Indian J Physiol Pharmacol 2015; 59(2) : 211–222

Original Article

Perinatal Exposure to Low-dose Nonylphenol Specifically Improves Spatial Learning and Memory in Male Rat Offspring

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Abstract

4-Nonylphenol (NP) has weak estrogen-like activity, and can therefore act as an endocrine disruptor. This study examined the effects of perinatal exposure to low-dose NP on learning and memory, general activity, and emotionality in male rat offspring. Dams were orally administered 1 or 10 mg/kg/day of NP or vehicle from gestational day 10 to postnatal day 14. The male offspring were evaluated using a battery of behavioral tests, including an appetite-motivated maze test (MAZE test) used to assess spatial learning and memory. In the MAZE test, times to reach goal (food) for both groups treated with NP were significantly shorter than those for the control group. In other behavioral tests (the open-field, elevated plus-maze, and step-through passive avoidance tests), NP did not affect any of each behavioral parameter. Thus, this study indicates perinatal exposure to low-dose NP specifically improves spatial learning and memory in male rat offspring.

Introduction

4-Nonylphenol (NP) is an endocrine disruptor with weak estrogenic activity and is a degradation product of nonylphenolethoxylate (NPE), which is used in industrial detergents, emulsifiers, and wetting agents. Many studies have reported that the degradation of NPE by bacteria in the environment generates short-chain NPE and NP, and these metabolites were found in the environment (1–4). The levels of NPE metabolites in the environment may be sufficient to disrupt endocrine function in wildlife and humans; studies have been concerned on the effects of NPE metabolites.

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(Received on March 14, 2014)

A number of studies have shown that NP can affect the reproductive, endocrine, and immune systems. For example, exposure to 30–100 and 100–350 mg/kg/day NP for several generations has accelerated vaginal opening, disrupted the estrogen cycle, decreased sperm number, and altered kidney and liver structures in rats (5). In addition, it has been reported that exposure to 50 mg/kg NP for several generations also affect the reproductive capacity in rat offspring (6).

Prenatal exposure to 80 and 200 mg/kg/day NP decreases all of the following: sperm counts in the tail of the epididymis, daily testicle sperm production, sperm activity, and normal sperm rate in male rat offspring (7). In addition, gestational exposure to NP affects body weight and some reproductive organ weights in the offspring of rats (7) and mice (8). Prenatal exposure to 80 and 200 mg/kg/day NP can affect the normal development of immune organs and inhibit the function of immune cells in male rat offspring (9). The neonatal treatment of male pups with NP reduced their reproductive capacity (10). However, some previous studies did not show any effect on the reproductive tract after neonatal exposure to NP (11, 12).

Several studies have addressed the effects of NP on the central nervous system (CNS). For example, prenatal exposure to 200 mg/kg/day NP impaired neurobehavioral development, as well as learning and memory in male rat offspring (7). Further, chronic administration of 100 and 200 mg/kg/day NP impaired the learning and memory and exploratory activity of male mice (13). It has also been shown that joint prenatal exposure to NP and estradiol might induce nervous development impairment in male rat offspring (14). However, perinatal exposure to NP did not affect the locomotor activity in the open-field test and running wheel activity in male rat offspring, when pregnant Sprague-Dawley (SD) rats consumed diets containing 25, 500 and 2000 ppm NP (15). Furthermore, perinatal exposure to 0.1 and 10 mg/kg/day NP did not affect the behavioral characteristics of male rat offspring, but it did affect their response to aversive stimulus and their monoaminergic neural pathways (16). These inconsistencies in the effects of NP on the reproductive system and CNS may be related to the dosage of NP used and the duration of NP treatment.

Bisphenol A (BPA) is also one of the most common environmental endocrine disruptors, and it has a very weak estrogenic activity. Several studies have reported that BPA has effects on the development and function of the CNS (17–19). Our previous study reported that perinatal exposure to a low-dose of BPA, but not to a high-dose of BPA, impaired spatial learning and memory without significantly changing the locomotor activity level of male rat offspring (20).

As above, the NP dose is almost higher than 10 mg/kg when NP affects reproductive system (5–8) and CNS (7, 13, 14) in rodents, and the effects of perinatal exposure to low-dose NP have not yet been clarified. Our previous study indicated that perinatal exposure to low-dose BPA impaired spatial learning and memory performance. Therefore, this study was designed to evaluate the effects of perinatal exposure to low-dose NP on learning and memory performance, the general activity and emotionality in male SD rats.

Materials and methods

Animals and treatments

Pregnant SD rats, at gestational day (GD) 6, were purchased from Kyudo Corp. (Saga, Japan). The animals were maintained under controlled room temperature (22±2°C), relative humidity (55±10%) and 12-h light:12-h dark cycles (lights on from 07:00 to 19:00). Food and water were freely available. NP at 1 or 10 mg/kg/day (Kanto Chemical Co., Inc., Tokyo, Japan) or vehicle was orally administrated to dams from GD 10 to postnatal day (PND) 14. NP was dissolved in corn oil. Oral administrations were performed after the rats were lightly anesthetized using halothane (Fluothane; Takeda Pharmaceutical Co., Ltd., Tokyo, Japan).

