, permeability and residual effect, but they were later suspected to be a causative agent of the colony collapse disorder that occurred in various parts of the world [Henry *et al.*, 2012; Whitehorn *et al.*, 2012; Gill *et al.*, 2012], as well as the sharp declines in fish [Yamamuro *et al.*, 2019] and bird [Hallmann *et al.*, 2014] populations. Moreover, effects of NNs on mammals, including humans, have also been reported; NNs exert excitatory effects on mammalian nAChRs similar to those of nicotine [Kimura-Kuroda *et al.*, 2012] and can cause clinical depressive disorders [Taira, 2014]. In order to prevent adverse effects on human health, the use of three NNs was temporarily banned in the EU in 2013. In Japan, on the other hand, the standards for pesticide residues of NNs in foods were more loosely regulated than in Europe and the United States, and in 2015 they were further eased. Therefore, the use of NNs is still increasing in Japan, and may be related to the aforementioned increase in patients with mental and developmental disorders. The need for an impact assessment of NNs at the international level is urgent. Colleagues in our laboratory have reported on the reproductive and neurodevelopmental disorders of birds and mammals caused by the NN clothianidin (CTD) [Tokumoto *et al.*, 2013; Hoshi *et al.*, 2014; Hirano *et al.*, 2015, 2018, 2019; Yanai *et al.*, 2017].

Here, I explore the effects of dinotefuran (DIN), an NN that is newer than CTD and has the largest domestic shipment volume among NNs, on the neurobehavioral function of mammals. Specifically, I consider the effects of this agent in the context of the impact of multiple stress factors on mammals. It has been reported that symptoms appear when various stress factors are combined with individual predispositions [Lazarus, 1993], and that the combined effects of several environmental factors enhance or suppress each effect [Tanida *et al.*, 2009; Hirano *et al.*, 2018]. Therefore, I multilaterally analyze the effects of environmental factors to which we are routinely exposed—namely, environmental chemicals and environmental stresses—on the neurobehavioral functions of mammals by examining the effects of combined exposure to DIN and stress on mammals.

Chapter I

Verification of the causal relationship between subchronic exposures to dinotefuran and depression-related phenotype in juvenile mice

Introduction

Endocrine disrupters, including insecticides, induce neurodevelopmental effects in humans and wildlife [World Health Organization (WHO) and United Nations Environment Programme (UNEP), 2012], and early-life exposure to pesticides is associated with adverse effects on the neurodevelopment and behavior of children [Roberts *et al.*, 2012]. Neonicotinoids (NNs), one of the newly developed pesticides, are now the most widely used pesticides in the world. They are a class of neuroactive insecticides chemically similar to nicotine. Currently there are seven major NNs in the market: imidacloprid (IMI), nitenpyram (NTP), acetamiprid (ACE), thiamethoxam (TMX), thiacloprid (THI), clothianidin (CTD) and dinotefuran (DIN). Although these NNs were developed as selective agonists to insect nicotinic acetylcholine receptor (nAChR), an *in vitro* study showed that IMI, ACE and nicotine cause similar excitatory effects through mammalian nAChRs [Kimura-Kuroda *et al.*, 2012], and several *in vivo* studies have revealed reproductive and neurobehavioral effects of CTD [Hoshi *et al.*, 2014; Hirano *et al.*, 2015, 2018; Yanai *et al.*, 2017]. Triggered by one case report of a 31-year-old depressed male who had been exposed to NNs due to the termite control of his dwelling [Taira, 2014], I focused on depression among mental and neurodevelopmental disorders.

The total number of people with depression in the world increased by 18.4% between 2005 and 2015 [GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016]. In Japan, the number of patients with depression has also been increasing over the last two decades [Ministry of Health, Labour and Welfare, 2017]. Although the exact cause(s) of depression and susceptibility to depression are not fully understood, many genetic and environmental factors are suspected; for example, a polymorphism in the promoter region of the serotonin (5-HT) transporter gene [Caspi *et al.*, 2003] and occupational pesticide exposure [Beard *et al.*, 2014] have been described. Moreover, depression is associated with sex differences [Altemus *et al.*, 2014] and interactions between genes and the environment [Strachan *et al.*, 2017]. Based on the above, it is apparent that various factors can be associated with depression.

The 'monoamine hypothesis' has been proposed as one of the explanations of causes of depression. According to this hypothesis, depression can be induced by a depletion of monoamine neurotransmitters: 5-HT, dopamine and noradrenaline [Coppen, 1967]. The alternation of the 5-HT neural function, in particular, involves in the pathophysiology of depression [Owens and Nemeroff, 1994]. The 5-HT neurons are controlled through nAChRs. The release of 5-HT is facilitated by α7 nAChR activation [Andreasen *et al.*, 2012], and 5-HT neuron excitability is increased by α4β2 nAChRs in the dorsal raphe

nucleus (DRN) in which most of the 5-HT neurons are located [Garduño *et al.*, 2012]. Many studies on cholinergic signaling or smoking have suggested the modulation of depression through nAChRs [Mineur and Picciotto, 2010; Picciotto *et al.*, 2015] and an association between nicotine and depression [Dierker *et al.*, 2015; Goesling *et al.*, 2015; Mineur *et al.*, 2016].

Two tests, the tail suspension test (TST) and forced swimming test (FST), are widely used for the evaluation of antidepressants in rodent models [Castagné *et al.*, 2010]. In these tests, the efficacy of drugs is evaluated by the length of the animal's immobility time, which is thought to reflect behavioral despair. The length of immobility time is decreased by many types of antidepressants, including selective 5-HT reuptake inhibitors.

DIN is the most widely used pesticide in Japan among the NNs for the control of insect pests on leafy vegetables, in residential and commercial buildings and for professional turf management and so on. However, there has been no animal experimental study on the involvement of DIN in depression. The present study was conducted to investigate the relationship between subchronic exposures to DIN and a depression-related phenotype by using behavioral tests such as TST and FST, and immunohistochemical analysis.

Materials and Methods

Experimental animals

Three-week-old male C57BL/6NCrSlc mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained as described elsewhere [Hirano *et al.*, 2018]. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 26-05-07) and carried out according to the Kobe University Animal Experimental Regulations.

DIN administration

Assuming the exposure situation in agricultural land, Water-soluble Arubarin® (contains 20% DIN; Mitsui Chemical Co., Ltd., Tokyo) was administered to mice in their drinking water for 5 weeks from the age of 3 weeks. The mice were divided into four groups (n = 6 mice in each): DIN-0 (vehicle as Control), DIN-100 (100 mg/kg/day), DIN-500 (500 mg/kg/day) and DIN-2500 (2,500 mg/kg/day) with reference to the no-observed-effect level (NOEL) of 550 mg/kg/day in the ICR mouse [Food Safety Commission of Japan, 2016]. Twice a week, the body weights of individual mice were determined and the water intakes were calculated from the decrement of the water weights placed in the bottle of each group.

TST and FST

On the last day of the 5 weeks of exposure to DIN, the TST and FST were performed as described elsewhere [Porsolt *et al.*, 1977; Steru *et al.*, 1985] with some modification. In the TST, the mouse was suspended from a hook of a white box 60 cm above the surface of a table, by a plastic tape set 1 cm away from the tip of the mouse's tail. The mouse was considered "immobile" when it was completely motionless. In the FST, the mouse was placed in an acrylic cylinder (40 cm height × 20 cm dia.) containing 20-cm-deep water kept at 23–25°C. The mouse was considered "immobile" when it remained floating in the water, except for movements to keep its head above the water. In both tests, after a 2-min acclimatization period, the immobility time was recorded from a side view by a video camera for 4 min. The percentage of immobility time during this 4-min period was calculated.

Tissue preparation

The day after TST and FST, all mice were deeply anesthetized with isoflurane by an inhalation anesthesia apparatus (BS-400T; Brain Science idea, Osaka, Japan) and transcardially perfused with 0.9% normal saline, followed by perfusion with ice-cold 4% paraformaldehyde in phosphate buffer. The brains were excised, weighed and postfixed with the same fixative overnight at 4°C. The brains were then dehydrated through a

graded series of ethanol followed by xylene, and embedded in paraffin. Serial sections of each brain were then cut at 10-μm thickness on a sliding microtome (SM2000R; Leica Microsytems, Wetzlar, Germany) and mounted on slide glasses (Platinum Pro; Matsunami Glass, Kishiwada, Japan). All sections were stored at −30°C until use for the following steps.

Histological and immunohistochemical analysis

To detect 5-HT on the DRN, the immunohistochemistry protocol was performed similar to that described [Hirano *et al.*, 2018]. Briefly, the rabbit polyclonal anti-5-HT antibody (20080, ImmunoStar, Hudson, WI, U.S.A.) diluted 1:80,000 in phosphate-buffered saline with 0.05% Tween-20 (PBST; pH 7.4) was used as the primary antibody, and goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in tris (hydroxymethyl) aminomethane-HCl buffer (EnVision®+; Dako, Glostrup, Denmark) was used as the secondary antibody. Finally, the brain sections were counterstained with hematoxylin, dehydrated with absolute ethanol, cleared by xylene and coverslipped with Eukitt® (O. Kindler GmbH, Freiburg, Germany). The 5-HT-positive cells were counted using three sections of the DRN: −4.48, −4.60 and −4.72 mm from the bregma according to the brain atlas [Paxinos and Franklin, 2001], and the average of these values was determined for each mouse.

Statistical analysis

Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan). All data were analyzed by one-way ANOVA followed by Dunnett's test. The results were considered significant when the P-value was <0.05.

Results

Body weights, brain weight and water intake

The body weight, brain weight and water intake data are shown in Table I-1. DIN did not significantly suppress these three parameters in all of the DIN-administered groups compared to the control group.

Table I-1 Body weight, brain weight and water intake

	Groupes			
	DIN-0	DIN-100	DIN-500	DIN-2500
Body weight (g)	25.16 ± 1.86	25.73 ± 1.57	25.54 ± 1.71	23.96 ± 0.93
Brain weight (g)	0.425 ± 0.022	0.439 ± 0.014	0.433 ± 0.012	0.427 ± 0.015
Water intake (g/day)	4.31 ± 0.59	4.74 ± 0.99	4.08 ± 0.58	3.41 ± 0.56

mean ± SD, n = 6

TST and FST

In the TST, the immobility time was significantly decreased in the DIN-500 group, and the median immobility time was lower in the DIN-100, DIN-500 and DIN-2500 groups compared to the DIN-0 group (Fig. I-1A). In the FST, no significant difference in the immobility time by DIN administration was observed compared with the DIN-0 group (Fig. I-1B). Compared to the DIN-0 group, the median immobility time was higher in the DIN-100 group but lower in the DIN-500 and DIN-2500 groups (Fig. I-1B). In both the TST and FST, DIN administration did not significantly increase the immobility time, which increases when mice show behavioral despair (Fig. I-1A and I-1B). An

antidepressant-like effect of both acute and chronic nicotine treatment had been suggested in studies using the TST and FST [Tizabi *et al.*, 1999; Vázquez-Palacios *et al.*, 2004; Andreasen and Redrobe, 2009], and these previous studies support our findings of DIN, which is chemically similar to nicotine.

