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NOTE

Toxicology

Influence of acute exposure to a low dose of systemic insecticide fipronil on locomotor activity and emotional behavior in adult male mice

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ABSTRACT. Fipronil (FPN) is a systemic insecticide that antagonizes the gamma-aminobutyric acid type A (GABA_A) receptors in insects. Recently, adverse effects of FPN on mammals have been reported, but most of those were caused by high doses of FPN and additives in the products. We investigated the effects of low-dose pure FPN on the emotional behavior of mice. Nine-week-old male mice conducted behavioral tests 24 hr after FPN administration by gavage at doses of 0.05 or 5 mg/kg based on the no-observed-effect level (NOEL), showed a significant increase in locomotor activity and dose-dependent responses on the time they spent in the central zone in the open field test. Pure FPN below the NOEL dose may affect the emotional behavior of mice.

KEY WORDS: behavioral test, fipronil, gamma-aminobutyric acid (GABA), insecticide, locomotor activity

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Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system that binds to GABA receptors and regulates a variety of signalings. When GABA binds to GABA_A receptors, it generates inhibitory potentials and suppresses neuronal excitement [12]. Thus, GABA has a mental stabilizing effect inducing relaxation and reducing anxiety [1, 22, 29], and decreased GABA levels have been suggested to be associated with many mental disorders such as anxiety or sleep disorder, depression, and schizophrenia [5, 17].

Fipronil (FPN), developed in the late 1980s, is a systemic insecticide that has a slow-acting and highly effective insecticidal effect against a wide range of insects with inhibitory action at the GABA_A receptor. Due to its unique mechanism of action, FPN has been shown to have high insecticidal activity against pests that are resistant to conventional insecticides, and it is effective at lower doses against a wide range of pests. FPN has a lower affinity to mammalian GABA_A receptors compared to those of insects [4, 15, 30]. Therefore, it is widely used as an insecticide, a flea larvicide for dogs and cats, an attractant, and an insect repellent.

However, following the global death and disappearance of honeybees since the 1990s, a chemical risk assessment conducted by the European Food Safety Commission concluded that FPN was toxic to honeybees [6]. For this reason, the use of FPN has been banned in the EU since 2013. In Japan, FPN has been shown to be more toxic to dragonflies than other insecticides and is suspected to be the main cause of the decline in red dragonfly (*Sympetrum frequens*) [11, 16]. Thus, in recent years there has been a concern that FPN may affect insects other than pests. According to a series of reports, dogs and cats have developed convulsions, epilepsy and, in the worst cases, have died after flea larvicide containing FPN was dripped onto their skin [19]. Adverse effects on mammals, including thyroid dysfunction in humans [13] and reproductive toxicity in rats [23] have also been reported. Furthermore, in 2017, FPN-contaminated eggs were found in more than 40 countries, including the EU and the US [20]. FPN and its metabolites were frequently detected in river water in Japan [24]. These reports have raised concerns around the world about the

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safety of FPN.

Because FPN has GABA-inhibitory effects, its involvement in mammalian psychiatric disorders has been a concern. Previous studies have reported effects on cognitive and emotional behavior in mammals. Exposure of rats to FPN formulations at a dose of 1 mg/kg/day for 1 week during gestation and lactation increased anxiety-like behavior and aggression [8], and exposure of adult rats to 30 mg/kg for 15 days elicited memory deficits [9]. However, the FPN concentrations in those experiments were very high relative to the no-observed-effect level (NOEL) of the insecticide evaluation report [8, 9, 26]. In addition, since the formulation contains repellents and organic solvents, the effects observed in the experiments may not be limited to FPN alone. In the present study, we investigated the effect of a single dose of pure FPN at a concentration close to NOEL on the emotional behavior of mice.