The pups in each litter were counted on PND 1 (the day of birth) and randomly culled to 10 pups on PND 3. This study did not evaluate the effects of perinatal

exposure to low-dose NP in female rat offspring. Therefore, male offspring were left as much as possible and the numbers of female offspring were regulated to total 10 pups in each litter. Offspring were weaned on PND 20 and successively kept. They were housed with a same-sex sibling after age 4 weeks. On the basis of perinatal exposure, the pups either belonged to the control, 1 mg/kg NP, and 10 mg/kg NP groups. At 6 weeks of age, male offspring were randomly selected from each litter and assigned to an appetite-motivated maze test (MAZE test) or other behavioral tests (the open-field, elevated plus-maze and step-through passive avoidance tests). The ratio of male offspring derived from each dam was same as much as possible. Six male offspring from each group were used for MAZE test, and the other six male offspring from each group were used for other behavioral tests. For the group of male offspring used in the MAZE test, the food was restricted to 12 g/day and water to 33.3 mL/day from 6 weeks of age to enhance their motivation for the rewards (condensed milk 20 g/100 mL water) used in the MAZE test. On the other hand, food and water were freely available for the male offspring used in the other behavioral test. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University, with the approval of the Institutional Animal Care and Use Committee.

Open-field test

The general behavior and emotionality of the rats were observed using the open-field test, as described by Hall (21). The open-field apparatus consisted of a gray circular floor, 60 cm in diameter, surrounded by 50-cm-high walls. The floor was divided into 19 equivalent sectors using black lines (Fig. 1). The form of divisions was slightly different, however dimension is about equal. The open-field was illuminated by a 100-W bulb placed 80 cm above the center of the floor and was divided into two regions: an outer ring (0–12 cm from the wall) and an inner ring (12–30 cm from the wall). Each rat was placed in the center of the open-field and was observed for 3 min. The total number of sectors crossed by the rat (ambulation), the number of line crossings inside the inner circle (inner circle movement), and the number of times each rat stood on its hind legs (rearing) were recorded to evaluate the general behavior and emotionality of the rats. Behavioral observations were performed three times at 2-h intervals. Seven-week-old rats were used for the open-field test.

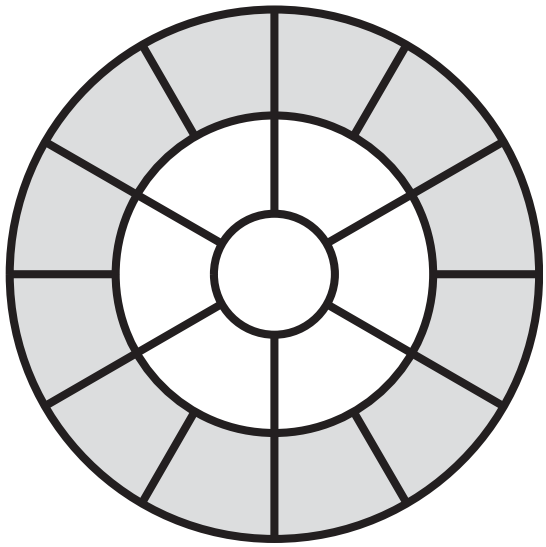

Fig. 1 : The bottom view of the open-field apparatus. White and gray segments represent the inner ring and outer ring, respectively. Open-field test were performed to evaluate general activity and emotionality in male offspring.

Elevated plus-maze test

Emotional behavior was tested using the elevated plus-maze test (22). A maze in the form of plus sign was elevated 60 cm above the floor, and the test was performed under bright light conditions. One set of opposing arms was enclosed completely by 60-cm-high walls, while the other set of opposing arms had no walls. The elevated plus-maze consisted of two open (50 × 10 cm) and two closed arms (50 × 10 × 60 cm), with all the arms connected by a central platform (14 × 14 cm). At the beginning of the test, each rat was placed in the center of the maze facing an open arm. Entries into and time spent in the open and closed arms were recorded for 5 min. The time spent in the open arms and the numbers of entries into the open arms were used as measures of anti-anxiety-like behavior. In this task, higher values indicate lower levels of anxiety. Seven-week-old rats were used for the elevated plus-maze test.

MAZE test

The MAZE test was used to assess spatial learning and memory. The apparatus and the experimental procedures were adopted from our previous study (20). The maze was constructed by inserting partitions of various sizes (50 × 15 cm, 50 × 30 cm, 50 × 45 cm, 50 × 60 cm) into a large compartment (90 × 90 × 50 cm) with an attached goal partition (15 × 15 × 50 cm). The apparatus was illuminated using three 100-W bulbs placed 100 cm above the floor and four different cues were placed on the walls.

