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NOTE

Toxicology

Peripubertal exposure to the neonicotinoid pesticide dinotefuran affects dopaminergic neurons and causes hyperactivity in male mice

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ABSTRACT. Although neonicotinoid pesticides are expected to have harmful influence on mammals, there is little animal experimental data to support the effect and mechanisms. Since acetylcholine causes the release of dopamine, neonicotinoids may confer a risk of developmental disorders via a disturbance in the monoamine systems. Male mice were peripubertally administered dinotefuran (DIN) referring to no observed effect level (NOEL) and performed behavioral and immunohistological analyses. In an open field test, the total locomotor activity was increased in a dose-dependent manner. The immunoreactivity of tyrosine hydroxylase in the substantia nigra was increased in DIN-exposed mice. These results suggest that exposure to DIN in peripubertal male mice causes hyperactivity and a disturbance of dopaminergic signaling.

KEY WORDS: dinotefuran, dopamine, hyperactivity, neonicotinoid, neurobehavioral effect

Neonicotinoids are modeled on the chemical structure of nicotine and have been used as pesticides in recent years. Since they have much higher affinity for the nicotinic acetylcholine receptors (nAChR) of insects than of mammals [17], they have been considered to have low toxicity to mammals. Nevertheless, it has been reported that neonicotinoids have nicotine-like excitatory effects on mammalian nAChR [9]. In addition, several studies have reported that neonicotinoids have various effects on the reproductive systems and neurobehavior of quails and mice [6–8, 16, 19]. nAChR is widely expressed in the central nervous system [4], and numerous functions of acetylcholine signals have been elucidated, including the induction of dopamine (DA) release [1].

DA, one of the monoamines, is a neurotransmitter synthesized from tyrosine. The major DA nerves in the midbrain belong to nigrostriatal pathway. In this pathway involved in motor function, the cell body exists mainly in the substantia nigra (SN), and the axon is projected to the striatum. In a previous study, sequential injections with paraquat resulted in a significant loss of dopaminergic neurons in the SN [11]. In addition, an *in vivo* study showed that neonicotinoid causes transient DA release in the rat striatum [2]. Therefore, the nigrostriatal pathway is considered to be a target of chemical substances in the environment.

The World Health Organization and United Nations Environment Programme (WHO/UNEP) have expressed alarm over the influence of pesticides have various influences on humans and ecosystems [18]. In addition, the American Academy of Pediatrics (AAP) published a report on the relationship between pesticides and developmental disorders [14]. Indeed, the number of children with developmental disabilities in Japan has more than doubled in the past decade based on the results of a survey on special needs education in FY 2016 [12]. It has been suggested that pesticides and other environmental chemical substances are responsible for the increase in developmental disabilities [10].

Dinotefuran (DIN) was developed in 2002 as one of the latest neonicotinoids; it has the largest domestic shipment volume in

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Table 1. The combination of blocking agents and antibodies used for immunohistochemistry

Detection	Blocking reagent	Primary antibody	Secondary antibody
TH	Blocking reagent A and B (Nichirei Bioscience, Tokyo, Japan)	Mouse monoclonal antibody against TH (MAB318; 1:5,000) (Merck Millipore, Darmstadt, Germany)	Histofine MAX-PO (M) (Histofine Simplestain system) (Nichirei Bioscience, Tokyo, Japan)
DRD_1	Blocking reagent A and B (Nichirei Bioscience, Tokyo, Japan)	Mouse monoclonal antibody against DRD1 (MAB5290; 1:1,500) (Merck Millipore, Darmstadt, Germany)	Histofine MAX-PO (M) (Histofine Simplestain system) (Nichirei Bioscience, Tokyo, Japan)
DRD_2	Blocking One Histo (Nacalai Tesque, Kyoto, Japan)	Rabbit polyclonal antibody against DRD2 (AB5084P; 1:1,500) (Merck Millipore, Darmstadt, Germany)	EnVision + System- HRP Labeled Polymer Anti-Rabbit (Dako, Glostrup, Denmark)

TH: Tyrosine hydroxylase, DRD_1: Dopamine receptor D_1, DRD_2: Dopamine receptor D_2.

Japan. While various effects of neonicotinoids have been reported, there has been little research on the effects of DIN on mammals. In this study, we aimed to analyze the effects of DIN exposure during the peripubertal period on the nigrostriatal pathway and behavior.

Male C57BL/6NCrSlc mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained as described elsewhere [7]. 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.

We divided the mice into four groups (n=6 in each) based on agricultural chemicals and animal drug evaluation form [3] in which 550 mg/kg/day DIN is no observed effect level (NOEL) in mice: DIN-0 (Control), DIN-100 (100 mg/kg/day), DIN-500 (500 mg/kg/day), and DIN-2500 (2,500 mg/kg/day). All mice were actively administered Water-soluble Arubarin® (consisting of 20% DIN; Mitsui Chemical Co., Ltd., Tokyo, Japan) dissolved in water from 3 to 8 weeks of age. We determined the body weights and water drinking volume in individual mice to estimate the amounts of the putative exposure of DIN twice a week.

