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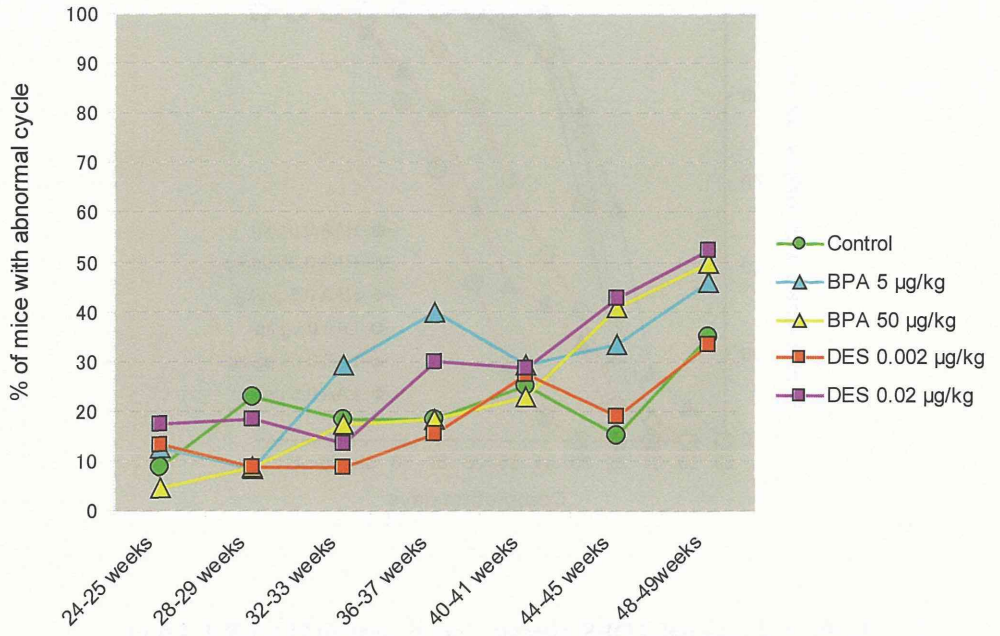

Fig. 1 Effects of neonatal BPA and DES exposure on estrous cycle in C57B/6J mice

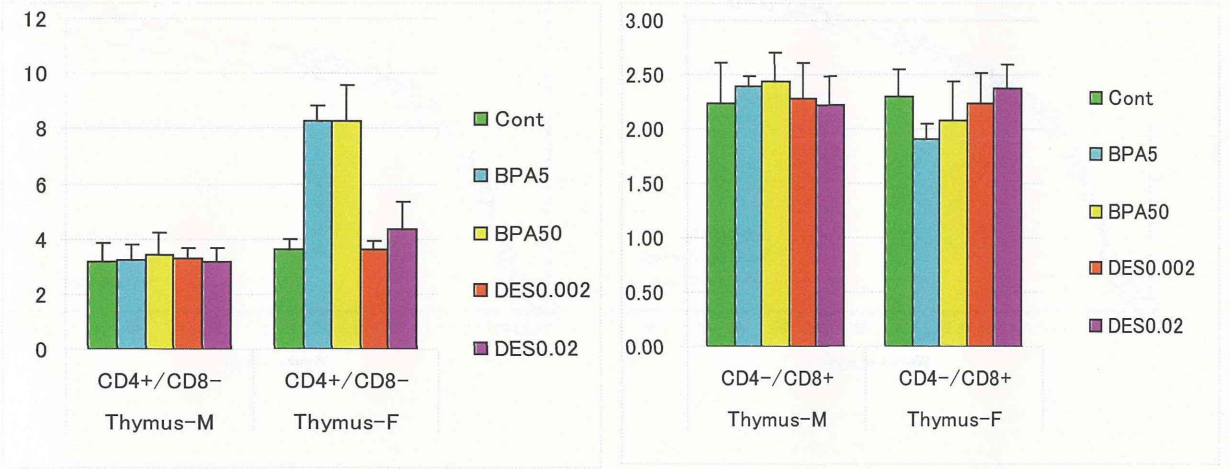

Fig. 2 Effects of neonatal BPA and DES exposure on T cell differentiation in C57B/6J mice