We used three types of MAZE, each of which had different levels of difficulty (Fig. 2). In each MAZE test, the route necessary to reach the goal was more complicated than the previous (MAZE (A) → MAZE (B) → MAZE (C)). MAZE (A) was performed at 8-week-old rats, MAZE (B) was performed at 10-week-old rats, and MAZE (C) was performed at 12 -week-old rats. The procedure of the MAZE test followed the order: habituation → training → testing. The rats were habituated to the apparatus and the reward at 7 weeks of age (habituation), and habituation was performed for 3 consecutive days. For every MAZE test, the rats were first trained the correct approach by using an apparatus that only had the correct approach (training). The rats were subsequently tested for 3 consecutive days from the day after training (testing): each rat was placed gently in the maze and allowed to find the goal and get the reward.

For training, the rats underwent three trials in a single day with a 1-min inter-trial interval. During training, if the rat did not reach the goal within 180 s, an experimenter guided the rat to the goal. For testing, the rats underwent three trials every day for 3 days, with a 1-min inter-trial interval. During testing, if the rat did not reach the goal within 300 s, an experimenter guided the rat to the goal. The recorded behavioral parameters were time-to-goal and error. Time-to-goal was defined as the latency required to reach the goal and to start consuming the reward. Error was defined as the number of entries into an incorrect area of the maze. Both parameters were recorded until rats get the reward or become a time limit, and used as measures of spatial learning and memory.

Step-through passive avoidance test

Fear-motivated learning and memory was measured using the step-through passive avoidance test (23). The passive avoidance apparatus (Shintecno Co., Ltd., Fukuoka, Japan) consisted of a small light chamber (10 × 20 × 12 cm) and a large dark chamber (30 × 30 × 30 cm) connected by a path (8 × 8 cm). The two chambers were separated by a guillotine door, and a grid floor elicited an electric current.

The step-through passive avoidance test was

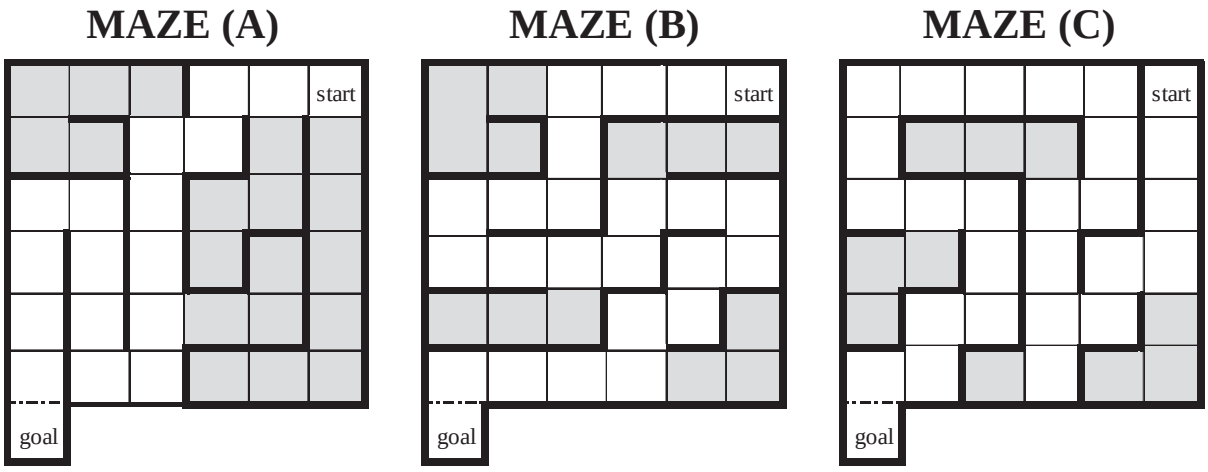

Fig. 2 : MAZE apparatus. In each MAZE test, the route necessary to reach the goal was more complicated than the previous (MAZE (A) → MAZE (B) → MAZE (C)). All MAZE test were performed to evaluate spatial learning and memory in male offspring. White and gray segments represent the correct approach and error areas, respectively.

performed for 3 consecutive days, and 11-week-old rats were used. On day 1, each rat was gently placed in the light chamber, and the door between the two compartments was opened 10 s later. Rats were allowed to enter the dark chamber for 90 s (acclimation). On day 2, each rat was gently placed in the light chamber and the door between the two compartments was opened 10 s later. Acquisition time, defined as the latency to enter the dark chamber, was measured. Upon entering the dark chamber, the door was closed and an electric shock (1 mA for 5 s) was delivered through the grid floor by using a shock generator (MSG-001; Toyo Sangyo Co., Ltd., Toyama, Japan). On day 3, each rat was gently placed on the light chamber and the door between the two compartments was opened 10 s later. The retention time, defined as the latency to enter the dark chamber, was measured. The upper time limit for entry was set at 300 s.