Histological and immunohistochemical findings

The immunohistochemical detection of 5-HT in the DRN is illustrated in Fig. I-2A. DIN administration did not significantly decrease the number of 5-HT-positive cells which decreases when mice are depressed. The median number of 5-HT-positive cells was lower in the DIN-100 group but higher in the DIN-500 and DIN-2500 groups compared to the DIN-0 group (Fig. I-2B)

Discussion

Regarding the phenotype of depression, in the present study the difference in the number of 5-HT-positive cells depending on the DIN dose showed the same trend as the immobility time in the FST, but not the same trend as the immobility time in the TST. Although both the TST and FST are used to study depression, they involve different neuronal mechanisms; monoamine metabolism changes follow the FST but not the TST [Renard *et al.*, 2003]. This difference in neuronal mechanisms could cause the different trends between the TST and FST. The tendency of the change was also observed in immobility time in the DIN-100 and DIN-2500 groups which differed between the TST and FST. Further studies should focus on the difference in the effects on depression-like behavior that is dependent on the DIN dose.

The 5-HT neurons project to many areas of brain (*e.g.*, the substantia nigra, amygdala and hippocampus). The 5-HT_{2C} receptor controls dopaminergic system in the brain [Di matteo *et al.*, 2001]. A disturbance of dopamine induces hyperlocomotion [Hagino *et al.*, 2015]. The release of 5-HT from DRN terminals in the amygdala may enhance conditioned fear [Graeff *et al.*, 1997]. Postsynaptic 5-HT_{1A} receptors in the hippocampus participate in the development of tolerance to aversive events [Guimarães *et al.*, 1993]. A change in the number of 5-HT-positive cells in the DRN can disturb the activities of these

destinations, which could cause behavioral changes. The behavioral tests focusing on these abnormalities are required to be conducted.

Tryptophan hydroxylase (TPH) is a rate-limiting enzyme of the biosynthesis of 5-HT. Nicotine administration inhibits TPH expression in dorsal and median raphe nuclei [Jang *et al.*, 2002]. Moreover, the administration of nicotine to adolescent rats alters the concentrations and functions of 5-HT receptors [Xu *et al.*, 2002], and the transcription of the 5-HT_{1A} receptor in the cerebral cortex and dorsal hippocampus is increased by nicotine administration [Kenny *et al.*, 2001]. Further research is needed of the effects of DIN on the 5-HT system, including TPH and 5-HT receptors.

The present analyses did not confirm that DIN alone cause depression-like indication. However, this study was performed under short-term conditions that may not reflect depression closely. The pathogenic mechanism of depression is still unknown and is assumed to be due to a combination of genetic, environmental and psychosocial factors. For example, chronic stress is a risk factor for depression [Mahar *et al.*, 2014]. Further investigations are needed to clarify the effects of DIN on mice exposed to stressful events.

Summary

It has been suggested that an increase in the use of pesticides affects neurodevelopment, but there has been no animal experiment showing a causal relation between neonicotinoids (NNs) and depression. In Chapter I, dinotefuran (DIN), the most widely used NN in Japan, was examined whether induces depression. Male mice were administered DIN between 3 and 8 weeks of age, referring to the no-observed-effect level (NOEL). The mice were then subjected to a tail suspension test (TST) and a forced swimming test (FST). After these tests, their brains were dissected for immunohistochemical analyses of serotonin (5-HT). Antidepressant activity in TST and no decrease in 5-HT-positive cells were observed. The subchronic exposure to DIN alone in juvenile male mice may not cause depression-like indication.

Figures and Figure legends

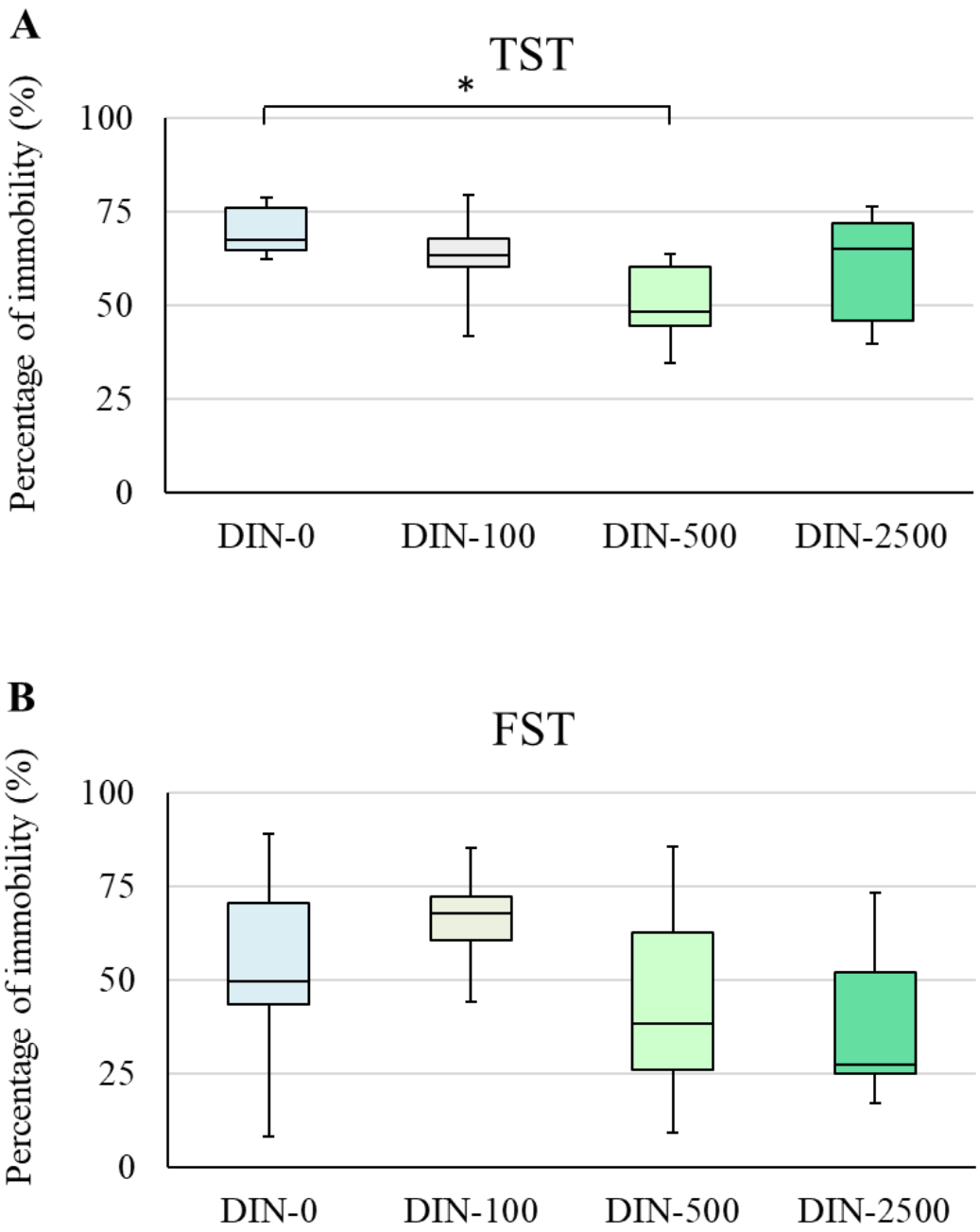

Fig. I-1. Effects of DIN exposure on the immobility time in the TST (A) and FST (B). Data are reported as a box-and-whisker plot. The bottom and top of the box are 25th and 75th quartiles respectively and the band inside the box is the median. The whiskers extend to the highest and lowest value. (A) In the DIN-500 mice, the immobility times were significantly decreased (Dunnett's test, *P<0.05). The medians of the immobility time were lower in the DIN-100, DIN-500 and DIN-2500 groups compared to the DIN-0 group. (B) The medians of immobility time were higher in the DIN-100 group and lower in DIN-500 and DIN-2500 groups than the DIN-0 group.