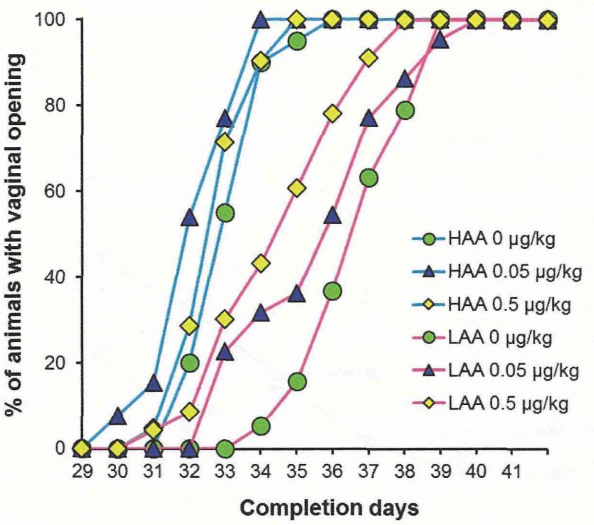

Fig. 3 Effects of neonatal DES exposure on puberty in HAA & LAA rats

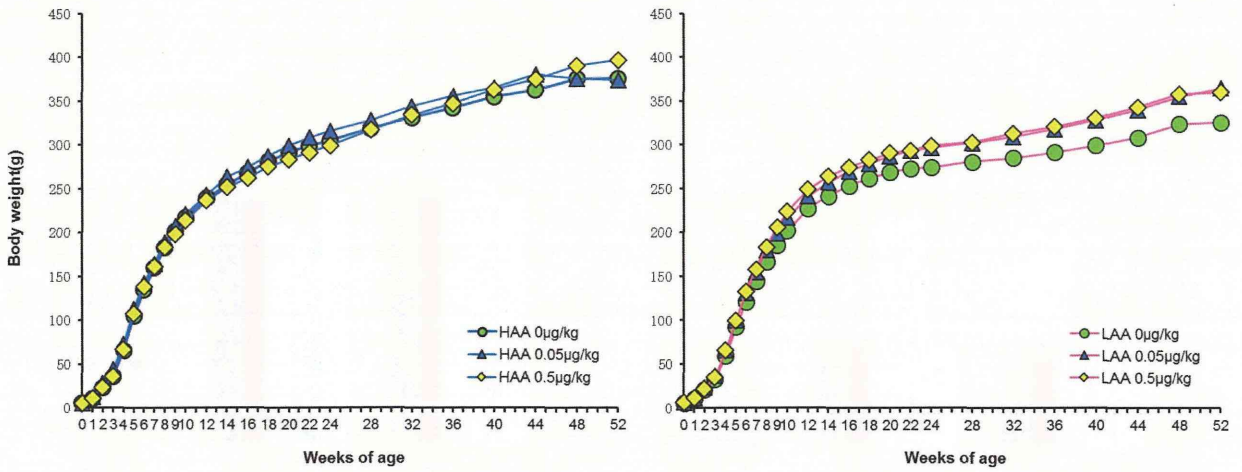

Fig. 4 Effects of neonatal DES exposure on body weight change in HAA & LAA rats

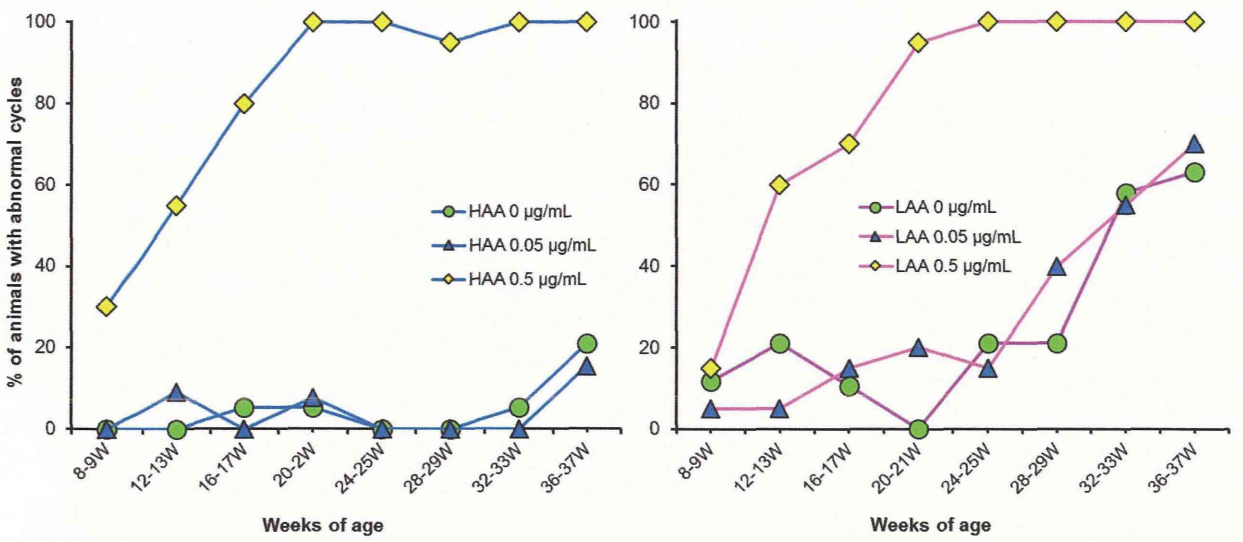

Fig. 5 Effects of neonatal DES exposure on estrous cycle in HAA & LAA rats

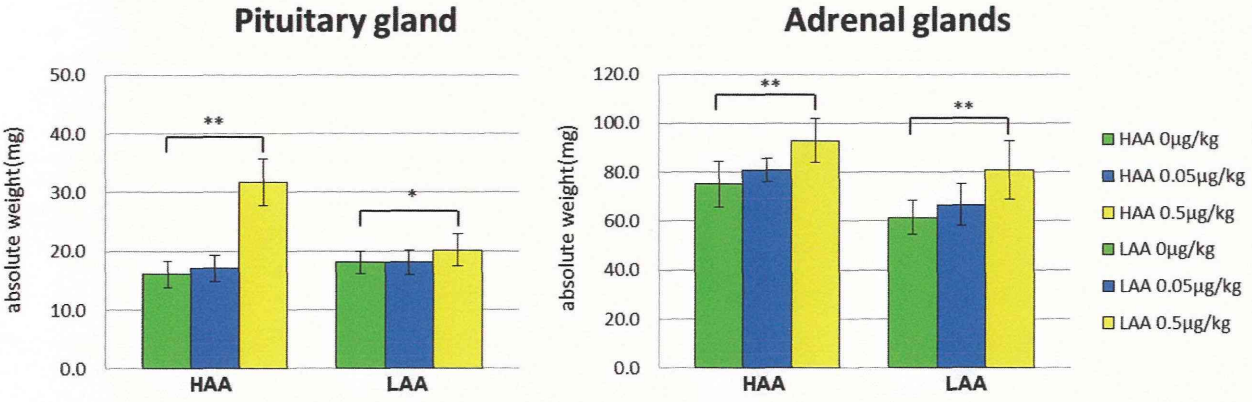

Fig. 6 Effects of neonatal DES exposure on weights of pituitary gland and adrenal glands in HAA & LAA rats

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Ⅲ. 研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷り

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Letter

Neurobehavioral evaluation of mouse newborns exposed prenatally to low-dose bisphenol A

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ABSTRACT — There have been few neurobehavioral toxicology studies on newborn animals. Thus, we developed a mouse newborn behavioral testing method for evaluating the risk of neurotoxicity of environmental toxicants, by means of determining the newborn's motor activity applying the tare function of an analytical balance. Motor activities including crawling, pivoting, righting or tremors of mouse newborns were evaluated. Tremors of newborns of dams exposed to bisphenol A at 2, 20 or 200 μg/kg/day on days 5 through 18 of gestation were significantly increased when evaluated on postnatal day 1, as well as those of newborns exposed prenatally to diethylstilbestrol at 0.5 μg/kg/day. We suggest that our developed testing method may provide a useful addition to neurobehavioral assessment in very young rodents exposed to environmental hormone mimics.