Male C57BL/6NCrSlc mice (7 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and maintained as described elsewhere [14]. This study was approved by the Institutional Animal Care and Use Committee (Permission #26-05-07) and carried out according to the Kobe University Animal Experimental Regulations. Fipronil (5-amino-1-(2,6-dichloro- α,α,α -trifluoro-p-tolyl)-4-trifluoromethylsulfanylpyrazole-3-carbonitrile, cas.No:120068-37-3) was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). We divided the mice into four groups: FPN-0 (vehicle as Control), FPN-0.05 (0.05 mg/kg/day) and FPN-5 (5 mg/kg/day) with reference to the no-observed-effect level (NOEL) of 10 mg/kg/day from a general pharmacology test in male mice [7]. FPN was suspended in 0.5% carboxymethylcellulose (10 ml/kg, FUJIFILM Wako Pure Chemical) by oral gavage. All administrations and behavioral tests were conducted in the light phase. Each behavioral test was performed 24 hr after administration of FPN, with a one-week interval. The open field test (OF) was used to evaluate locomotor activity and anxiety-like behavior in a novel environment. In the OF mice were acclimated to the testing room conditions (450 lux, $23 \pm 2^\circ\text{C}$) in their home cage for 1 hr prior to the start of the test. The mouse was placed in a corner of an open field ($60 \times 60 \times 40$ cm, Tom Products, Tokyo, Japan) with its nose pointed at the wall and was free to explore for 10 min. All of the mouse's activities were recorded by a video camera. At the end of each trial, the mouse was moved to a new cage, and all materials and testing surfaces were cleaned with Serius soft acidic water (NDX-1500 PLB, Serius Soft Acidic Water Generator Hygiene Managing System, OSG, Osaka, Japan). Total distance, moving speed (total distance [cm] / total movement duration [sec]), and the time spent in the center zone (30×30 cm) and travelling were recorded.

Next, the elevated plus maze test (EPM) was performed to evaluate locomotor activity and anxiety-like behavior under the fear condition of no walls at high altitude. In the EPM, mice were acclimated to the testing room conditions (10–20 lux, $23 \pm 2^\circ\text{C}$) in their home cage for 1 hr before the start of the test. The apparatus consisted of two opposing open arms without walls (30×5 cm), two closed arms with walls (same size, 15 cm wall), and a center zone (5×5 cm) and formed a cruciform made of acrylic plate (Tom Products). The mouse was placed in the center zone with its nose pointed at the open arm and was free to explore for 10 min. All of the mouse's activities were recorded by a video camera. At the end of each trial, the mouse was moved to new cage, and all materials and testing surfaces were cleaned with Serius soft acidic water. Total distance, percentage of open arm entries, and time spent in the open arms and travelling were recorded. The results of each behavioral test were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan). All data were analyzed by Smirnov-Grubbs test for outlier and one-way ANOVA followed by Dunnett's test or Kruskal-Wallis test followed by Steel test. Levene's test was used to evaluated the homogeneity of variance. The results were considered significant when the P -value was <0.05 .

The purpose of this study was to evaluate the effects of acute low doses (less than the NOEL-dose) of FPN on the behavior of mice. In the OF, the total distance and moving speed were significantly greater in the FPN-5 group than in the control group (Fig. 1A and 1B). These results indicated that FPN increased locomotor activity. Previously it was

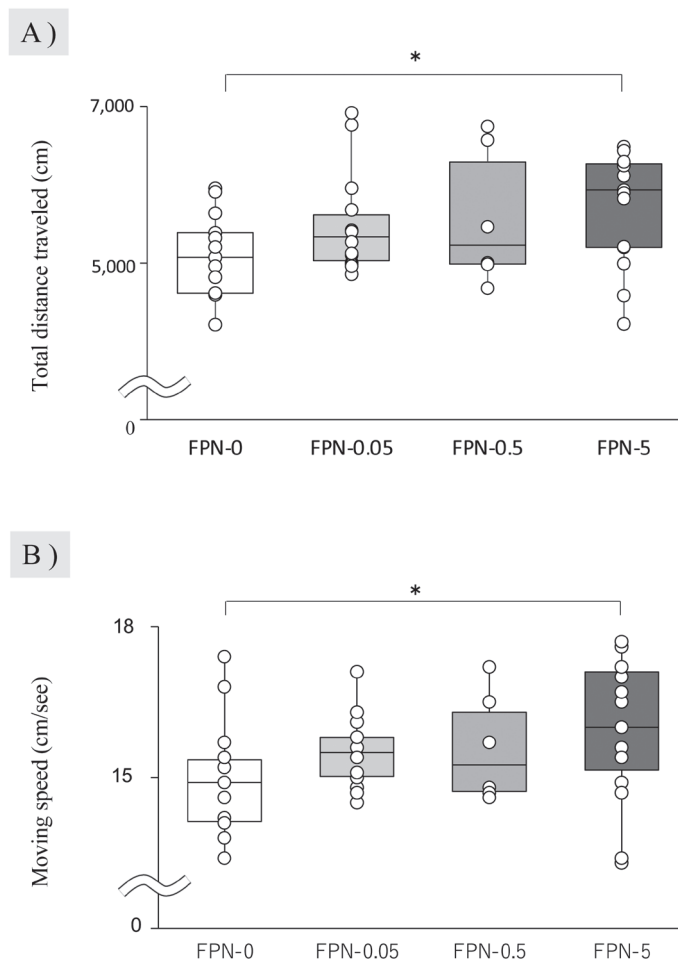


Fig. 1. Effects of fipronil (FPN) exposure on total distance travelled (A) and moving speed (B) in the open field test (OF). Data are reported in the form of a box plot, and each result is plotted. FPN-0 (n=14); FPN-0.05 (n=14); FPN-5 (n=15); A: in the FPN-5 mice, the total distance travelled is significantly greater than in the FPN-0 group (Dunnett's test, $*P<0.05$). B: the moving speed is significantly faster than that in the FPN-0 group (Dunnett's test, $*P<0.05$).