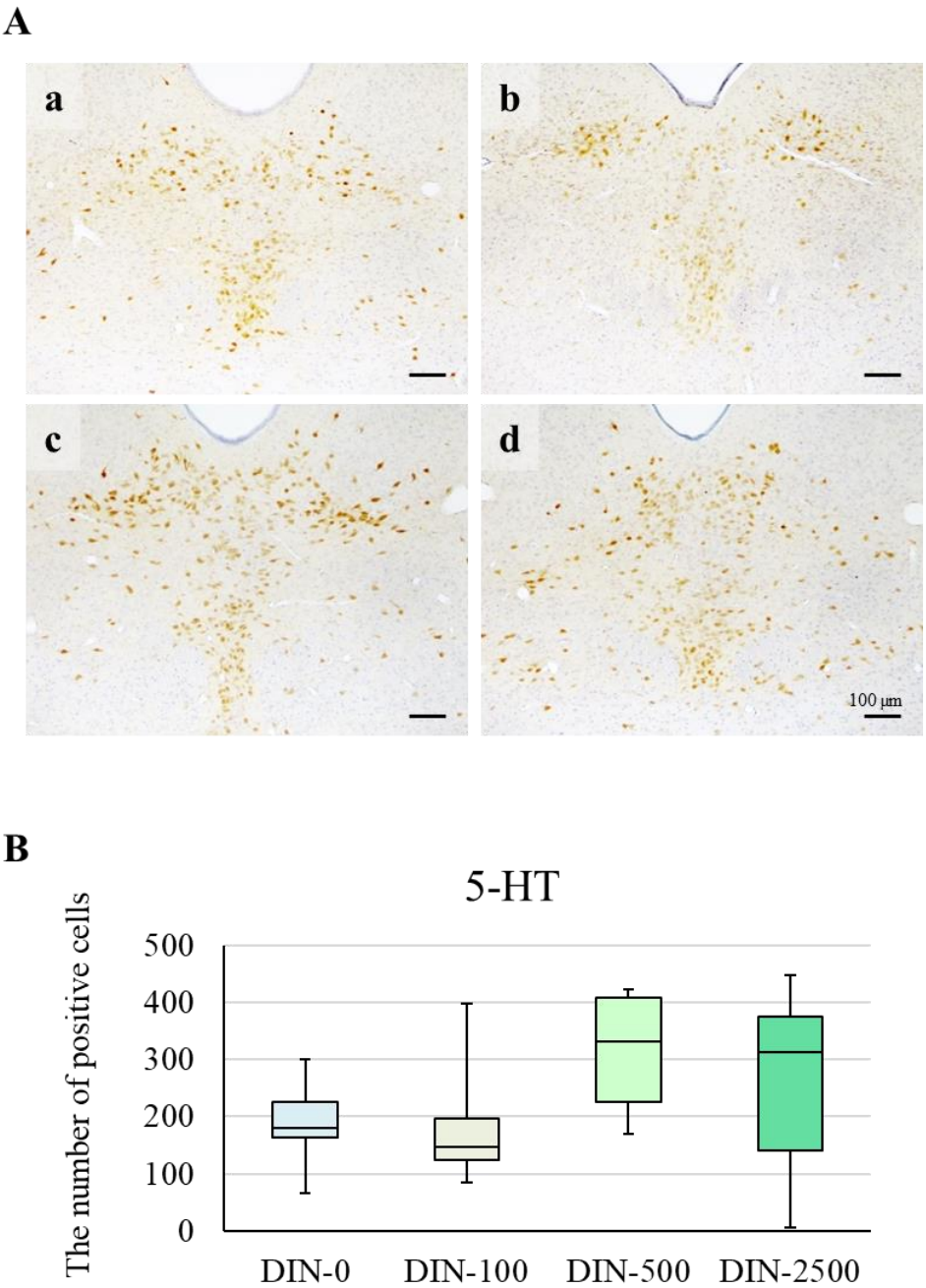

Fig. I-2. Representative immunohistochemistry for 5-HT of the DRN in the mice of the DIN-0 (A-a), DIN-100 (A-b), DIN-500 (A-c) and DIN-2500 (A-d) groups. (B) The numbers of 5-HT-positive cells. Data are reported as a box-and-whisker plot. The bottom and top of the box are 25th and 75th quartiles respectively and the band inside the box is the median. The whiskers extend to the highest and lowest value. The median numbers of 5-HT positive cells were lower in DIN-100 and DIN-2500 groups and higher in the DIN-500 group compared to the DIN-0 group. The between-group differences were not significant (Dunnett's test, *P<0.05).

Chapter II

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

Introduction

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 that NNs were delivered to the brain [Ford *et al.*, 2006]. 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 [Kimura-Kuroda *et al.*, 2012]. Hirano *et al.* demonstrated that CTD induces anxiety-related behavior with human-audible vocalization in male mice [Hirano *et al.*, 2015, 2018]. Moreover, clinical cases of

depressive disorder caused by IMI ingestion have been reported [Taira, 2014].

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 chapter I, I 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 [Coppen, 1967]. The biosynthesis of 5-HT and DA are rate-limited by tryptophan hydroxylase 2 (TPH2) and tyrosine hydroxylase (TH), respectively. DIN increases the locomotor activity during the OFT and does not increase the likelihood of behavioral despair during the TST and FST. In mice under a condition without forced stress, DIN enhances the intensity of TH positivity in the substantia nigra (SN) but does not decrease the number of 5-HT-positive cells in the dorsal raphe nuclei (DRN) [Takada *et al.*, 2018; Yoneda *et al.*, 2018]. These results suggest 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 [Hirano *et al.*, 2015]. The 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 [Willner *et al.*, 1987]. Here, the effects of the combined exposure to DIN and CUMS on mice were examined using three behavioral tests (*i.e.*, OFT, TST and FST), immunohistochemical evaluations of 5-HT, TPH2 and TH in brain, and the analysis 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 [Food Safety Commission of Japan, 2016], The mice were divided 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, the body weight of each mouse was measured.

Extraction and analysis of DIN in blood samples

A 100-*μl* aliquot from a whole blood sample was fortified with 100 *μl* of a deuterium-labeled DIN standard (dinotefuran-d3, 100 ppb). Three m*l* 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^{TM}, 100 mg/3 m*l*; GL Science, Tokyo, Japan) and a primary-secondary amine (PSA) cartridge (InertSep^{TM}, 500 mg/6 m*l*; GL Science) which had been preconditioned with 5 m*l* 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-μl 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 analyzes. 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 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 II-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 $R^2 = 0.998$.

Table II-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

Measurement of corticosterone in plasma

Plasma corticosterone levels were determined using commercially available ELISA kits (501320; Cayman Chemical, Ann Arbor, MI, U.S.A.) 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 cortisone). The absorbance was read at 405 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, U.S.A.). The levels of corticosterone were determined by comparing samples to the standard curve generated with the kit.

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, U.S.A.) 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.

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 [Steru *et al.*, 1985]. 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.

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 [Porsolt *et al.*, 1977]. 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 [Takada *et al.*, 2018]. Serial sections of each brain were then cut at 10-μm 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 the ventral tegmental area (VTA), immunohistochemistry was performed according to the protocol described elsewhere [Takada *et al.*, 2018]. The combinations of blocking agents and antibodies used for the detection of each protein by

immunohistochemistry are listed in Table II-2. The data were evaluated using following criteria: −, 50-80%; ±, 100%; +, 120-150%; ++, 150-180% compared to DIN-0 group.

Table II-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, U.S.A.)	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, U.S.A.)	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 analyzes 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 II-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 the DIN-0 group.

Table II-3. Body weight and brain weight

	Weight (g)	Non-stressed	Stressed
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

mean ± SEM

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 concentration of DIN were increased. Moreover, higher values were observed in the DIN-2500+CUMS group than in the DIN-2500 group (Fig. II-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 those of corticosterone in the DIN-0, DIN-500 and DIN-2500 groups, respectively [F (1, 34) = 9.742, $P < 0.01$] (Fig. II-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. II-2A). The locomotor activities and the anxiety-like behaviors were evaluated by the total travel distance and the time spent in the center square, respectively (Fig. II-2B and II-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-500 and DIN-2500 groups, as compared to those in the DIN-0 group (Fig. II-2B and II-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. II-2B and II-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 group (Fig. II-2B and II-2C).

TST

The mean percentage of immobility time was shorter in the DIN-2500 group than in

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

Immunohistochemical findings

Immunohistochemical analyses were conducted of the DRN and MRN visualization of 5-HT (Fig. II-4) and TPH2 (Fig. II-5) and of the SN and VTA visualization of TH (Fig. II-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 in the DIN-0 group (Fig. II-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. II-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 in the DIN-0 group (Fig. II-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. II-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 in the DIN-0 group (Fig. II-6). The positive intensity of TH was higher in the SN in the DIN-500, DIN-2500 and DIN-0+CUMS groups than in the DIN-0 group (Fig. II-6). The increase in the positive intensity of TH in the SN was counteracted in the DIN-500+CUMS group (Fig. II-6).

Discussion

Levels of exposure concentrations to DIN in the human body have risen steadily over the past 20 years in Japan [Ueyama *et al.*, 2015]. Recently, reports from scientific organizations such as the WHO/UNEP and the American Academy of Pediatrics have suggested a causal relationship between pesticide exposure and developmental disorders [Elsabbagh *et al.*, 2012; WHO and UNEP, 2012]. 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 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 the monoaminergic systems in developing male mice. The results in this study are summarized in Fig. II-7.

The detection of DIN in the blood confirmed 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 study has

reported that stress reduced circulating nicotine levels [Winders *et al.*, 1998], 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-like behavior was decreased by CUMS. The increase of locomotor activity and the decrease of anxiety-like 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 induces hyperactivity [Grønli *et al.*, 2005] and early life stress induces ADHD like behaviors [Bock *et al.*, 2017] 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 has reported that nicotine administration suppresses depression-like behaviors in FST induced by chronic

unpredictable stress [Biala *et al.*, 2017]. 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 the 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, the sensitivity to the antidepressant effects of 5-HT uptake inhibitors is greater in the TST than in the FST [Steru *et al.*, 1987]. Monoamine metabolism changes are known to follow the FST, but not the TST [Renard *et al.*, 2003]. Several 5-HT_{1A} agonists generally decrease the duration of immobility in the FST, whereas they increase the duration of immobility in the TST [Castagné *et al.*, 2010].

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 the 5-HT neurons in the DRN is known to be increased by presynaptic α4β2 nAChR on glutamatergic neuronal endings [Garduño *et al.*, 2012], and nAChR partial agonists have been shown to augment the antidepressant effects of 5-HT medications [Mineur *et al.*, 2015]. DIN might act on presynaptic α4β2 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 in the DIN-0 group. These results are supported by the previous report which chronic stress increases 5-HT in the rat brain [Adell *et al.*, 1988]. The increase 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.

I 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 [Hunter *et al.*, 2010]. Also, CHRNA7 coding the α7 subunit of nAChRs contains a stress hormone response element [Leonard *et al.*, 2002]. 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 [Bang and Commons, 2012; Challis *et al.*, 2013]. 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 [Ochoa *et al.*, 1989]. Exposure to nicotine for a long period of time also causes desensitization of nAChRs [Picciotto *et al.*, 2008]. 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. The following facts were reported that nicotine increases the expression of the TH gene [Hiremagalur *et al.*, 1993], and CTD directly injected into the brain evokes a striatal DA surge via nAChRs [Faro *et al.*, 2012]. 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 in the DIN-0 group. These results are supported by those of previous reports; chronic stress induces loss of dopaminergic neurons in the VTA [Sugama and Kakinuma, 2016] and chronic corticosterone enhancement aggravates SN neurodegeneration in mice [Burtscher *et al.*,

2019].

The activity of midbrain DA neurons is thought to be strongly regulated by 5-HT_{2A} receptors [Ikemoto *et al.*, 2000]. The high positive intensity of TH immunostaining was not seen 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 [Gervais and Rouillard, 2000]. Moreover, the nicotine exposure can increase sensitivity to stress and promote the long-lasting activity in DA neurons [Morel *et al.*, 2018].

