Table 5 Major toxicity findings for trityl chloride in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	60	300	600†	0	12	60	300
Male								
Death	0/12	0/12	0/12	0/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	↓
ALT, Total cholesterol	/	–	–	–	/	–	–	↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	–	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	0/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	0/6	no data	0/6	0/6	3/6	6/6
Female								
Death	0/12	0/12	0/12	1/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	–
ALT, Total cholesterol	/	–	–	–	/	–	–	-,↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	↑	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	2/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	4/6	no data	0/6	0/6	5/6	6/6

Only critical data are shown in this table. †indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase $P < 0.05$. ↓indicates significant decrease at $P < 0.05$. Relative liver weights were increased by 11% for males and 8% for females at 60 mg/kg, and 29% for both sexes at 300 mg/kg in the newborn main study and by 44% for males and 46% for females at 600 mg/kg in the newborn dose-finding study. Body weight depression in males (13%) and an increase of relative liver weights (32% for males, 40% for females) were observed at 300 mg/kg in the young main study.

Therefore, pUETLs of 400–500 and 300 mg/kg/day are proposed as appropriate for newborn and young rats, respectively.

1,3,5-Trihydroxybenzene (Table 6)

The newborn investigation was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and at 0, 20, 100, and 500 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 250, 500, and 1000 mg/kg for dose-finding and at 0, 30, 100, 300, and 1000 mg/kg for the main study.

Common changes were observed in the thyroids and liver. The only toxic change in newborn main study was hypertrophy of thyroid follicular cells with increase in relative thyroid weights in both sexes at 500 mg/kg. Increased relative liver weights in females were not accompanied by any histopathological changes. Although decrease of adrenal weight and histopathological alterations such as vacuolization and pigmentation were noted at the end of the dosing and recovery-maintenance periods, these were always slight and not dose-dependent. There were no chemical-related changes with other examinations, including developmental parameters, in newborn rats. In the young study, similar effects on the thyroids and liver were found at 1000 mg/kg, but the incidence of thyroid histopathological changes was slightly less than in newborn animals at 500 mg/kg.

pNOAELs of 100 and 300 mg/kg/day for newborn and young rats can be considered appropriate because of the lack of data with dose settings between 100 to 500 mg/kg in the newborn, and no histopathological examination at 500 mg/kg in the young dose-finding study. The degree of toxicity at 1000 mg/kg for young rats was almost equal to that at 500 mg/kg for newborn rats. Therefore, pUETLs of 500 and 1000 mg/kg/day are proposed as equivalents for newborn and young rats, respectively.

DISCUSSION

More than 100 000 industrial chemicals are now in use around the world and sufficient toxicity information is available for only a small proportion. The Japanese government started the Existing Chemical Safety Program to obtain minimal toxicity data sets from 28-day toxicity studies using young rats for high production volume chemicals lacking toxicity information. For the present six targeted chemicals, we found toxicity information for only two chemicals by literature search. Daniel *et al.* (1993) reported no toxic effects of 2-chlorophenol on oral administration to male and female Sprague Dawley rats at up to 257 mg/kg for 10 days or 150 mg/kg for 90 days. Our results were consistent with their data, as we found no toxicity at 500 mg/kg in young dose-finding study (14 days administration) and at 200 mg/kg in the young study (28 days), while further providing information on CNS effects at higher doses. As for (hydroxyphenyl)methyl phenol, consisting of bisphenol D, E, and F isomers, bisphenol F has been reported to have estrogenic potential evidenced by several *in vitro* and *in vivo* experiments (Hashimoto *et al.* 2001; Yamasaki *et al.* 2002; Stroheker *et al.* 2003). However, we could not establish any such activity in this study. Our results are reasonable because oral administration of bisphenol F increased relative uterus weights only at more than 100 mg/kg, but not 50 mg/kg given during PNDs 22–25 (Stroheker *et al.* 2003), while our highest dose of (hydroxyphenyl)methyl phenol was equivalent to 30 mg/kg of bisphenol F.