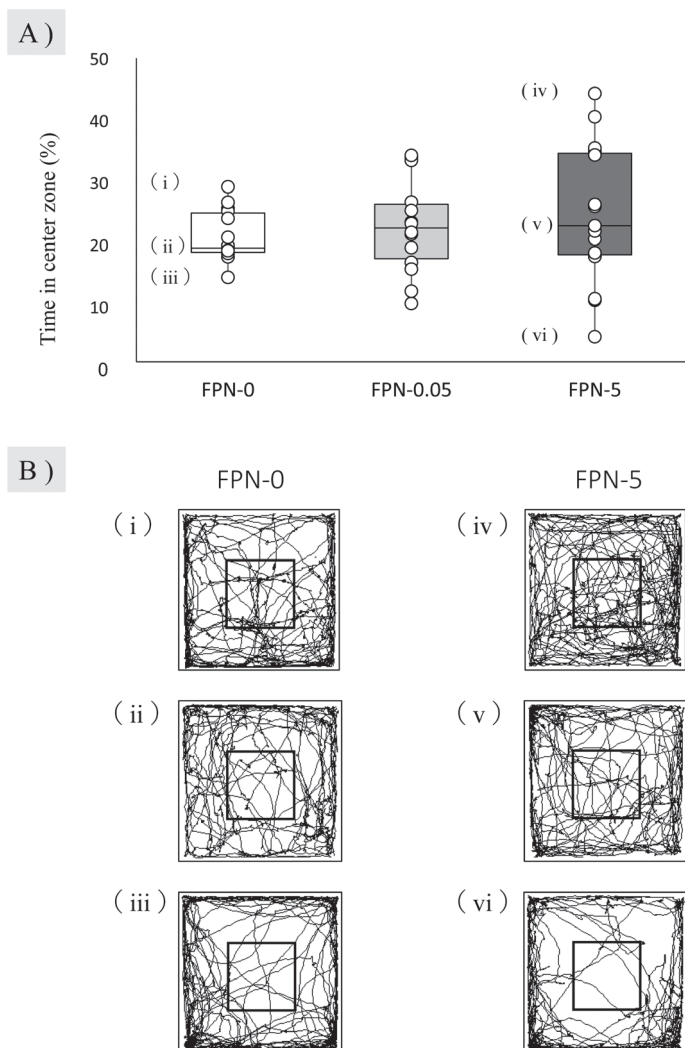


Fig. 2. Effects of fipronil (FPN) exposure on the time spent in the center zone (A) and the trajectory map of mice as illustrated by video tracking software (B) in the open field test (OF). Data are reported in the form of a box plot, and each result is plotted. FPN-0 (n=14); FPN-0.05 (n=14); FPN-5 (n=15); A: in the administration group, the time spent in the center zone is not significantly different compared to that in the FPN-0 group by Steel test. However, the homogeneity of variance was denied by Levene's test ($P=0.038$). It gives the impression that the variation increases in a dose-dependent manner. B: (i)~(vi) in the figure corresponds to (A). Compared to the control group (i)~(iii), the individuals in the FPN-5 group showed various trajectory such as short (iv), similar (v) or short stay time (vi).