Key words: Behavioral assessment, Bisphenol A, Tremor, Mouse newborn

INTRODUCTION

Studies with rodents show that prenatal exposure to some neurotoxicants adversely affects neonatal orientation, attention and motor maturity, as well as activity level, executive function, response inhibition and sensory processing later in life (Schneider *et al.*, 2011). Although there have been a vast number of animal toxicological studies carried out on pregnant animals including embryos/fetuses and mature animals, there is a paucity of reports on animal toxicology studies utilizing newborn animals a few days after birth. To evaluate the neurotoxic effects of chemicals on newborns in rodents, we very recently developed a mouse newborn neurobehavioral testing method; it involves quantitative determination of a newborn animal's activity automatically using the tare function of an analytical balance. We have demonstrated that developmentally 10 mg/kg methylnitrosourea-treated ICR mice showed increases in motor behavior including crawling, pivoting, righting or tremors when estimating this activity by this testing method on postnatal day 1 (Nagao *et al.*, 2013).

Bisphenol A (BPA) is one of the environmental endocrine disruptors released by plastics and resin known to interfere with hormonal responses. During fetal life, the intrauterine environment is critical for normal development, and even small changes in the levels of hormones may lead to changes in brain histology and function, and consequently in behavior. Nuclear estrogen receptor binding assays indicate that BPA has at least a 10,000-fold lower affinity for the two estrogen nuclear receptors than 17β-estradiol. In isolation, these results would suggest that BPA at environmentally relevant levels of exposure would not pose a public health problem (Zoeller *et al.*, 2012). However, low doses of BPA administered perinatally can modify explorative behavior and anxiety in rat (Fujimoto *et al.*, 2013). It has been shown that perinatal BPA exposure disrupted sexually dimorphic behavior in the postnatal developmental period and in adult mice, when evaluated by the elevated plus maze test and the open field test (Gioiosa *et al.*, 2013; Nakamura *et al.*, 2012). In addition, mice treated with BPA prenatally were hyperactive, like attention-deficit hyperactivity disorder (ADHD), in these behavioral tests. The mode of action underlying these effects is unclear. Recently, we demonstrated that histological changes of the cortical plate were found in mouse fetuses exposed to a low dose of BPA (Komada *et al.*, 2012), and that prenatally BPA-treated mice showed hyperplasia of layer 6b and abnormal neuronal distribution in the neocortex of newborn (our unpub-

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lished data). Thus, the purpose of this study is to clarify the effect of prenatal low-dose BPA exposure on the neurobehavior of mouse newborns.

MATERIALS AND METHODS

Animals and housing

ICR mice purchased from CLEA (Osaka, Japan) were used. The experimental protocols were approved by the Animal Care and Use Committee of Kinki University. Mice were kept under SPF conditions and a constant light-dark cycle (dark period from 7:00 pm to 7:00 am) at 24 ± 1°C and 55 ± 5% relative humidity. Food (CE-2, CLEA, Osaka, Japan) and drinking water were available *ad libitum*. Water was available via glass bottles with Teflon seals during the experimental period. Pregnant mice were housed individually throughout the study in polypropylene plastic tubs with stainless steel lids and corncob bedding. Ten-week-old mice were allowed to copulate overnight at a 1:1 female to male ratio. The next morning, females with vaginal plugs were regarded as pregnant, and the day of gestation was designated as embryonic day (E) 0. Pregnant mice were allowed to give birth. The day of birth was designated as postnatal day (PND) 0. Pups were weighed on PND1.

Chemicals, dose selection and treatment regimen

Bisphenol A (BPA, Sigma-Aldrich, St. Louis, MO, USA) was suspended in 0.5% CMC-Na and four to five pregnant mice in each group were administered BPA at 2, 20 or 200 μg/kg/day by oral gavage on E6 through E18. The dose solution was prepared once per 5 days and analyzed prior to dosing. Administration occurred at a defined time (12:00 pm). In a previous study, we showed that hyperplasia of the cortical plate and the promotion of neurogenesis were identified in mouse fetuses exposed to BPA at 200 μg/kg/day (Komada *et al.*, 2012). Methylnitrosourea (MNU, Sigma Chemicals, St. Louis, MO, USA) was used as a potent developmental neurotoxicant in rodents, and dissolved in physiological saline immediately before use. Five pregnant mice received an intraperitoneal injection on E13 (12:00 pm) at a dose of 1 mg/kg because E13 is a part of the organogenesis period of the mouse neocortex and the peak of neurogenesis in the primordium (Komada *et al.*, 2010). We chose to expose an additional group of dams to diethylstilbestrol (DES, Sigma Chemicals) at 0.5 μg/kg. We chose this dose because the estrogenic potency of DES has typically been 100- to 1,000-fold times higher than that of BPA, and this dose of 0.5 μg/kg is 400 times lower than our high dose of BPA (Vom Saal and Welshons, 2006). Five pregnant mice were administered DES orally on E6 through E18. Eight untreated pregnant mice served as controls because statistical significances in various toxicological endpoints were not detected between the untreated and 0.5% CMC-Na-treated or physiological saline-treated mice (Komada *et al.*, 2010, 2012).