Table 6 Major toxicity findings for 1,3,5-trihydroxybenzene in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)		
	0	100	500	0	300	1000
Male						
Relative organ weight						
Liver	/	–	–	/	–	↑
Thyroids	/	–	↑	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	4/6	0/6	0/6	2/6
Female						
Relative organ weight						
Liver	/	–	↑	/	–	↑
Thyroids	/	–	(↑)	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	5/6	0/6	0/6	4/6

Only critical data are shown in this table. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase $P < 0.05$ (except in parentheses where statistical significance was not attained). Numbers are for animals with the feature in the total examined. Increase of relative organ weights at 500 mg/kg in the newborn main study was observed for thyroids (39% for males, 24% for females) and liver (9% for females). Increase of relative organ weights at 1000 mg/kg in the young main study was observed for thyroids (14% for males, 19% for females) and liver (23% for males and 9% for females).

Table 7 Comparative susceptibility of newborn and young rats to the six chemicals

	Newborn study		Young study		pNOAEL	pUETL
	pNOAEL	pUETL	pNOAEL	pUETL	Young/Newborn	Young/Newborn
	mg/kg/day		mg/kg/day			
2-Chlorophenol	40	200–250	200	1000	5.0	4.0–5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
p-(α,α-Dimethylbenzyl) phenol	30	300	100	700–800	3.3	2.3–2.7
(Hydroxyphenyl) methyl phenol	100	140–160	40	1000	0.4	6.3–7.1
Trityl chloride	60	400–500	12	300	0.2	0.6–0.8
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0

Although there has been no reports for p-(α,α-dimethylbenzyl) phenol, it causes endocrine disruption and possible antiestrogenic activity, when administered to newborn female rats in this study. Therefore, further studies on this chemical should be conducted to elucidate the mechanisms, because the present investigation did not indicate any effects on sexual differentiation such as preputial separation, vaginal opening and the estrous cycle.

For our focus on the comparative sensitivity of newborn and young rats to chemicals, two toxicity endpoints, pNOAEL and pUETL, were newly defined as appropriate, considering the entire data sets from both main and dose-finding studies. We believe that this alternative assessment approach allowed us to make more realistic comparisons between newborn and young rats under the same experimental conditions as far as possible.

The ratios of pNOAELs for chemicals between newborn and young rats may provide an additional UF value in risk assessment according to susceptibility of newborn rats, because regulatory limit values for chemicals to protect public health of humans, including infants, are derived from the division of NOAEL by UFs. The data in Table 7 indicate newborn rats to be 1–5 times more susceptible to four of the tested chemicals, 2- and 4-chlorophenols, p-(α,α-dimethylbenzyl) phenol and 1,3,5-trihydroxybenzene, than young rats in terms of the pNOAELs, similar to the results of previous analyzes of five phenolic chemicals, 4-nitro-, 2,4-dinitro-, 2,4,6-trinitro-, 3-methyl- and 3-amino-phenols (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004). Immaturity in the detoxification potential of phase 1 and phase 2 enzymes in newborn animals may be the major cause of higher toxicity in newborn rats (Rich & Boobis 1997; Gow *et al.* 2001), because these chemical classes are probably direct toxicants. In the case of (hydroxyphenyl)methyl phenol, the pNOAEL (100 mg/kg/day) for newborn rats was 2.5 times higher than that (40 mg/kg/day) for young rats, but it can be speculated that values are in practice rather similar because the toxicity for young rats at the high dose, 200 mg/kg, was only slight (Table 4). As for trityl chloride, newborn rats were obviously less susceptible (0.2 for the pNOAEL ratio). Similar results were

also reported from our previous analysis for bromoalkanes (Hirata-Koizumi *et al.* 2005) and may be explained by mechanisms of action and metabolic characteristics of newborn rats. As this class of chemicals possibly requires metabolism to act as toxicants, the relatively mature metabolic enzyme status of young rats would be expected to provide toxic intermediates by metabolic activation to a greater extent than in newborn rats, as evidenced by data for previously reported chemicals (Onkenhout *et al.* 1986; Kennedy *et al.* 1993). Other compounds such as acetaminophen, bromobenzene, and carbon tetrachloride have also been shown to not produce liver injury in neonatal animals at doses that are hepatotoxic to adults (Gregus & Klaassen 1998).