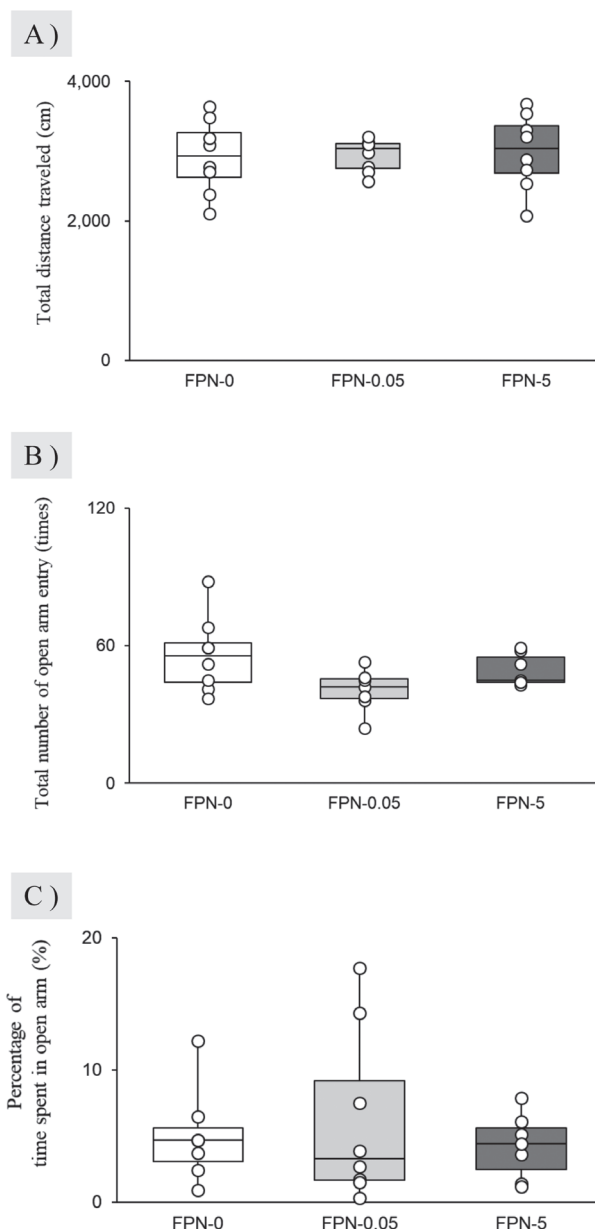


Fig. 3. Effects of fipronil (FPN) exposure on the time spent in the total distance (A), the percentage of open arm entries (B), and the time spent in the open arm (C) in the elevated plus maze test (EPM). Data are reported in the form of a box plot, and each result is plotted. FPN-0 (n=8); FPN-0.05 (n=8); FPN-5 (n=8); no significant differences are observed in any of the evaluation items by Dunnett's test.

reported that rats exposed to a flea larvicide containing 280 mg/kg FPN by the dermal route exhibited hyperactivity characterized by increased locomotor activity, similar to the present results [26]. However, the FPN used in the previous study was at very high doses and contained other additives, such as repellants and surfactants. Recently, insecticide formulations have been shown to be more toxic than drugs [18]. For example, Roundup® (Monsanto Co., St. Louis, MO, USA), a glyphosate product, is nearly 100 times more toxic than the glyphosate alone, and the tendency was observed for neonicotinoid insecticide products. In this study pure FPN was used, suggesting that it caused hyperactivity at a dose lower than the NOEL concentration in the current toxicity test. By contrast, these changes in locomotor activity were not observed in EPM (Fig. 3A).

There was no significant difference between the groups in the time spent in the central zone of the OF (Fig. 2A), and the percentage of open arm entries and the time spent in the open arm in the EPM (Fig. 3B and 3C). However, some mice in the administration group in the OF showed strong anxiety after a short time spent in the central zone and conversely increased exploratory behavior to

the central zone (Fig. 2A and 2B). The mice showed various emotional behaviors after FPN administration, and the variation of the time they spent in the central zone increased in a dose-dependent manner. Regarding the interpretation of the experimental results, in the absence of statistical significance, it is inappropriate to assume that there is no physiological significance [28], and we believe that not only the magnitude of the mean, but also the change in variance should be considered. Nakamura and Suzuki [21] showed that data variation was associated with increased anxiety. That is, increased anxiety leads to data variability, and the same is likely to be the case in the present study. In addition, previous studies have reported increases in anxiety-like behavior with high doses FPN [8]. Our results suggest that the effects of FPN below the acute NOEL dose on anxiety-like behavior in adult male mice may vary depending on the sensitivity and environment.

It was also shown that the acute administration of pure FPN at a dose below NOEL could affect the emotional behavior of mice. There is particular concern about increased locomotor activity, but the mechanism is not clear. An extreme decrease or increase in dopamine leads to an increase in locomotor activity [10]. In addition, GABA and GABA_A receptors are closely related to the inhibition of dopamine release [27]. Oral gavage and stereotaxic injection of FPN into the striatum and substantia nigra has been reported to reduce tyrosine hydroxylase (TH) expression and dopamine levels in rat [2, 3, 25]. FPN may affect dopamine release in the brain and induce hyperactivity. Therefore, it is necessary to investigate the effects of FPN administration on dopamine release and the mechanism underlying increased locomotor activity.

The present results showed, for the first time, that FPN below a NOEL dose may affect the locomotor activity and emotional behavior of adult male mice. In particular, this study investigated the effects of pure FPN, rather than FPN-containing products, on behavior. These results should contribute to the accuracy of future evaluations of the safety of FPN.

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

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