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 [Tanida *et al.*, 2009]

The present study showed that DIN changed the corticosterone levels, the behaviors and the 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 [Rodrigues *et al.*, 2010], CTD has been shown to significantly increase anxiety-like behavior [Hirano *et al.*, 2015] and the levels of 5-HT, GABA and DA have been shown to be significantly reduced by IMI administration [Abd-Elhakim *et al.*, 2018]. The effects on the 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.

Summary

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. Neurobehavioral effects of DIN on mice under non-stressful conditions were demonstrated in Chapter I. For further toxicity assessment in Chapter II, the effects of DIN on mice exposed to stressful conditions were investigated. After subacutely administering a no-observed-effect-level (NOEL) dose of DIN and/or chronic unpredictable mild stress (CUMS) to mice, three behavioral tests (*i.e.*, open field test [OFT], tail suspension test [TST] and forced swimming test [FST]) were conducted. 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.

Figures and Figure legends

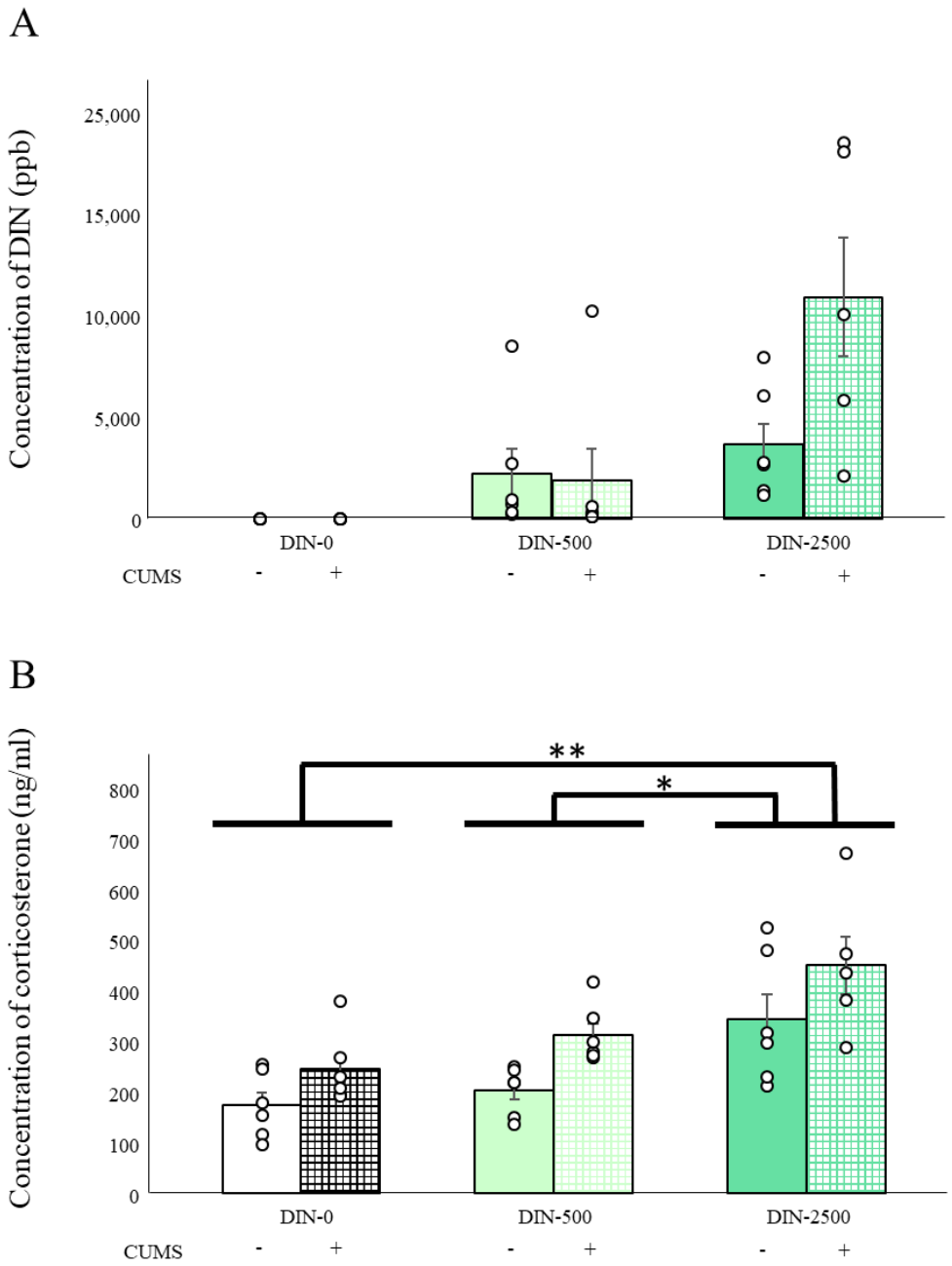

Fig. II-1. The level of DIN (A) and corticosterone (B) in blood samples in each DIN exposed group with or without CUMS (mean ± SEM). (A) DIN was not detected in the DIN-0 and DIN-0+CUMS groups, and the blood corticosterone levels in the other groups were dependent on the concentrations of DIN administered. The level of DIN in the DIN-2500+CUMS group was higher than that in 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 those in the DIN-0, DIN-500 and DIN-2500 groups, respectively. The level of corticosterone showed high in a DIN-level-dependent manner.

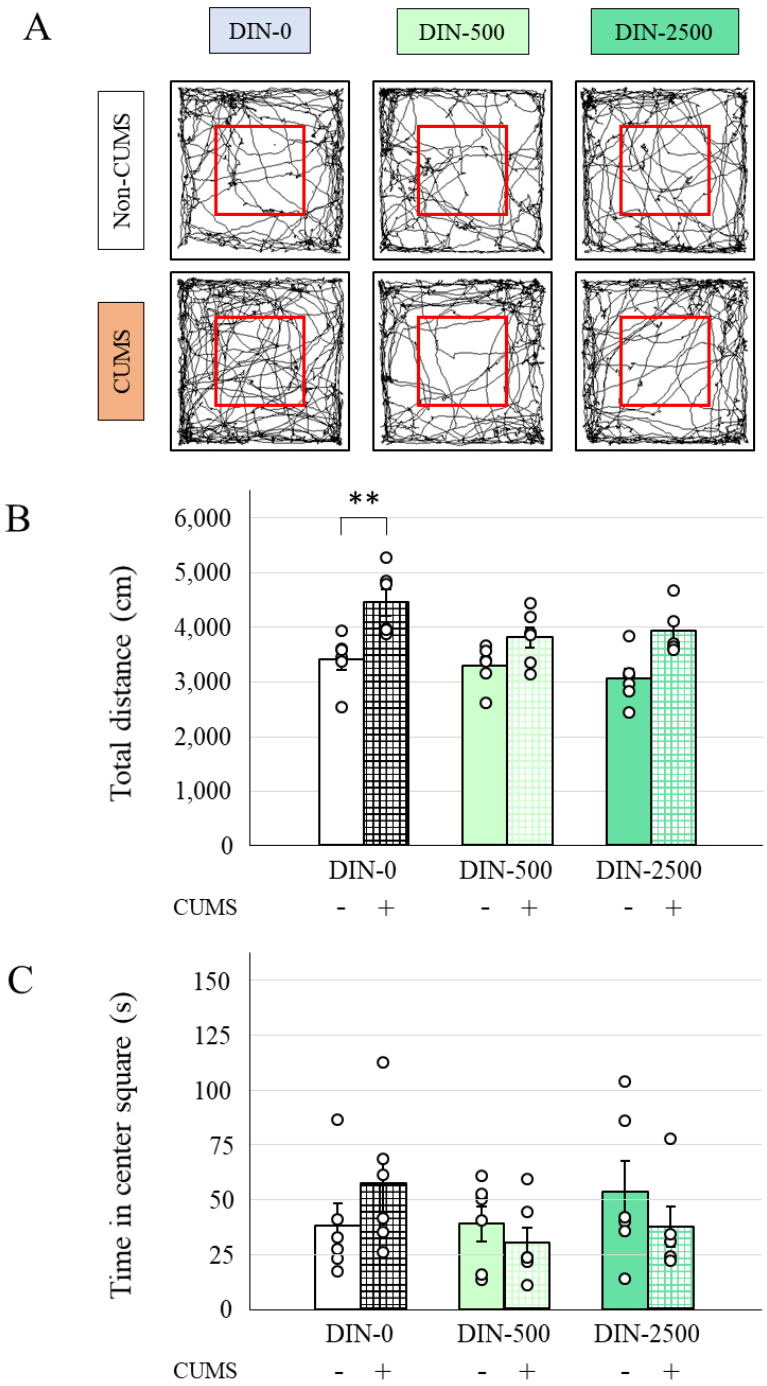

Fig. II-2. Behavioral effects of combined exposure to DIN and CUMS in the OFT. (A) The representative trajectory maps of each group are shown. (B) The total travel distances of each group are shown (mean ± SEM); these distances are considered to reflect locomotor activities (Tukey-Kramer's post hoc test,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