Statistical analysis

All data are expressed as means±S.E.M. Statistical significance between groups was analyzed using one or two-way analysis of variance (ANOVA) by using post-hoc Dunnett's multiple comparison tests (Stat View, SAS, Cary, NC, USA). One-way ANOVA was performed in elevated plus-maze test and step-through passive avoidance test, and two-way ANOVA was used to analyze the behavioral parameters in the MAZE test and open-field test were analyzed using. The threshold for statistical significance was set at $P<0.05$.

Results

Effect of Perinatal Exposure to NP on Performance in the Open-Field Test

For the control group (n=6), ambulation, rearing, and inner circle movement values gradually decreased over time (Fig. 3).

Ambulation values declined less rapidly in the 1 mg/kg NP group (n=6) than those observed in the control

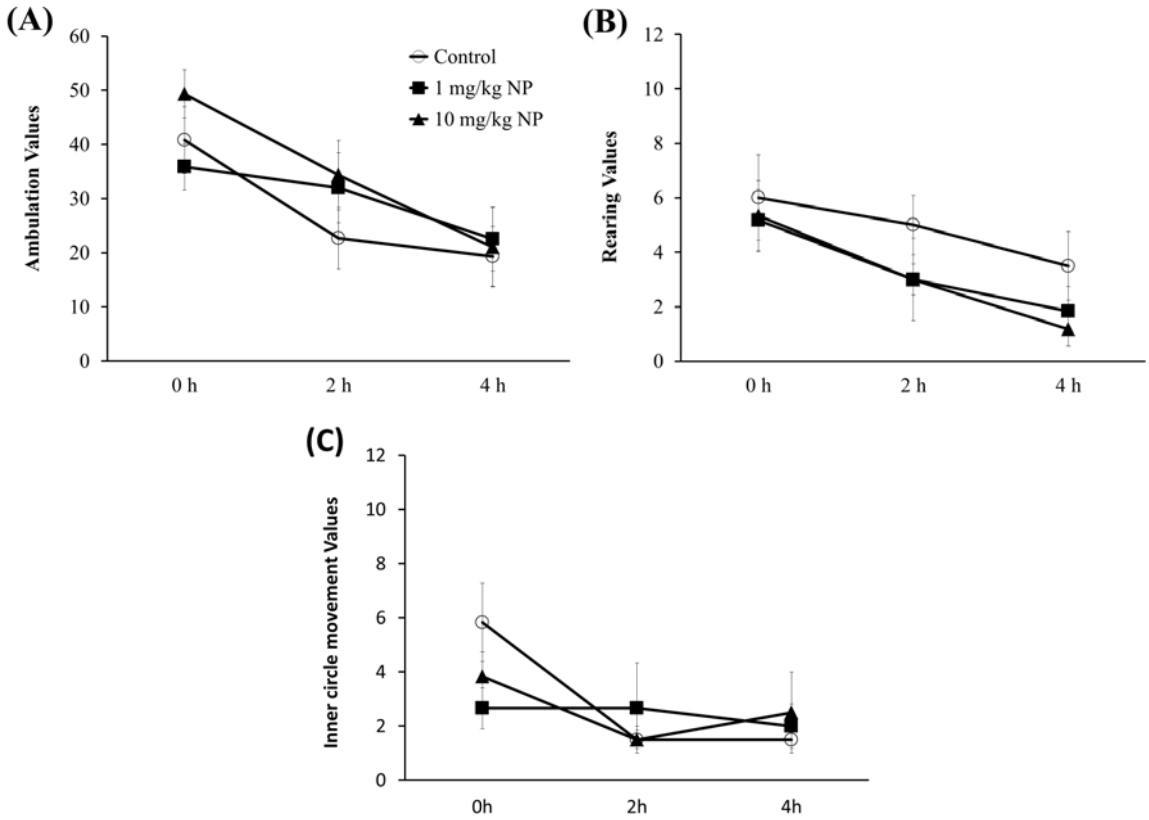

Fig. 3 : The effects of perinatal exposure to NP on (A) Ambulation (the total number of sectors crossed in the arena), (B) Rearing (number of times a rat stood on its hind legs), and (C) Inner circle movement (the number of line crossings inside the inner circle) during the open-field test in 7-week-old male rat offspring. Dams were treated with oral NP administration at 1 or 10 mg/kg/day or vehicle from GD 10 to PND 14. The results are expressed as the mean±S.E.M. (n = 6 per group).

group. However, no significant differences were observed between the two groups (Fig. 3A). In the 10 mg/kg NP group (n=6), ambulation values were slightly higher than those in the control group during early tests (0 h and 2 h), and there were no significant differences compared to the control group (Fig. 3A). Compared to the control group, the 1 mg/kg NP groups showed lower rearing values during later tests (2 h and 4 h). However, the differences were not significant when compared to the control group (Fig. 3B). In the 10 mg/kg NP group, rearing values were lower than those observed in control group during later test. However, none of differences were significant compared to the control group (Fig. 3B). The 1 mg/kg NP group showed lower inner circle movement values compared to the control group during the initial test (0 h). However, no significant difference was observed between the two groups (Fig. 3C). The 10 mg/kg NP groups also had lower inner circle movement values compared to the control group during the initial test (0 h). However, the differences were not significant (Fig. 3C).