Determination of newborn activity using an electric balance

For the evaluation of motor activity, 3 male and 3 female newborns per dam were used. Recently, we developed a mouse newborn behavioral testing method for evaluating the risk of neurotoxicity of chemicals (Nagao *et al.*, 2013). Briefly describing this method, an electric balance (Tuning-fork analytical balance, HTR-80, Shinko Denshi Co., Ltd., Tokyo, capacity 80 g, readability 0.0001 g, repeatability (σ) 0.0001 g, interface RS232C, D-SUB9P) on a shock-proof stage was used to evaluate the absolute values obtained from the range of fluctuations between weighing values resulting from the movement (crawling, righting, pivoting, tremors specific to newborns) of newborns on PND 1 from 12:00 pm to 1:00 pm, based on the results in our preliminary study. The absolute value was defined as the activity of a newborn, and the total activity of a newborn was the sum total of the absolute values for 6 min. The large movements (crawling, righting and pivoting) and small movements (tremors) of newborns were defined as motor activities showing an absolute value of 0.0002 or more and an absolute value of 0.0001, respectively, based on the results of our preliminary studies. In those studies, the data on various types of activities detected in this behavioral testing method were analyzed and the difference between the weighing value and that 0.1 sec beforehand of the newborn showing tremors was found to be 0.0001. The unstable weighing values obtained by the movement of the newborn in the plastic dish (94/16, Greiner Bio-One GmbH, Austria) on the pan of the balance were recorded by a personal computer every 0.1 sec via WinCT (Windows Communication Tools) software (version 3.00, A&D Company Ltd., Tokyo, Japan). The plastic dish was exchanged for new one after each determination of newborn activity for 6 min. The possibility of reflection was checked using a fixed weight (1 g and 5 g) on the balance for 3 min before the measurement of newborn activity in order to ensure that the weights of newborns on the balance did not reflect drift in the value of the balance. The room in which the measurement was carried out was maintained under the same experimental conditions as the animal room.

Statistics

For the effects of BPA, the data on newborn activity were analyzed via two-way analysis of variance (ANOVA) with Treatment (BPA at doses of 2, 20 and 200 μg/kg/day vs. Control) as a factor. Whether the repeated measure ANOVA detected significant interactions or not, one-way ANOVA was followed by tests for simple main effects, and detailed multiple comparisons were made using Tukey's honestly significant difference post hoc tests, given corresponding significant F-values. For the effects of DES or MNU, the data were analyzed by the Student's t-test. Statistical evaluation of data was performed using JMP (version 9.0; SAS Institute Inc, Cary, NC, USA). All data used the litter average as the statistical unit, and statistical significance was assumed for probability levels of 0.05 or less.

RESULTS AND DISCUSSION

The doses of BPA (2-200 μg/kg/day) used in the present study were well below the published No Observed Adverse Effective Level (NOAEL) dose, namely, 5 mg/kg/day (NTP, 2001). There were no adverse changes in general conditions and spontaneous delivery of dams exposed to low-dose BPA, DES or MNU, and the numbers of live newborns on PND 0 in the chemically treated groups were comparable to those in the controls. Body weights of male and female newborns on PND 1 in the BPA-, DES- or MNU-treated groups were comparable to those in the controls (data not shown). As for the behavioral changes of the newborns on PND 1, the total activities (the sum total of the absolute values) for 6 min in the BPA-treated groups were comparable to those in the control group in both sexes. The ANOVA for males and females revealed main effects of treatment [$F_{3,19} = 0.094$, $p = 0.963$, and $F_{3,16} = 0.791$, $p = 0.517$, respectively]. No significant differences in the total activities were detected between the DES-treated group ($p = 0.065$ and 0.098, respectively) or MNU-treated group ($p = 0.062$ and 0.373, respectively) and the controls in males and females. Total absolute values of 0.0001 (tremors) for 6 min in males of 2, 20 and 200 μg/kg/day BPA-treated groups [$F_{3,17} = 25.060$, $p < 0.0001$] and in females of the 2 and 200 μg/kg/day BPA-treated groups [$F_{3,17} = 15.589$, $p < 0.0001$] were significantly increased compared with those in the control group. There were significant differences in tremors between the DES-treated group and the controls in both sexes ($p < 0.0001$). However, no significant difference was found between the MNU-treated group and the controls in male and female newborns ($p = 0.895$ and 0.928, respectively, Fig. 1).