On the last day of the 6-week experimental period, an open field test and a Y-maze test were conducted to evaluate the locomotor activity, the anxiety-like behavior and the working memory during the light phase. In an open field test, the mice were placed on the corner of an open field (60 × 60 × 30 cm) under LED illumination. The total distance traveled and time spent in the center zone (30 × 30 cm) were measured over 60 min using Image J software (National Institutes of Health, Bethesda, MD, U.S.A.). In a Y-maze test, the mice were placed at the end of one arm (40 cm long ×15 cm high ×8 cm wide) under LED illumination. All their activities were recorded by a video camera over the subsequent 10 min, and then the total instances of alternation behavior were counted. Alternation behavior was defined as successive entry into the three arms and expressed as the ratio of actual alternations to possible alternations (defined as the total number of arm entries minus two), multiplied by 100.

On the day following the 6 weeks of exposure to DIN, all mice were deeply anesthetized with isoflurane using inhalation anesthesia apparatus (BS-400T; Brain Science idea, Osaka, Japan) and perfused intracardially with ice-cold 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. The brains were excised, weighed and immersed in the same fixative solution for 6 hr at 4°C. The brains were then dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. Cross sections were cut at 10-μm-thickness, and each section was mounted on a slide glass (Platinum Pro; Matsunami Glass, Kishiwada, Japan).

To detect tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra, and dopamine receptor D_1 (DRD_1) and dopamine receptor D_2 (DRD_2) immunoreactivity in the striatum, we performed the immunohistochemistry protocol as described elsewhere [6]. The combination of blocking agents and antibodies used for the detection of each protein by immunohistochemistry is listed in Table 1.

Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan). Differences were considered statistically significant if $P<0.05$. One-way ANOVA analysis followed by the Dunnett's test or Kruskal-Wallis test followed by Steel test were used to determine differences between groups.

We measured locomotor activity for 60 min in an open field test and found that DIN dose-dependently increased the total distance traveled (Fig. 1A), with the effect being significant for DIN-2500. By contrast, we found no significant effect of DIN on the time spent in the center zone (Fig. 1B). In this test, the total distance traveled and the time spent in the center zone respectively indicated hyperactivity and anxiety. These results suggest that DIN causes hyperactivity but has no significant effect on anxiety-like behavior.

In the Y-maze test, there was no significant difference in the spontaneous alternation behavior among the four groups (Fig. 1C). This result suggests that peripubertal exposure to the DIN does not impair working memory.

The results of the immunohistochemical analyses of the brain visualizing TH are shown in Fig. 2A–D. TH immunoreactivity was enhanced in the DIN exposure group relative to the control group. But we found no effect of DIN on DRD_1 or DRD_2 immunoreactivity in the striatum (Fig. 2E–L). TH is the rate-determining enzyme of DA synthesis, so it is inferred that the increase in the intensity of TH positivity found in this study reflects the increase in DA synthesis by DIN.

Our results indicate that peripubertal exposure of DIN affects the DA nervous system and induces hyperactivity in male mice. Regarding the relationship between locomotor activity and DA content, it has been reported that hyperactivity is observed under

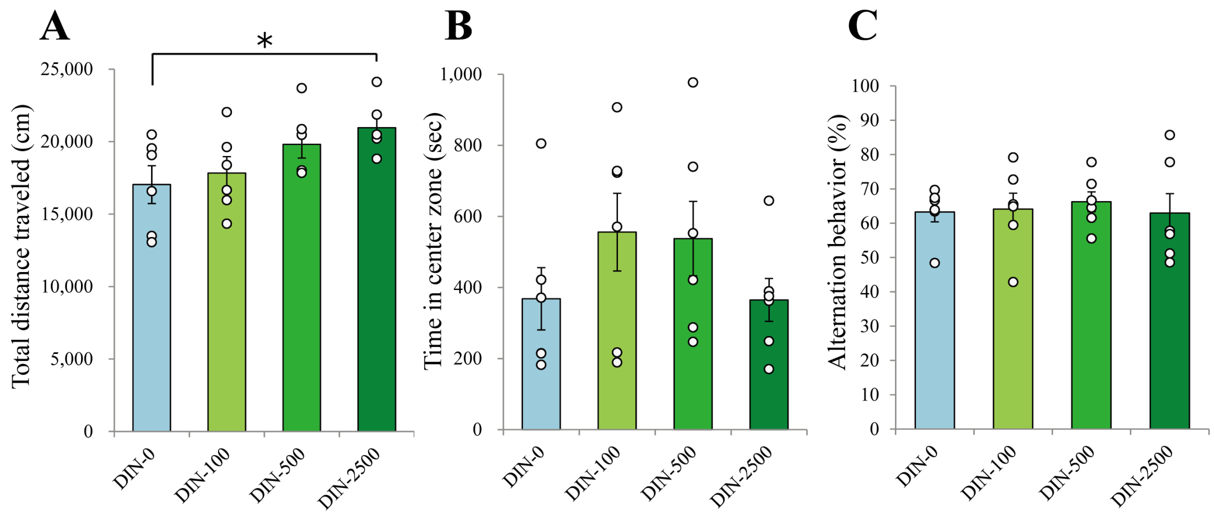

Fig. 1. Behavioral effects of peripubertal exposure to DIN on mouse behavior in an open field test and a Y-maze test. Total distances traveled were significantly increased in the DIN-2500 compared to the DIN-0 (A), but time spent in the center zone was not changed (B) in the open field test. There were no significant effects among all groups in the percentage of alternation behavior (C). Columns indicate the mean ± SE of each group and circles show the individual values of mice (n=6 in each), **P*<0.05 by one-way ANOVA followed by Dunnett's *post-hoc* test (A and B) or Kruskal-Wallis test followed by Steel's *post-hoc* test (C).