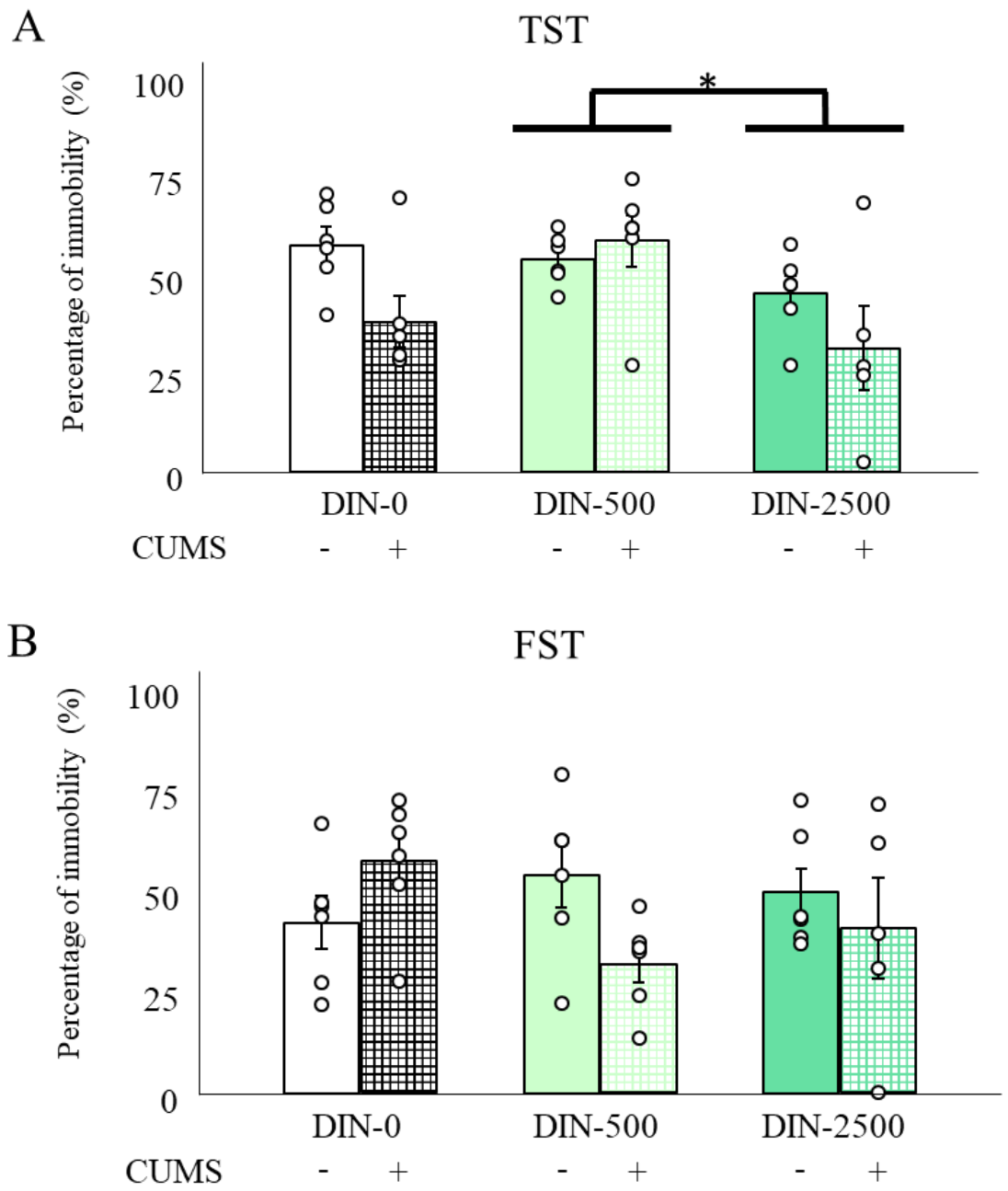

Fig. II-3. Behavioral effects of combined exposure to DIN and CUMS in the (A) TST and (B) FST (mean ± SEM). Immobility behaviors during the two tests are considered as reflective behavioral despair.

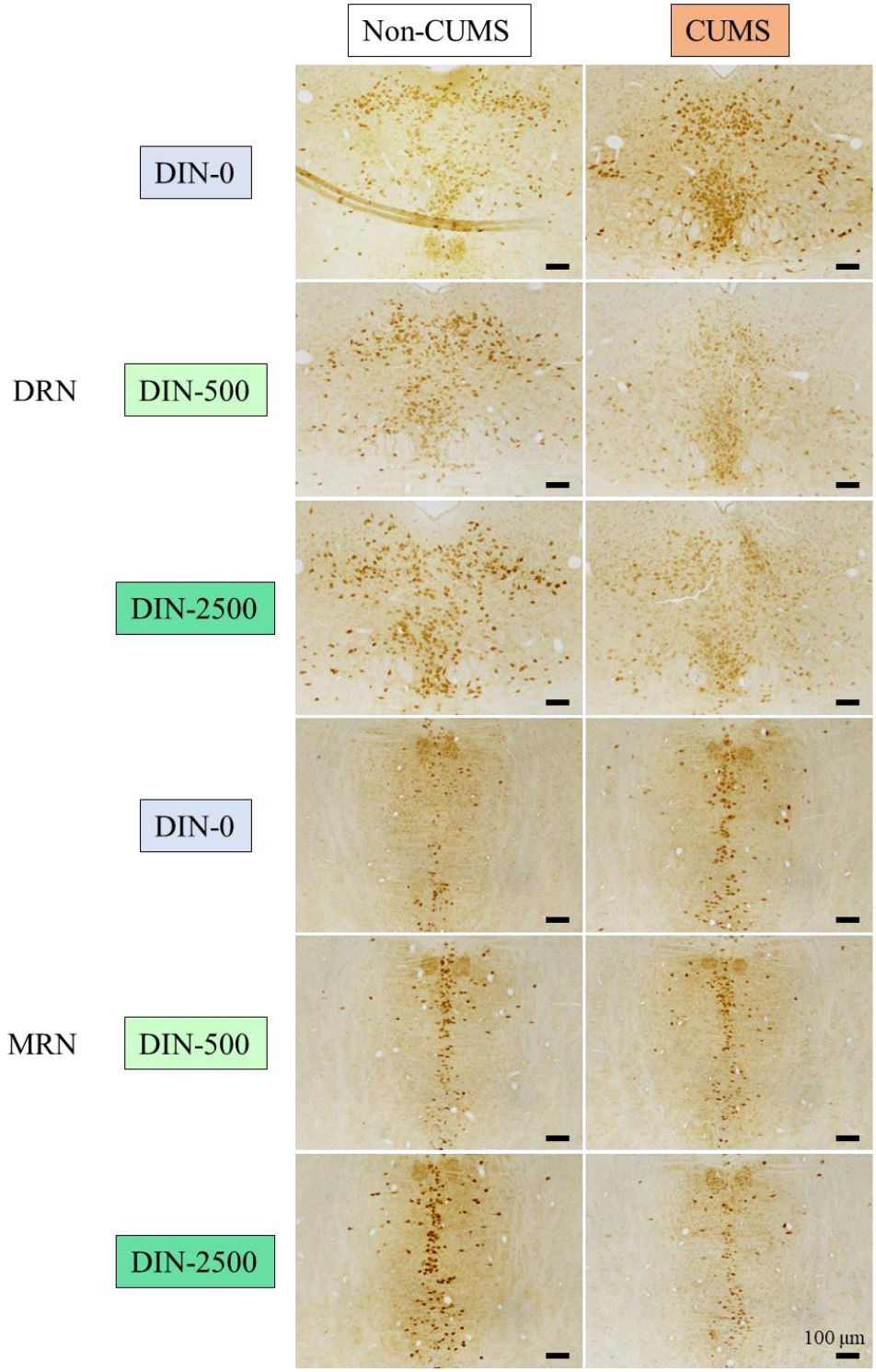

Fig. II-4. Representative immunohistochemistry for 5-HT in the DRN and MRN in all groups. The positive intensities of 5-HT in the DRN and MRN were increased in the DIN-500, DIN-2500 and DIN-0+CUMS groups, as compared to those in the DIN-0 group. The increase in the DIN-0+CUMS group was counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups.

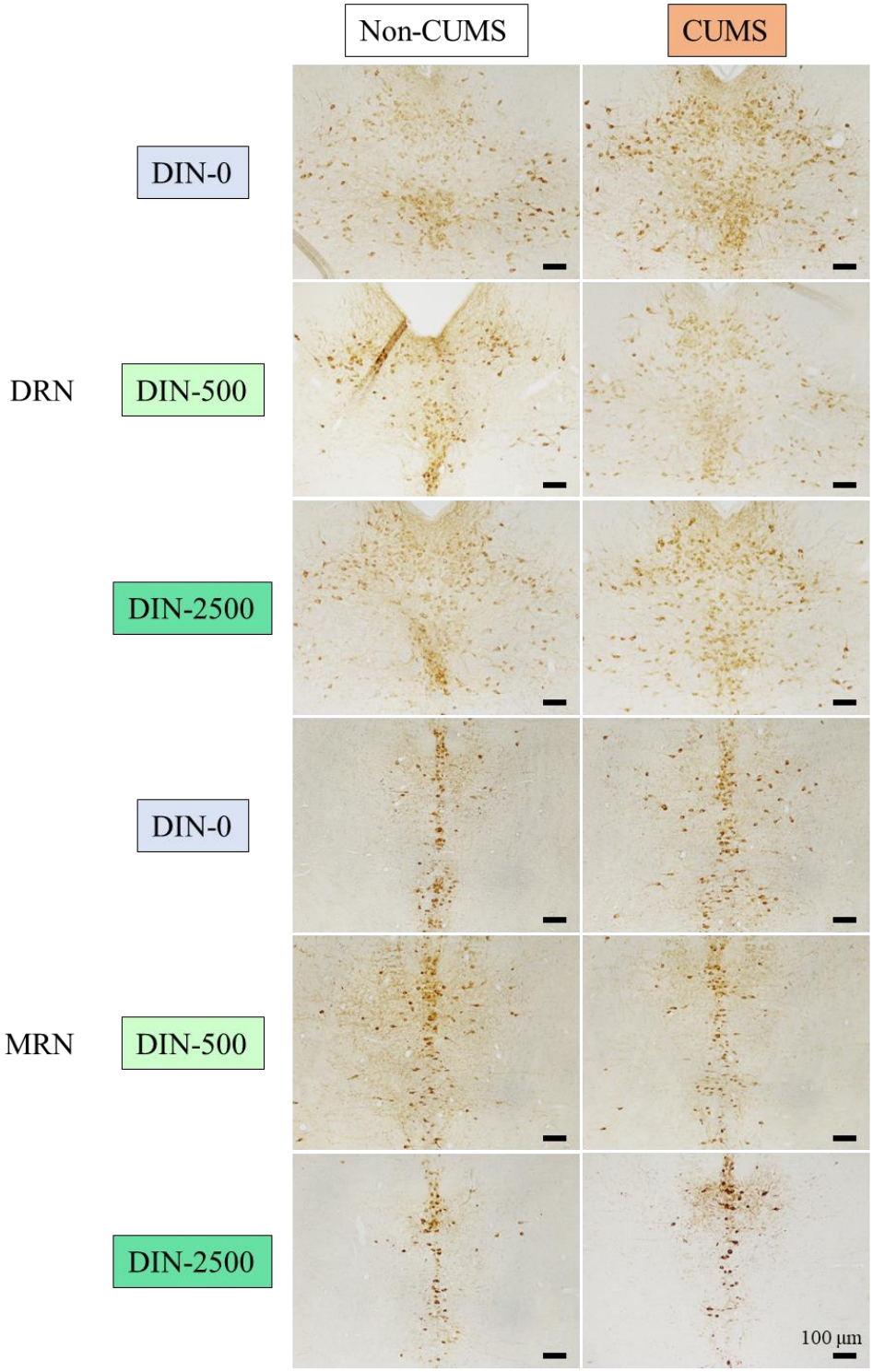

Fig. II-5. Representative immunohistochemistry for TPH2 in the DRN and MRN in all groups. The positive intensities of TPH2 in the DRN and MRN were increased in the DIN-500 and DIN-0+CUMS groups, as compared to those in the DIN-0 group. The increase in the DIN-0+CUMS group was counteracted in the DIN-500+CUMS and DIN-2500+CUMS groups.