The present study clearly demonstrated the increased

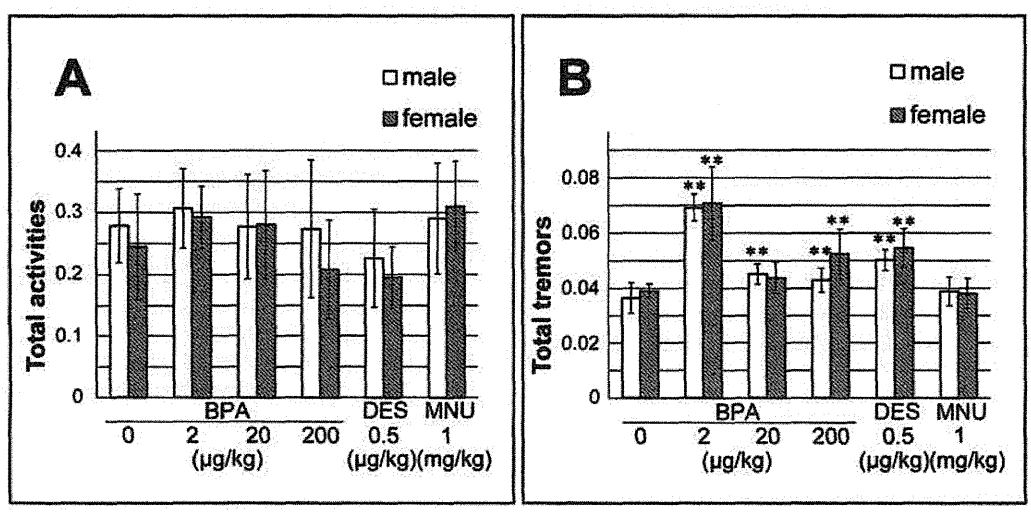

Fig. 1. Total activities (A) from crawling, pivoting and righting and total tremors (B) of PND 1 newborns from dams in the control group, the BPA-treated groups, the DES-treated group and the MNU-treated group. Vertical lines represent standard deviations. *Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
Total activities of a newborn was sum total of the absolute values for 6 min, and total tremors was sum total of the absolute values of 0.0001 (see Nagao *et al.*, 2013).

tremors in newborn infants prenatally exposed to BPA (2, 20 and 200 μg/kg/day) or DES (0.5 μg/kg/day), while no significant increase in large movements such as crawling, righting or pivoting was detected. Tremors have been reported in association with specific risk factors (such as maternal pathologies, maternal use of drugs or substance abuse, low birth weight or prematurity, electrolyte abnormalities, sepsis or brain lesions, or perinatal complications including low Apgar scores, as well as perinatal asphyxia) (Rosman *et al.*, 1984; Armentrout and Caple, 2001), but they have also been reported in two-thirds of low-risk term-born neonates (Parker *et al.*, 1990) during the first few days after birth. Tremors associated with neurological signs are generally thought to be part of a more complex neurological pattern, and are often associated with brain lesions or other risk factors (Futagi *et al.*, 1999). In the present study, the total activities or tremors of newborns exposed to MNU (1 mg/kg) on E13 were comparable to those in the controls, whereas a significant increase in the total activities was found in newborns exposed to MNU at a fairly high dose (10 mg/kg) (Nagao *et al.*, 2013). The discrepancy between the previous findings and the present ones may be attributed to the applied dose of MNU, because mouse newborns treated with MNU at 10 mg/kg on E13 showed microcephaly.