Effect of Perinatal Exposure to NP on Performance in the Elevated Plus-Maze Test

Compared to the control group (n=6), the number of open arm entries in both of the NP groups (n=6 each) were not significantly different (Fig. 4A). The number of closed arm entries in the 1 mg/kg NP groups was slightly lower than those in the control group. However, the differences was not significant when compared to the control group (Fig. 4A). The 10 mg/kg NP group also showed slightly lower the number of closed arm entries compared to the control group. However, no significant difference was observed between the two groups (Fig. 4A). The times spent in both the arms in the 1 and 10 mg/kg NP groups were not significantly different from those in the control group (Fig. 4B).

Effect of perinatal exposure to NP on performance in the MAZE test

In the control group (n=6), the time-to-goal did not change in the MAZE (A) test with each consecutive day of testing. However, in the MAZE (B) and (C) tests, the time-to-goal decreased with each consecutive day of testing. In the 1 mg/kg NP group (n=6), the time-to-goal was significantly shorter than that observed in the control group for all three difficulties of the MAZE test ($P<0.01$ on days 2 and 3 in the MAZE (A) test, day 1 in the MAZE (B) test, and for the entire testing duration in the MAZE (C) test; $P<0.05$ on day 3 in the MAZE (B) test; Fig. 5A). In addition, the 1 mg/kg NP group had a significantly lower time-to-goal than that observed in the 10 mg/kg NP group (n=6) on day 3 in the MAZE (A) test ($P<0.01$; Fig. 5A). In the 10 mg/kg NP group, the time-to-goal was significantly shorter than that in the control group in the MAZE (B) and (C) tests ($P<0.01$ on day 1 in the MAZE (B) and (C) tests;

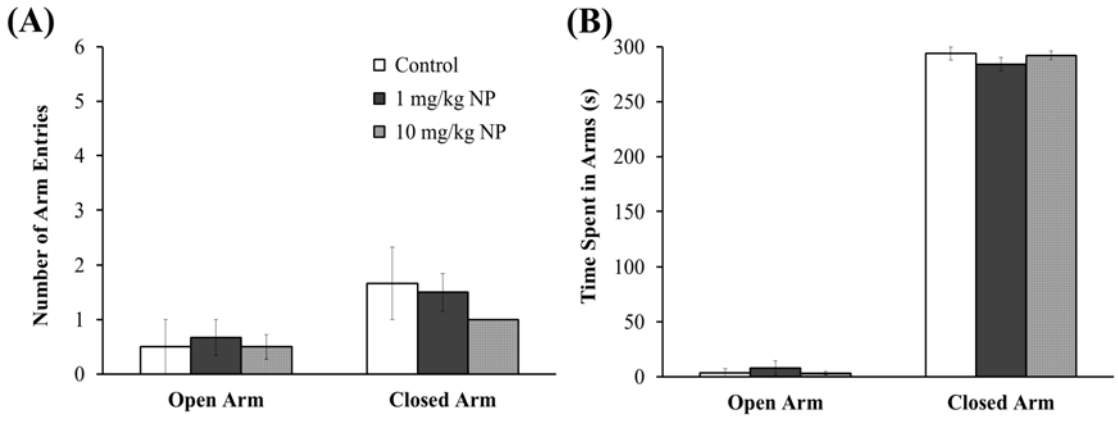

Fig. 4 : The effects of perinatal exposure to NP on (A) the number of entries to an arm and (B) the time spent in each arm during the elevated plus-maze test in 7-week-old male rat offspring. Dams were treated with oral NP administration at 1 or 10 mg/kg/day or vehicle from GD 10 to PND 14. The results are expressed as the mean±S.E.M. (n = 6 per group).

P<0.05 on day 3 in the MAZE (B) test, and days 2 and 3 in the MAZE (C) test; Fig. 5A). In the MAZE (A) test, error for the control group decreased on day 2 and increased slightly on day 3. However, in the MAZE (B) and (C) tests, error for the control group gradually decreased with each consecutive day of testing (Fig. 5B). Compared to the control group, the 1 mg/kg NP group showed a slightly higher error on day 2 in the MAZE (A) test, days 1 and 2 in the MAZE (B) test, and day 2 in the MAZE (C) test; however, the differences were not significant (Fig. 5B). For the 10 mg/kg NP group, error was slightly higher than those observed in the control group on days 2 and 3 in the MAZE (A) test. However, there were no significant differences when compared to the control group (Fig. 5B).