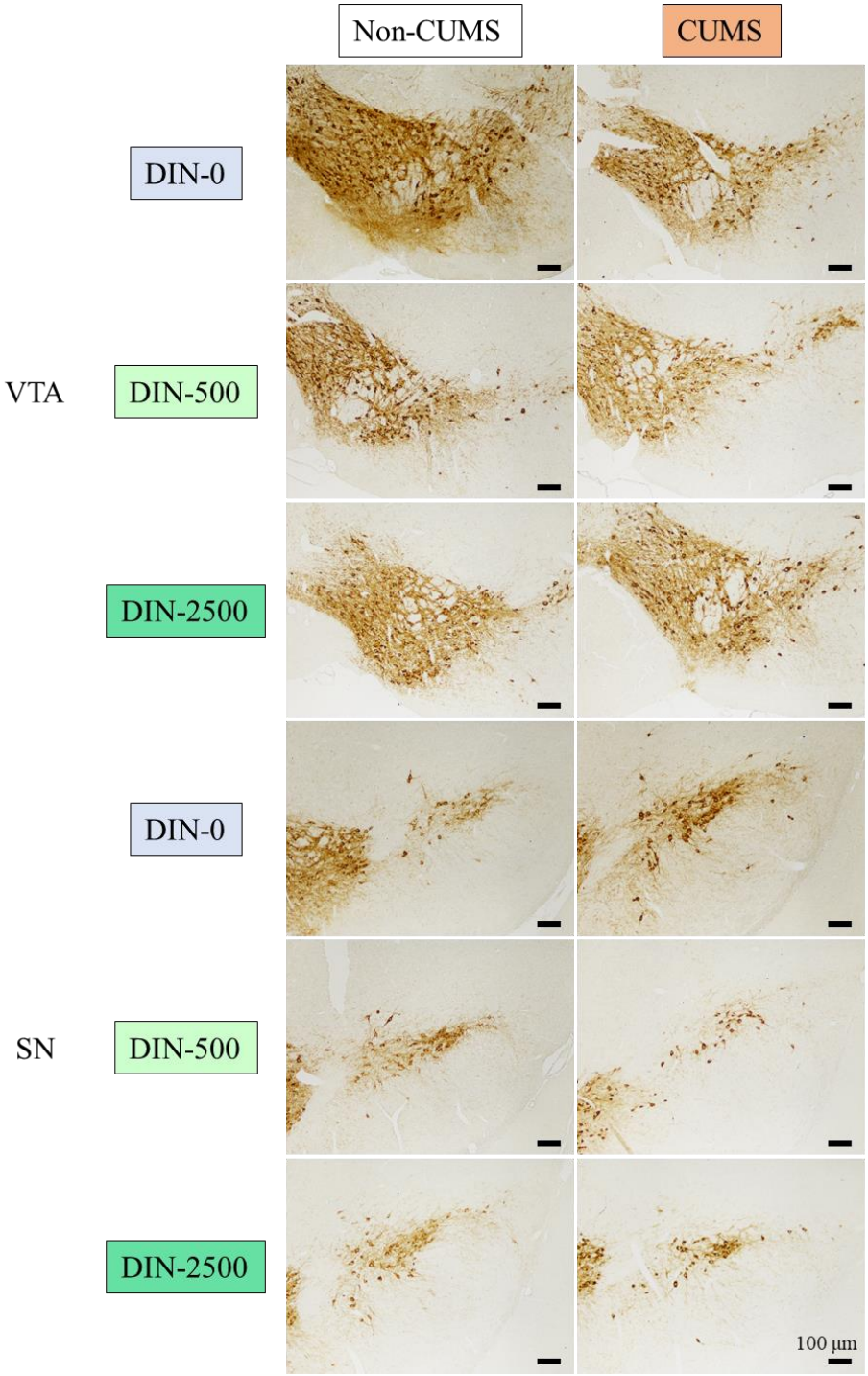

Fig. II-6. Representative immunohistochemistry for TH in the VTA and SN in all groups. The positive intensities of TH were decreased in the VTA and were increased in the SN in the DIN-500, DIN-2500 and DIN-0+CUMS groups, as compared to the corresponding values in the DIN-0 group. The increase in the 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.

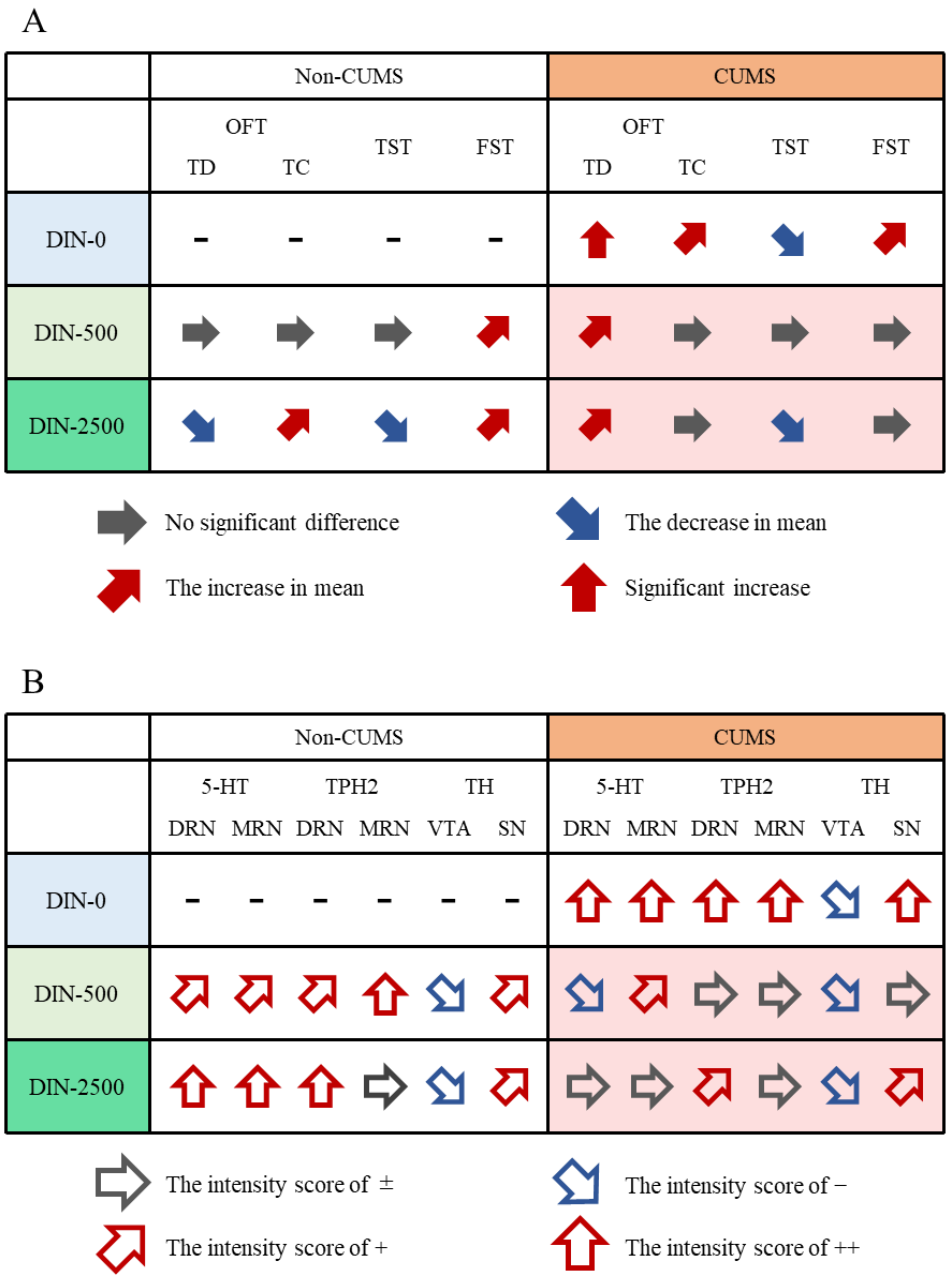

Fig. II-7. The summary of the results of the (A) behavioral tests and (B) immunohistochemical analyses. (A) The arrows in this figure represent the differences compared to DIN-0 group. (B) The positive intensities are indicated as follows: −, 50–80%; ±, 100%; +, 120–150%; ++, 150–180% compared to DIN-0 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.

CONCLUSION

This study could trigger a reassessment of the safety and credibility of NOEL doses calculated from the conventional toxicity test, demonstrating the importance of evaluating neurobehavioral changes of mammals which are currently excluded from the criteria for the evaluation of the safety and risk of pesticides. In addition, it suggests new endpoints for the evaluation of chemical substances with neurological effects, and provides much information toward the future development of risk assessment systems and biomarkers for environmental chemicals.

In Chapter I, the effects of subacute administration of DIN were examined on newly weaned mice under non-stressful conditions. The immobility time in TST was significantly decreased and the number of 5-HT-positive cells tended to increase. These results indicated that NOEL dose DIN administration for a short period induced the neurobehavioral changes of juvenile mice under non-stressful conditions. Some of these findings have already been published in the *Journal of Veterinary Medical Science*.

In Chapter II, the effects of DIN on mice were examined under stressed conditions. DIN decreased the immobility time of TST, increased the immobility time of FST and increased the positive intensity of 5-HT and TPH2 in the DRN and TH in the SN in non-stressed mice. Moreover, CUMS increased the total movement distance of OFT,

decreased the immobility time in TST, increased the immobility time in FST and increased the positive intensity of the expression of 5-HT and TPH2 in the DRN and TH in the SN. On the other hand, the changes observed above were suppressed in mice exposed to both DIN and CUMS, even though the levels of the corticosterone increased in the mice exposed to either stressor alone. These results indicate that DIN induces a "pleiotropic neurotoxic effect" which differs depending on the stress condition of mammals and an "endocrine disrupting effect" which increases stress hormone levels. Some of these findings have already been published in the *Journal of Veterinary Medical Science*.