The ratios of pUETLs, doses inducing the same degree of toxicity in newborn and young rats, were almost the same as for pNOAELs with the direct toxicants, as shown in Table 7. However, newborn rats were considerably more susceptible to (hydroxyphenyl)methyl phenol when considering the pUETL, due to the much steeper dose–response curve in newborn rats, with a 100 mg/kg/day pNOAEL and half the animals dying at 200 mg/kg, compared with a 40 mg/kg/day pNOAEL and only one death in 12 animals at 1000 mg/kg for young rats. Although young rats showed stomach hyperplasia in addition to hepatotoxicity at 1000 mg/kg, the cause of newborn deaths at 200 mg/kg was unclear. With regard to trityl chloride, the pUETL for young rats was almost the same as for newborn although the latter were less susceptible. Such an anomaly has also been found for bromoalkanes previously analyzed. Another example of a chemical for which susceptibility differs at low and high doses is chlorpyrifos, the maximum tolerated dose in 17-day-old rats being reported to be five times less than that in adults following oral exposure (Moser & Padilla 1998), but the differential sensitivity not appearing in low-dose exposure (Pope & Liu 1997). Thus as there are several chemicals of which dose–response curve in newborn rats was obviously steeper than that in young rats, pUETL ratios should be also taken into account for the susceptibility of newborn rats as the second endpoint marker.

In conclusion, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL in most cases. One exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

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ORIGINAL ARTICLE

Elevated susceptibility of newborn as compared with young rats to 2-*tert*-butylphenol and 2,4-di-*tert*-butylphenol toxicity

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ABSTRACT **In order to determine the susceptibility of newborn rats to 2-*tert*-butylphenol (2TBP) and 2,4-di-*tert*-butylphenol (DTBP) toxicity, studies were conducted with oral administration from postnatal days (PND) 4 to 21 and the findings were compared with results for young rats exposed from 5 or 6 weeks of age for 28 days. In the newborn rats, specific effects on physical and sexual development and reflex ontogeny were not observed. While there were no clear differences in toxicological profiles between newborn and young rats, the no-observed-adverse-effect levels (NOAELs) differed markedly. For 2TBP, clinical signs such as ataxic gait, decrease in locomotor activity and effects on liver, such as increase in organ weight, were observed and the NOAELs were concluded to be 20 and 100 mg/kg/day in newborn and young rats, respectively. Based on hepatic and renal toxicity (histopathological changes and increase in organ weight with blood biochemical changes), the respective NOAELs for DTBP were concluded to be 5 and 20 mg/kg/day. Therefore, the susceptibility of newborn rats to 2TBP and DTBP was found to be 4–5 times higher than that of young rats.**

Key Words: 2, 4-di-*tert*-butylphenol, 2-*tert*-butylphenol, susceptibility of newborn rats

INTRODUCTION

Protection of humans against disease and injury caused by chemicals in the environment is the ultimate goal of risk assessment and risk management (Landrigan *et al.* 2004). However, the focus has long been solely on adult exposure and toxicity and the fetus via maternal transfer, with little consideration given to early childhood. In the past decade, stimulated especially by the 1993 US National Research Council (NRC) report *Pesticides in the Diets of Infants and Children* (NAS 1993), recognition that special consideration is required for children in risk assessment has grown. The NRC report noted that 'children are not little adults', because of their unique patterns of exposures to environmental hazards and their particular vulnerability.

For the susceptibility of children to environmental chemicals, the early postnatal period (the suckling period) is of particular note. During this period, the infant could be exposed to various chemicals not only through mothers' milk, but also directly, by having chemical-contaminated baby food, mouthing toys or household materials, and so on; however, current risk assessment gives no consideration to toxic effects resulting from direct exposure to chemicals. An approach that adequately takes into account the susceptibility of infancy is urgently required. However, because there is no standard testing protocol intended for direct exposure of preweaning animals (newborn animals) to chemicals, and toxicity studies using newborn animals are complicated by practical difficulties regarding grouping, direct dosing, and general and functional observation, there is only limited information on susceptibility of the newborn at the present.

We therefore have established a new protocol for repeated dose toxicity studies using newborn rats (newborn rat studies) (Koizumi *et al.* 2001) for systematic application. Results have been compared with those of 28-day repeated dose toxicity studies using young rats (young rat studies) to provide a basis of analyzing susceptibility. Since young rat studies are routinely conducted as one of a battery of minimum toxicity tests and data are stored for many chemicals, comparative analyzes should provide important information for considering effects of direct exposure to chemicals during the suckling period.