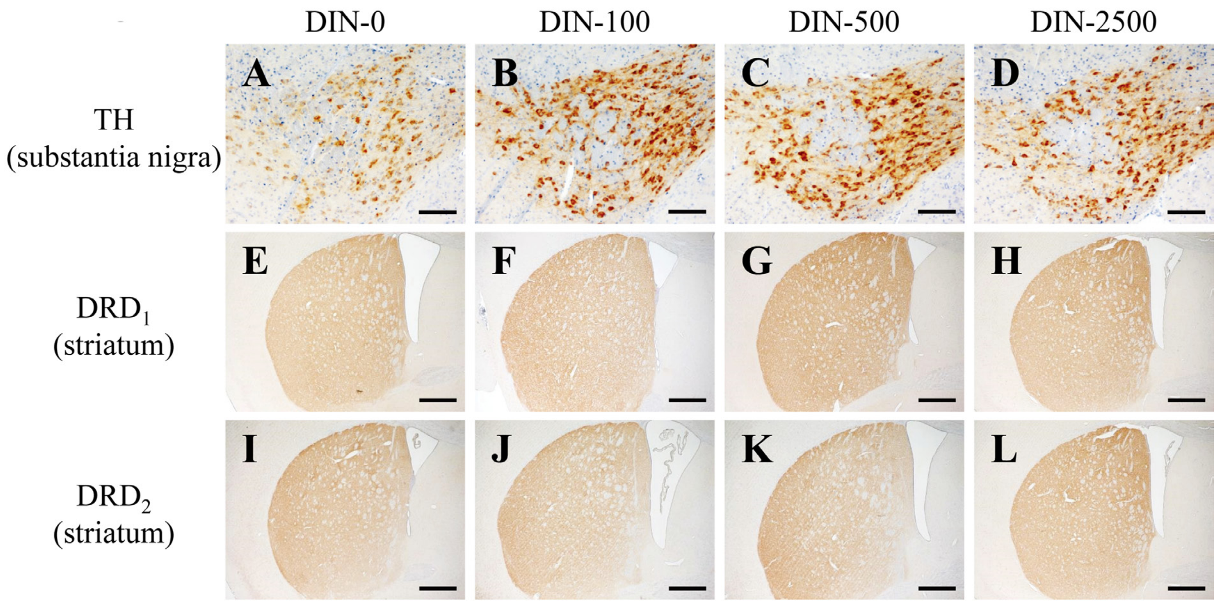

Fig. 2. Representative immunohistochemistry for TH, DRD_1 and DRD_2 in sections of the mouse brain. In the substantia nigra, enhancement of the intensity of TH positivity was observed in the DIN exposure group (A–D). No significant effect on the intensity of TH positivity was observed in DRD_1 (E–H) or DRD_2 (I–L) of the striatum. Bar=100 μm (A–D), 500 μm (E–L).

extreme decreases or under any increases in the amount of DA [5]. In our present study, it was suggested that the promotion of DA synthesis by DIN caused hyperactivity. Attention deficit hyperactivity disorder (ADHD), one of the developmental disorders, is characterized by hyperactivity, inattention and impulsivity. A previous study reported that mice exposed to fetal nicotine exhibited a wide range of ADHD-like symptoms, including hyperactivity and deficiency in working memory [20, 21]. In another study, ADHD-like symptoms such as hyperactivity and working memory disorder were seen in mice exposed to pyrethroid pesticide, and at the same time the expression levels of DA transporter and DRD_1 increased [13], suggesting that ADHD and the DA nervous system are closely related. However, our experiments revealed no effect on DRD_1, DRD_2 or working memory. From these results, it seems that although DIN is not solely responsible for the induction of ADHD symptoms, it may be one of the causes.

Regarding anxiety-like behavior, no significant effect was observed in this study. A previous study reported that *in utero* and lactational exposure to acetamiprid, one of neonicotinoids, caused an anti-anxiolytic effect [15]. In contrast, another study showed that acute exposure of clothianidin, one of neonicotinoids, causes anxiety-like behavior [7]. These disparate results suggest that the behavioral effects differ according to the type of neonicotinoids.

This study is the first report to analyze the neurobehavioral effects of DIN on mice. The results indicate that DIN enhanced TH positivity and increased locomotor activity. However, the detailed mechanism by which DIN enhances DA synthesis remains to be clarified. In addition, further studies are needed because DIN is likely to affect various pathways other than the nigrostriatal pathway. The ongoing results of such studies will help to clarify the relationship between pesticides and developmental disorders.

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