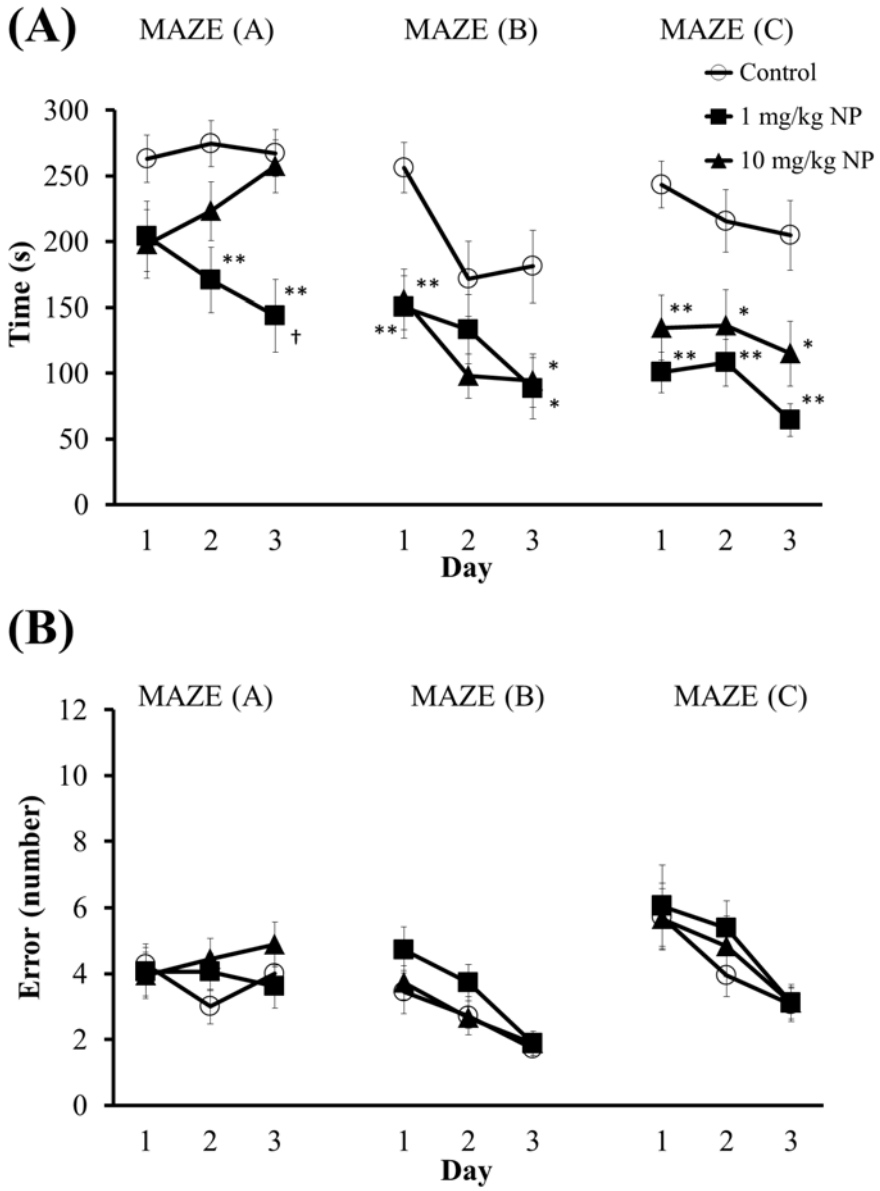

Fig. 5 : The effects of perinatal exposure to NP on (A) Time-to-goal, defined as the latency required to reach the goal and start eating the reward, and (B) Error, defined as the number of entries into the incorrect area, in the MAZE test in 8-, 10-, and 12-week-old male rat offspring. Dams were treated with oral NP administration at 1 or 10 mg/kg/day or vehicle from GD 10 to PND 14. The results are expressed as the mean±S.E.M. (n = 6 per group). *P<0.05 and **P<0.01 indicate significant differences from the control rats. †P<0.01 indicates significant differences from the 10 mg/kg/day NP group rats.

Effect of perinatal exposure to NP on the performance in the step-through passive avoidance test

During the training session for the step-through passive avoidance test, the 1 mg/kg NP group (n=6) showed slightly shorter latency compared to the control group (n=5). However, no significant difference was observed between the two groups (Fig. 6).

In the 10 mg/kg NP groups (n=6), latency was slightly shorter than those in the control group during the training session. However, the difference was not significant compared to the control group (Fig. 6).