The motor behaviors of newborns are regulatory behaviors and are orchestrated and coordinated by the neocortex. It is considered that the increase in motor behaviors was caused by neocortex anomalies, for example, layer formation, neuronal positioning and projection. Layer anomalies of neocortex were identified in developmentally BPA-treated mouse newborns, suggesting that these anomalies might relate to the abnormal motor behaviors of newborns (our unpublished work). We will present these results elsewhere in the near future. In addition, we showed that the maternal BPA oral dosing was related to hyperplasia of the cortical plate during the development of mouse telencephalon (Komada *et al.*, 2012). Taking these findings together, it is reasonable to suggest that the increased tremors of newborns exposed *in utero* to BPA may be related to the cerebral cortex damage induced by this compound.

Our goal was to assess the developmental effects of BPA on motor activity at an early life stage in mice using a very low dose of BPA, with a range comparable to that in humans. The developed newborn behavioral testing technique is a practical approach to solve one of the challenges in assessing early effects of neurotoxicants and it may provide a useful addition to neurobehavioral assessment in very young rodents. As for the advantages of our method, it is considered that there is a possibility of early detection of neurobehavioral abnormalities/disorders and early medical treatment by means of the analyses of newborn activity by this testing technique, as well as the measurement of newborn activity without stress such as pain.

In conclusion, prenatal exposure to low-dose BPA led to early-stage motor hyperactivity in mouse newborns. Although the mechanisms of the hyperactivity in newborns exposed to BPA *in utero* remain unknown, it is tempting to speculate that BPA might underlie the recent increase in the number of children with neurobehavioral disorders including ADHD and autism, which is based on organic functional disorder of the central nervous system. As the next step of our neurobehavioral studies using this technique, the relationship between brain damage including layer abnormalities of the neocortex and the behavioral abnormalities of newborns should be clarified. However, it should be taken into account that the early stage of postnatal brain development in the mouse is congruent with the development of the brain in the human fetus in the later part of gestation.

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Long-term exposure of 3T3 fibroblast cells to endocrine disruptors alters sensitivity to oxidative injury

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Abstract

When Swiss 3T3 fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP)within a range of 0.1 nM to 100 nM for 30 ~ 45 days, increased resistance to oxidative injury was observed. Western blot analysis revealed concomitant increased expression of bcl-2 protein and reduced histone methylation levels in cells after BPA or NP exposure. Using a heterologous expression system, we found that both chemicals could stimulate G protein-coupled receptor 30 (GPR30), a transmembrane estrogen receptor predominantly expressed in 3T3 cells, at lower concentrations which showed increased survival effects. Taken together, these results suggest that BPA or NP exposure might cause alterations in cellular activity against oxidative stress possibly through GPR30.

Introduction

A wide variety actions of chemicals with potential endocrine disrupting activities have been reported on not only endocrine functions but also neuronal and immune functions *in vitro* and *in vivo* [1,2]. For examples, two representative endocrine disruptors, bisphenol A (BPA) and nonylphenol (NP), have been demonstrated to show various *in vitro* effects, including increased cell proliferation in cancer cells, enhanced differentiation of adipocytes, induction of cytokine production in immune cells, extracellular-regulated kinase phosphorylation in neural or pituitary cells, inhibition of cell death in neuronal cells (references in [2]).

These various effects of BPA or NP might be mediated by binding to estrogen receptors, ERα or ERβ, or estrogen-related receptor γ, ERRγ [3,4,5]. Indeed, BPA shows inhibition of estrogen binding to ERα and ERβ with a Ki of 195 and 35 nM, respectively, and higher binding affinity to recombinant ERRγ with a Kd of 5.5 nM. Recent findings further suggest that G protein-coupled receptor 30 (GPR30) or G protein coupled estrogen receptor 1 is a transmembrane estrogen receptor existing in plasma membranes as well as endoplasmic reticulum, and experimental evidence indicates that BPA and NP bind to GPR30 to activate cyclic AMP (cAMP) production [6,7,8]. Interestingly, endogenous estrogen is also known to show neuronal cell protection from oxidative injury through activation of cAMP pathway [9]. In addition to these receptor-mediated actions of BPA, BPA has been implicated to act on non-receptor target, such as DNA, leading to induction of DNA double strand breaks, or unidentified pathway, leading to neurotoxicity [10,11].