In conclusion, the effects of DIN on the mammalian monoamine nervous system and behavior are various due to changes in the environment and can be counteracted in some cases. The present study provides valuable data for DIN safety studies because only a few studies have examined the effects of DIN on mammals. In addition, counteraction of the effects of environmental chemicals or stress induced by combined exposure to these stressors suggests the multifaceted nature of environmental chemicals. These results will contribute greatly to the study of the effects of environmental chemicals on mammals, including humans.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Nobuhiko Hoshi (Department of Animal Science, Graduate School of Agricultural Science, Kobe University), my supervisor, for his invaluable input throughout this research and for giving me this precious opportunity to study. I am also very grateful to Professor Hiroshi Kamisoyama and Professor Ushio Kikkawa (Department of Animal Science, Graduate School of Agricultural Science, Kobe University), who read this thesis and helped to improve the quality of the writing. I would also like to thank Associate Professor Yoshinori Ikenaka and Mr. Nimako Colins (Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University) for helping me to analyze the DIN in blood samples. I am deeply indebted to Professor Hiroshi Kitagawa, Assistant Professor Toshifumi Yokoyama, Assistant Professor Youhei Mantani (Department of Animal Science, Graduate School of Agricultural Science, Kobe University) and Assistant Professor Tetsushi Hirano (Division of Drug and Structural Research, Life Science Research Center, University of Toyama) for their helpful advice and suggestions for this research. I thank my laboratory members for their continuous support during this study. Finally, I also wish to give my thanks to my parents for their continuous encouragement, understanding, and warm support in my life.

This work was partly supported by Grants-in-Aid for Scientific Research A (#18H04132 to YI) and B (#19H04277 to NH) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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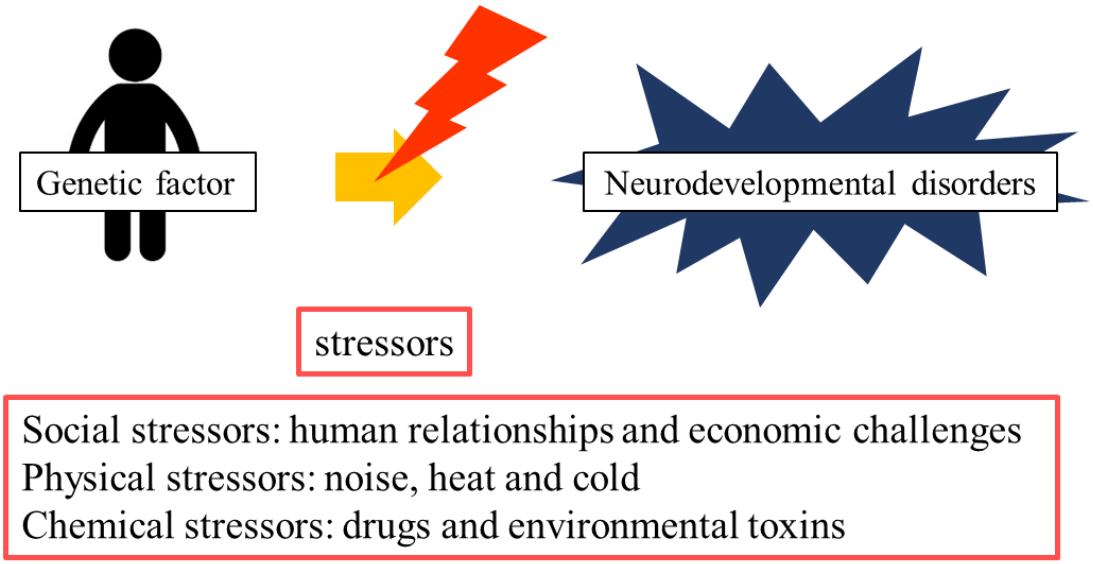
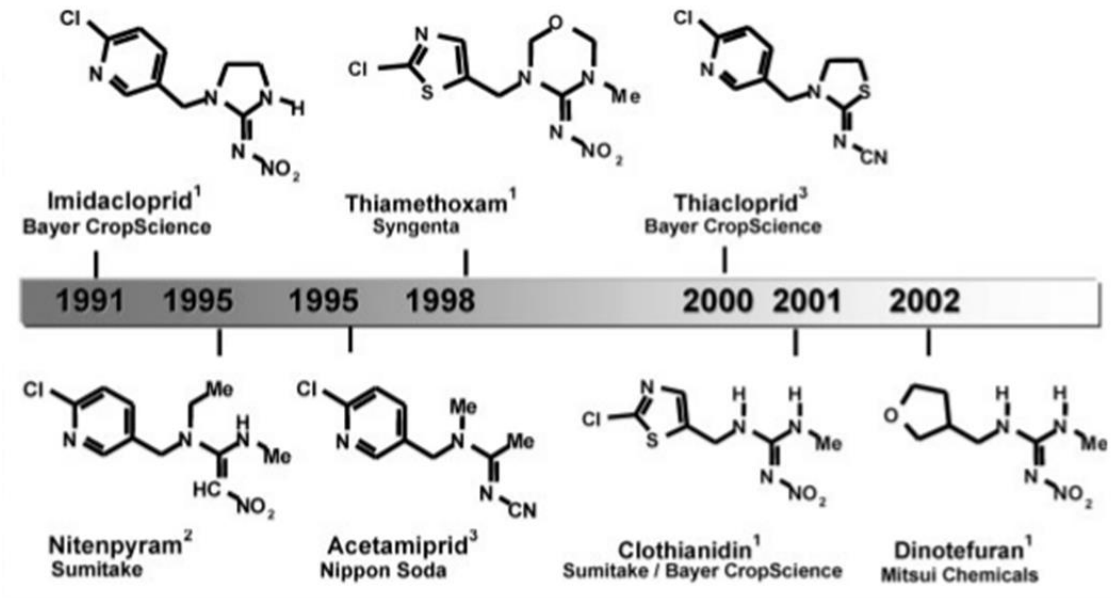
APPENDIX FIGURES

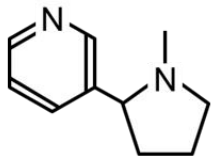
App. 1. Stressors in modern society.



App. 2. Types pf NNs.

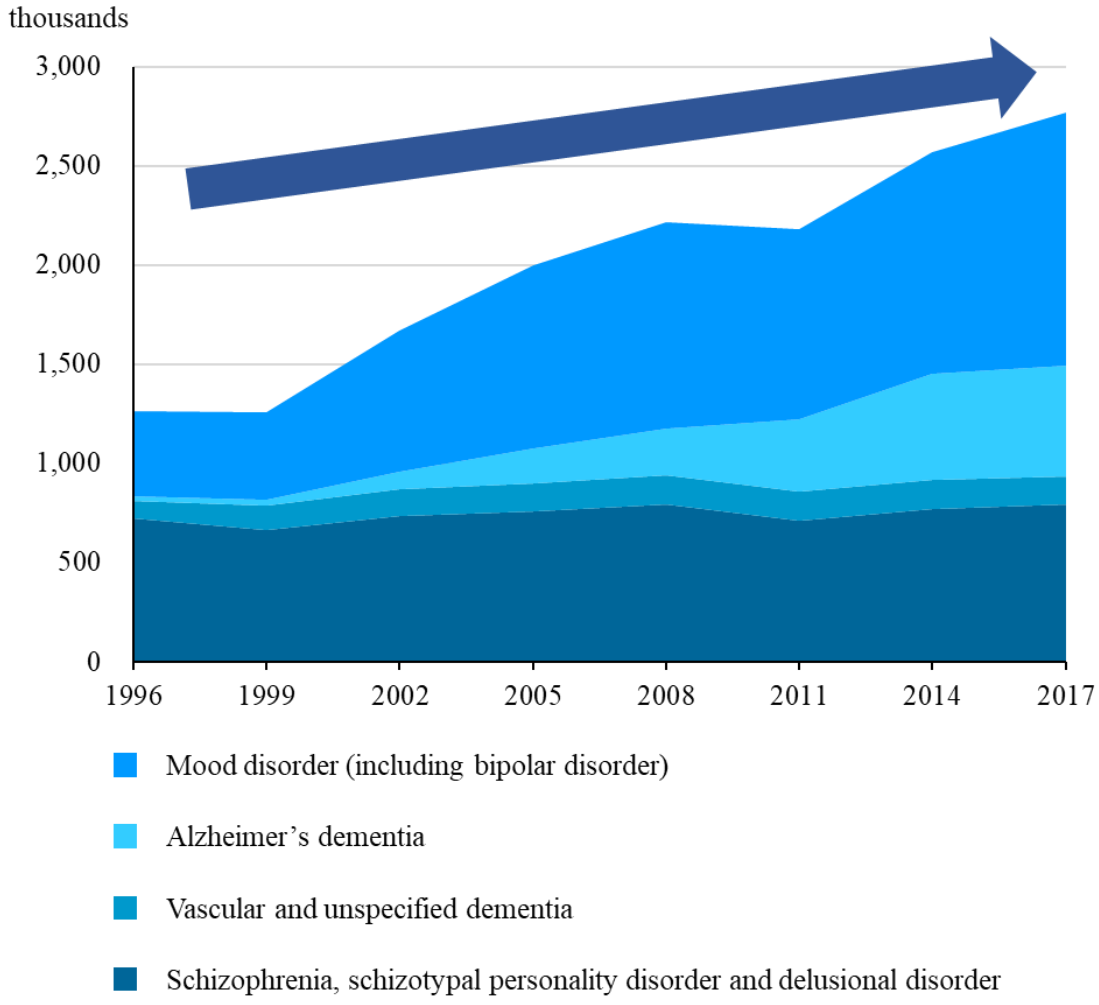


[Elbert *et al.*, 2008]