Compared to the control group, the 1 mg/kg NP group had a slightly shorter latency during the testing session. However, no significant difference was observed between the two groups. In the 10 mg/kg NP group, latency was not altered during the testing session. Therefore, the rats perinatally exposed to NP did not show any significant change in retention (24 h after foot shock) (Fig. 6).

Discussion

This study investigated the effects of perinatal exposure to low-dose of NP, an estrogen-related endocrine disruptor, on learning and memory performances, general activity, and emotionality in male rat offspring. Dams were orally administered NP or vehicle from GD 10 to PND 14, which is a critical and sensitive period for CNS development in the offspring. The “no observed adverse effect level” (NOAEL) of NP in reproduction is reported to be 10 mg/kg/day in the next generation (6). Thus, to evaluate the effects of NP, doses of 10 and 1 mg/kg/day, which is one-tenth of the NOAEL dose, were used. Dams were orally administered at NP (1 or 10 mg/kg/day) or vehicle from GD 10 to PND 14. Doerge DR, et al. reported that NP transferred to fetal brain and serum through the placenta after oral NP administration to pregnant rats (24). It was reported that maternally injected NP may be transferred to neonates through breast milk in rat (25). In addition, some studies reported that NP was detected in human breast milk (26, 27). Therefore, we supposed

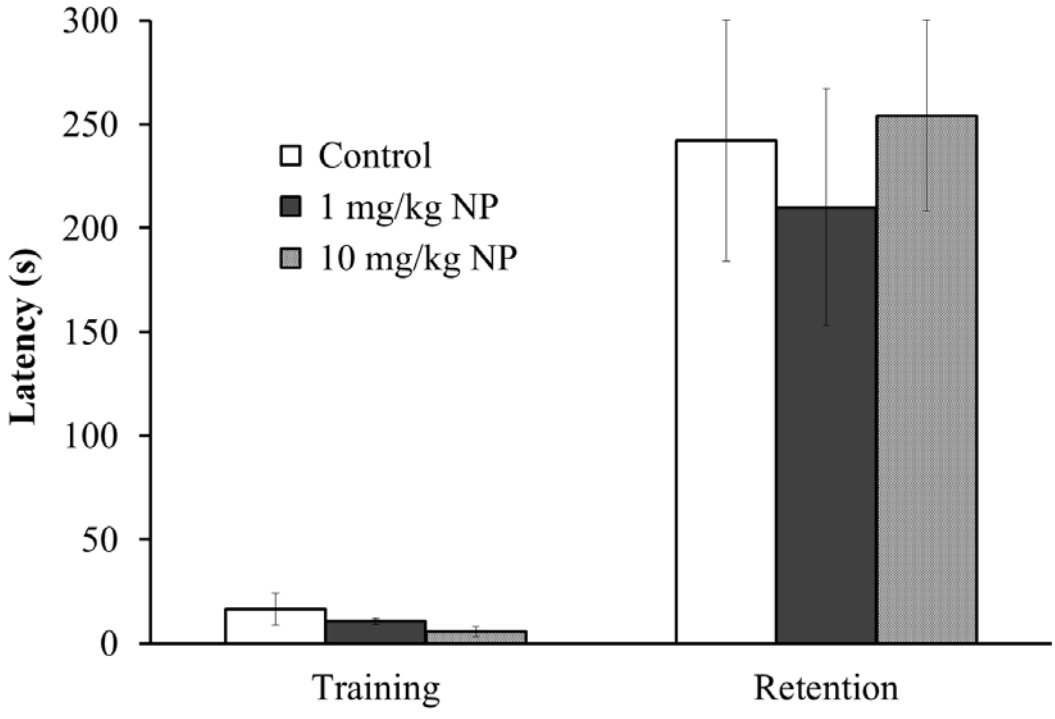

Fig. 6 : The effects of perinatal exposure to NP on the latency to enter the dark chamber in the step-through passive avoidance test in 11-week-old male offspring rats. Dams were treated with oral NP administration at 1 or 10 mg/kg/day or vehicle from GD 10 to PND 14. The results are expressed as the mean±S.E.M. (controls: n = 5 and NP groups: n = 6 each).

the offspring were exposed to NP via placenta or milk in this study.

The present study demonstrated that perinatal exposure to 1 and 10 mg/kg/day of NP improved spatial learning and memory in male offspring (Fig. 5). This improvement in spatial learning and memory was considered specific because locomotor activity (measured in the open-field test), emotionality (measured in the elevated plus-maze test), and fear-motivated learning and memory (measured in the step-through passive avoidance test) were not affected in the male offspring (Figs. 3, 4, and 6). This is the first study to show that perinatal exposure to NP improves spatial learning and memory in male rat offspring.