Recent research on endocrine disruptors has tended to focus on their actions at lower concentrations or doses as well as cellular mechanisms [12]. In the present

study, we examine whether long-term exposure to BPA or NP at lower concentrations induces the alterations of cell survival or responses to oxidative injury in non-neuronal cells. For this purpose, we employ Swiss 3T3 fibroblast cells, which are non-tumor cells and widely used in pharmacological and toxicological studies [13,14,15,16,17].

Materials and Methods

Chemicals; BPA was purchased from Sigma-Aldrich Chemicals (Tokyo, Japan). NP was from Wako Pure Chemicals Industry (Osaka, Japan).

Cell culture; Swiss 3T3 fibroblast cells or rat hepatoma RH7777 cells were maintained in phenol red-free Dulbecco's modified Eagle's medium (D-MEM, Wako Pure Chemicals Industry) containing 10% charcoal-stripped fetal calf serum (FCS). In some experiments, cells were cultured in D-MEM containing phenol red and FCS. For chemical exposure, cells were plated at approximately 30,000 cells per well of a 24-well plate and cultured in the presence of BPA or NP at the concentrations indicated. Every 3~4 day, cells were split, replated in a fresh well, and cultured with the same compounds until cell death or growth assay. In one-day exposure experiment, cells were cultured for 2 days and then BPA was added for further 1 day, followed by cell death assay. Establishment of RH7777 cells expressing FLAG-tagged GPR30 was performed by retroviral infection followed by single cell cloning, as shown previously [18].

RT-PCR; Total RNAs were extracted from cells, treated with RNase-free DNase (Invitrogen, Tokyo, Japan) and then reverse-transcribed with oligo$(dT)_{12\text{-}18}$ and reverse transcriptase (cDNA synthesis kit, Roche, Tokyo). The resulting cDNAs were amplified by PCR using GoTaq DNA polymerase (Promega, Tokyo, Japan) for *Esr1*, *Esr2*, *Esrrα*, *Esrrβ*, *Esrrγ*, or *GPR30*, which encoded ERα, ERβ, ERRα, ERRβ, ERRγ, or GPR30, respectively. The combinations of primers used were as follows: ERα-s1 (sense, tgccctactacctggagaac) and ERα-as1 (antisense, ccaacaaggcactgaccatc) for *Esr1*,

producing a 579-bp product; ERβ-s1 (sense, cagccctgttactagtcc) and ERβ-as1 (antisense, tctctcctggatccacac) for *Esr2*, producing a 264-bp product; mrEsrra-s1 (sense, gacagtccaaagggttcctc) and mrEsrra-as1 (antisense, gcttggtgatctcacactca) for *Esrrα*, producing a 316-bp product; mrEsrrb-s1 (sense, tggcagatcgggagcttgtg) and mrEsrrb-as1 (antisense, aggcgagagtgttcctcatcc) for *Esrrβ*, producing a 205-bp product; mrEsrrg-s1 (sense, atgcccaagagactgtgctt) and mrEsrrg-as1 (antisense, cagcatgcccactttgagac) for *Esrrγ*, producing a 216-bp product; GPR30mrh-s1 (sense, ggctttgtgggcaacatcc) and GPR30mrh-as1 (antisense, gacgctgctgtacatgttgatctg) for *Gpr30*, producing a 219-bp product. The cycling protocol was 60 s at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C, followed by 7 min at 72°C at the end of the cycling. PCR products were analyzed on 1% agarose gel electrophoresis.

Cell death assay; Cells were plated at approximately 8,000 cells per well of a 48-well plate, and cultured for 1 day. Cells were then washed and cultured in serum-free D-MEM without chemicals. After 3 hours, H_2O_2 was added and further cultured for two days. The number of viable cells was counted using Cell Counting Kit-8 (Dojin Chemistry Lab., Kumamoto, Japan), according to the manufacturer's protocol. The experiments were always done in triplicate or quadruplicate and repeated at least three times.

Cell growth assay; Cells were plated at approximately 8,000 cells per well of a 48-well plate, and further cultured in growth medium without chemicals. The number of viable cells was counted using Cell Counting Kit-8. The experiments were done in triplicate or quadruplicate and repeated two times.