We have already reported analytical results for eight chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane, 1,1,2,2-tetrabromoethane, 2,4,6-trinitrophenol, and tetrabromobisphenol A) (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005). The susceptibility of newborn rats to the toxicity of the first four agents was four times higher than that of their young counterparts at a maximum. For 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, while the doses causing clear toxicity were lower in newborn rats, doses at which toxic signs began to appear were paradoxically higher in the newborn case. These six chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups. For the other two chemicals, there were marked differences in toxicity profile between the newborn and young rats. Especially, in the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn case.

In the present investigation, two *tert*-butylphenols, 2-*tert*-butylphenol (2TBP), and 2,4-di-*tert*-butylphenol (DTBP), were chosen for comparative toxicity analysis. 2TBP has been used in the production of agricultural chemicals, aroma chemicals, and resins (New Chemical Index 2001), and DTBP in the production of antioxidants and ultraviolet absorbers (Chemical Products' Handbook 2004). For either chemical, there is no available toxicity information on human. Regarding toxicity to experimental animals, results from young rat studies of both chemicals are available in

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Toxicity Testing Reports of Environmental Chemicals of the Japanese government (MHLW 2001a, 2001b), but no other data have been reported regarding repeated dose toxicity. Since the young rats were only evaluated for toxicity profiles and no-observed-effect levels, we re-evaluated the results for a more practical evaluation index, the no-observed-adverse-effect level (NOAEL), which could serve as the basis for determining tolerable daily intake (TDI) or acceptable daily intake (ADI) for risk assessment, and conducted comparative analyzes with newborn rats.

MATERIALS

2-*tert*-Butylphenol (2TBP, CAS no. 88-18-6, purity: 99.97%) and 2,4-di-*tert*-butylphenol (DTBP, CAS no. 96-76-4, purity: 99.67%), obtained from Dainippon Ink and Chemicals, Incorporated (Tokyo, Japan), were dissolved in olive oil and corn oil, respectively. The test solutions were prepared once a week as stability for eight days had been confirmed. All other reagents used in this study were specific purity grade.

METHODS

All studies were performed under Good Laboratory Practice conditions and in accordance with 'Guidance for Animal Care and Use' of Panapharm Laboratories Co., Ltd, Research Institute for Animal Science in Biochemistry and Toxicology, or Mitsubishi Chemical Safety Institute Ltd.

Animals

In the newborn rat studies of 2TBP and DTBP, pregnant SPF Sprague-Dawley rats [Crj:CD(SD)IGS] were purchased at gestation days 13–15 from Charles River Japan Inc. (Yokohama, Japan), and allowed to deliver spontaneously. All newborn were separated from dams at postnatal day (PND) 3 (the date of birth was defined as PND 0), and pooled according to sex. At the same time, 12 foster mothers were selected among dams, based on the nursing condition. Each foster mother suckled four male and four female newborn, assigned to each of the four dose groups, including the controls, up to weaning on PND 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study Design) were individually maintained for nine weeks.

In the young rat studies, 4–5 week-old males and females of the same strain were obtained from the same supplier as for the newborn rat studies, and used at ages of 5–6 weeks after acclimation.

All animals were maintained in an environmentally controlled room at 20–26°C with a relative humidity of 40–70%, a ventilation rate of more than ten times per hour, and a 12:12 h light/dark cycle. They were allowed free access to a basal diet (MF: Oriental Yeast Co. Ltd, Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (sterile tap water or well water treated with sodium hypochlorite) throughout.

Study design

1. 18-day repeated dose toxicity study in newborn rats (newborn rat study)

Newborn rats (12/sex/dose) were administered the test substances by gastric intubation on PNDs 4–21. On PND 22, six males and six females in each treated group were sacrificed for autopsy (the scheduled-sacrifice group). The remaining animals in all groups (6 rats/sex/dose) were maintained for nine weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group).