Three types of MAZE test with progressive levels of difficulty were used in this study. The time-to-goal and error for the control group changed very little over the duration of MAZE (A) test; however, the time-to-goal and error decreased with each consecutive day of testing in MAZE (B) and (C) tests. The results indicate that control offspring have good learning performance. Time-to-goal in the 1 mg/kg NP group was significantly shorter than in the control group in the MAZE test (Fig. 5A). Thus, the results from this study suggest that perinatal exposure to 1 mg/kg/day NP improves spatial learning and memory in male rat offspring. The time-to-goal in the 10 mg/kg NP group was also significantly shorter than in the control group (Fig. 5A). The results suggest that a dose of 10 mg/kg/day NP also improves spatial learning and memory; however, with slightly weaker efficacy than that by the 1 mg/kg/day NP dose. Thus, our results show that perinatal exposure to low-dose NP affects the spatial learning and memory performance in male rat offspring.

In our previous study, we discussed the non-monotonic dose-response relationship between spatial learning and memory and perinatal BPA exposure (20). Hormones and agents like environmental endocrine disruptors tend to display non-monotonic dose-response relationships, such as those indicated by U-shaped or inverted U-shaped curves (28–32). The current results of NP exposure are consistent with the results of the above studies. Jie et al. (7) reported that 200 mg/kg/day NP exposure during the embryo organogenesis period caused changes to the neuronal ultra-structure of the hippocampus and reduction in learning and memory in male rat offspring in a water maze test; however, 50 and 100 mg/kg/day NP did not affect learning and memory performance. Thus, it might be considered that the effect of NP on improving spatial learning and memory weakened gradually as the dosage increased, and finally, an excess amount of NP, in this case 200 mg/kg/day, impaired spatial learning and memory.

In this study, we showed that exposure to NP caused an improvement in spatial learning and memory. In contrast, our previous study showed that BPA impaired spatial learning and memory (20). Many studies have shown that estrogen improves learning and memory (33-35), has neuroprotective effects (36-38), enhances spine and long-term potentiation (35, 39-41), and plays an important role in learning and memory. It is well known that the estrogenic activity of NP is stronger than that of BPA. A study has shown that the estrogenic activity of NP is equal or higher than that of BPA in both *in vitro* and *in vivo* assays (42). Additionally, Nishihara et al. (43) reported that the estrogenic activity of NP (the concentration showing 10% activity of 10^{-7} M 17β-estradiol is 2×10^{-7}) is higher than that of BPA (3×10^{-6}) as measured using a yeast two-hybrid assay. Moreover, the relative binding affinity of NP to the estrogen receptor (ER; mean IC_{50} in the ER competitive-binding assay is 2.40×10^{-6}) is higher than that of BPA (1.17×10^{-5}) (44). As described above, the estrogenic activity and ER relative binding affinity of NP are higher than that of BPA. Furthermore, a previous study showed that 10-100 nM BPA significantly enhanced long-term depression (LTD) in both the CA1 and CA3 areas of the hippocampus but suppressed LTD in the dentate gyrus (DG). However, 100 nM NP suppressed LTD by approximately 10% in the CA1 area but enhanced LTD in CA3 and DG (41). The effects of NP and BPA on LTD in CA1 and DG are opposite, and it has been proposed that the hippocampal formation is very important for the processing of certain aspects of spatial learning and memory (46). Therefore, we suggest that these differences between NP and BPA

might have different effects on spatial learning and memory in male rat offspring. Therefore, the effects of perinatal exposure to low-dose NP on brain development might cause an improvement in spatial learning and memory. In addition, Corrieri et al. (47) showed that perinatal exposure to 17β-ethynylestradiol, a xenoestrogen, enhanced spatial learning and memory in male rat offspring. Thus, we suppose that the estrogenic activity of NP might contribute to improving spatial learning and memory performance.

The step-through passive avoidance test measured fear-motivated learning and memory of male rat offspring. A short latency before entering the dark chamber during the retention trial suggested impairment of fear-motivated learning and memory. Exposure to NP did not significantly alter retention; thus, perinatal exposure to NP did not affect fear-motivated learning and memory in male rat offspring (Fig. 6). A previous study showed that perinatal exposure to NP did not affect behavioral characteristics in the step-through passive avoidance test (16).

In the open-field test and the elevated plus-maze test, no significant differences were observed between the control and NP groups (Figs. 3, 4). Therefore, perinatal exposure to NP did not affect general behavior and emotionality. Our results are consistent with those of other studies (15, 16).

In conclusion, perinatal exposure to low-dose NP specifically improves spatial learning and memory without changing the general activity, emotionality, or fear-motivated learning and memory in male rat offspring. Notably, the effect of 1 mg/kg/day NP, a tenth of the NOAEL dose, was effective. Therefore, the CNS may be affected by low-dose NP that does not affect the reproductive system. The present results strongly suggest that the CNS is more sensitive to perinatal NP exposure than is the reproductive system in male rat offspring.

Acknowledgments

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (22510069).

Conflicts of interest

The authors indicated no potential conflicts of interest.

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