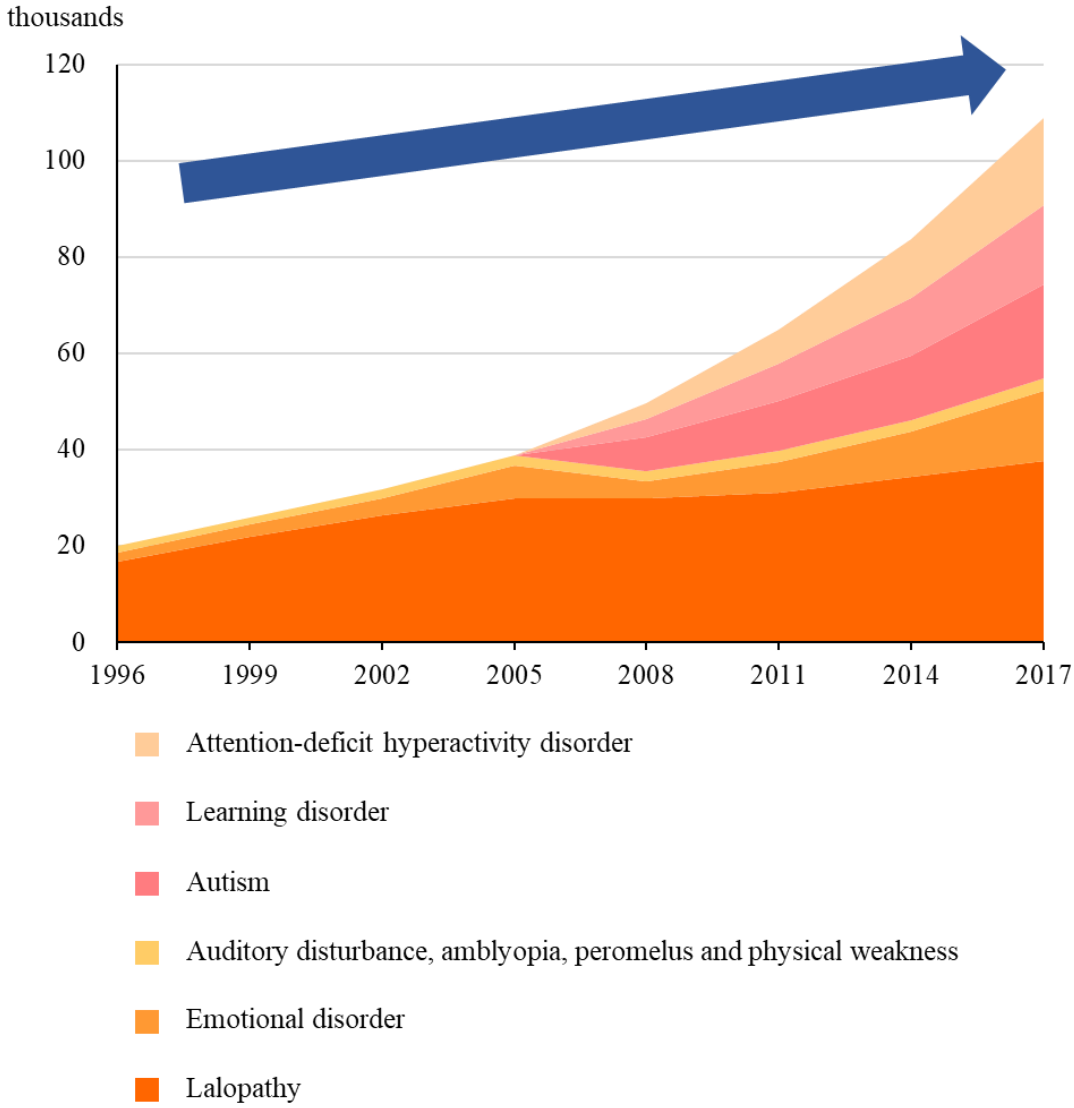
nicotine

App. 3. Total number of patients with mental illness.



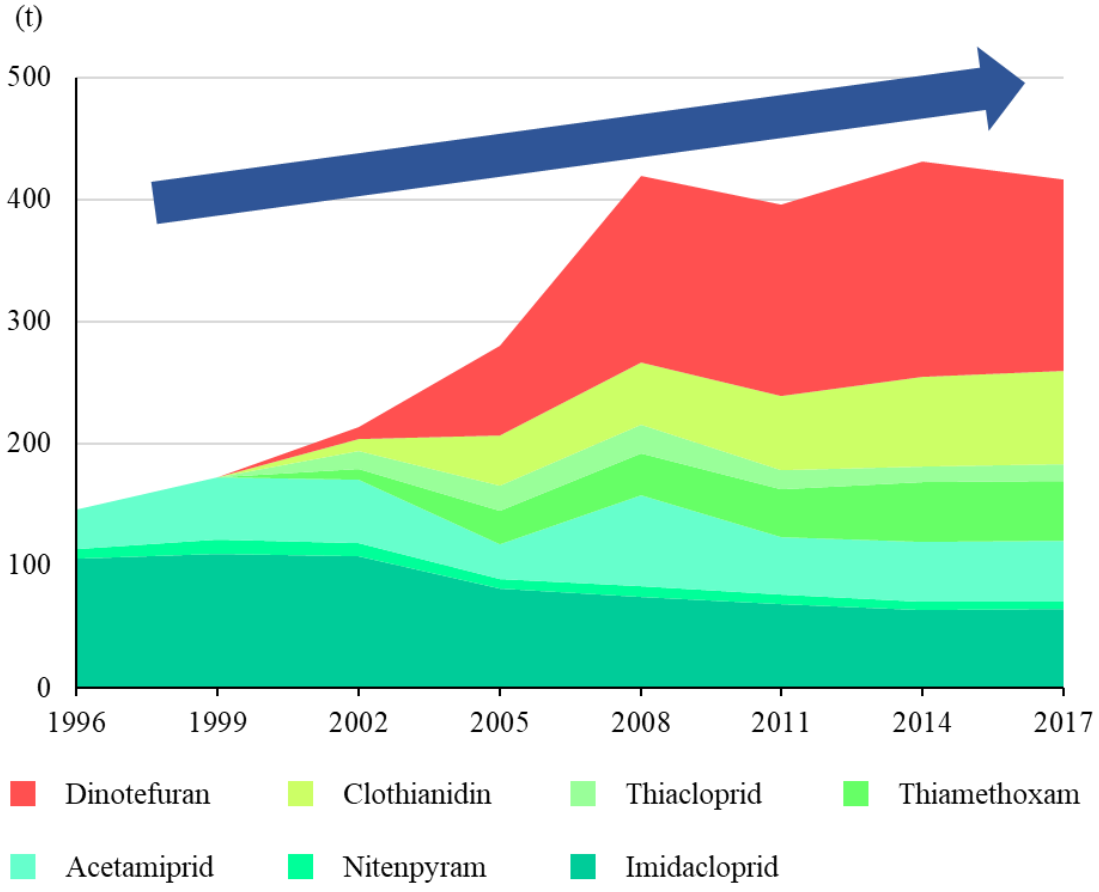
[Ministry of Health, Labour and Welfare, 2019]

App. 4. The number of pupils receiving special needs education.



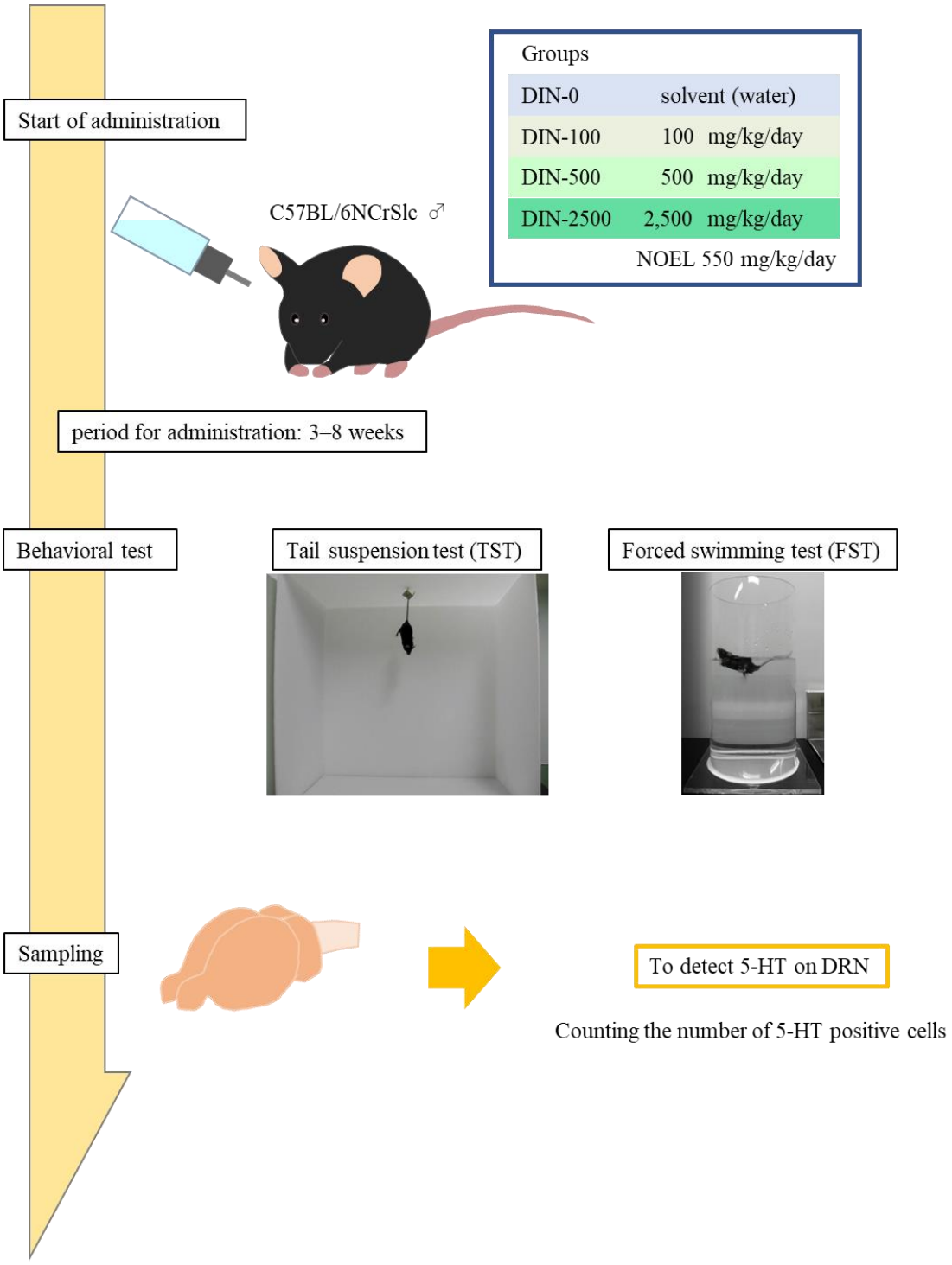
[Ministry of Education, Culture, Sports, Science and Technology, 2019]

App. 5. Domestic shipments of NNs.



[National Institute for Environmental Studies, 2019]

App. 6. The protocol in Chapter I.



App. 7. The protocol in Chapter II.