Based on the results of dose-finding studies conducted prior to the main study, the dose, which would show clear toxicity, was selected as the top dose, that without potentially toxic effects as the lowest dose, and the medium dose was set between them. In the dose-finding study for 2TBP (oral administration from PNDs 4–21), some clinical signs and suppressed body weight gain were observed at 200 mg/kg and an increase in relative liver weight at 60 mg/kg and more. For DBTP (oral administration from PNDs 4–17), all of the four males and four females died at 500 mg/kg, and the death of one of the four males, an increase in serum total cholesterol and phospholipid, and increase in relative liver weight were noted in the 100 mg/kg group. Therefore, the doses were set at 0, 20, 60, or 200 mg/kg/day for 2TBP and at 0, 5, 40, or 300 mg/kg/day for DTBP.

During the study, the rats' general condition was observed at least once a day (details of clinical signs noted in this study are described in 'Glossary of terms for toxicity testing' [NIHS 1994]). Body weight and food consumption (only the recovery-maintenance period) was examined once or more a week. As developmental parameters, fur appearance, incisor eruption, pinna detachment and eye opening were assessed for physical development, and testes descent or preputial separation and vaginal opening for sexual development (OECD 2004). In addition, reflex ontogeny, such as visual placing reflex, and surface and mid-air righting reflexes, were also examined (Adams 1986; Jensh & Brent 1988). Urinalysis (color, occult blood, pH, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity, and volume of the urine) was conducted in the last week of the recovery-maintenance period.

At PNDs 22 and 85, blood was collected from the abdominal aorta under ether anesthesia (for 2TBP) or from the postcaval vein under pentobarbital sodium anesthesia (for DTBP) after overnight starvation for the scheduled-sacrifice and recovery-maintenance groups, respectively. One portion was treated with EDTA-2K and examined for hematological parameters, such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, part of the blood was treated with 3.8% sodium citrate, and blood clotting parameters such as prothrombin time (PT) and activated partial thromboplastin time (APTT) were examined. Serum from the remaining portions of blood for both the scheduled-sacrifice and recovery-maintenance groups were analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio [A/G ratio], glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen [BUN], creatinine, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, γ-glutamyl transpeptidase [γ-GTP], calcium, inorganic phosphorus, sodium, potassium, and chlorine). Following collection of blood, all animals were sacrificed by exsanguination, and all organs and tissues were macroscopically examined. Then, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The above-listed organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopy. For other groups, organs with macroscopically abnormal findings or in which chemical-related effects were evident on microscopic examination for the highest dose group, were similarly investigated.

2. 28-day repeated dose toxicity study in young rats (young rat study)

Five to six week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed following the last treatment (the scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals was six for each sex/dose for both scheduled-sacrificed and recovery cases.

The doses were selected in the same way as the newborn rat studies. In the 12-day dose-finding study for 2TBP, ataxic gait was observed at 300 mg/kg and more, and increase in relative liver and kidney weight at 500 mg/kg. For DTBP, with 14-day administration, the death of one of the four females, various changes in some blood biochemical parameters, increase in relative liver weights and light gray macules on kidneys were found at 500 mg/kg. Increase in serum phospholipid and relative liver weights were also demonstrated in the 100 mg/kg group. Based on the results, the doses were determined at 0, 4, 20, 100, or 500 mg/kg/day for 2TBP and at 0, 5, 20, 75, or 300 mg/kg/day for DTBP. Recovery groups were set at 0, 100, 500 mg/kg/day for 2TBP and 0, 300 mg/kg/day for DTBP.

During the study, rats were examined for general condition, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights, and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances).

Statistical analysis

Data for body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological, blood biochemical findings (except for differential leukocyte count), and organ weights were analyzed by the Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups ($P < 0.01$ or 0.05). If not homogeneous or for qualitative urinalysis data and differential leukocyte count, the data were analyzed using Steel's multiple comparison tests (Steel 1959), or tests of the Dunnett type (Hollander & Wolfe 1973) ($P < 0.01$ or 0.05). For reflex ontogeny, and physical and sexual development parameters in the newborn rat studies, the χ^2-test (Fisher 1922) was conducted ($P < 0.01$ or 0.05).

RESULTS

2-*tert*-butylphenol (2TBP)

Newborn rat study

Various clinical signs such as decrease in locomotor activity, ataxic gait, deep respiration, and muscle weakness were observed throughout the dosing period in the 200 mg/kg group, as shown in Table 1. With 60 mg/kg, transient decrease in locomotor activity was noted on the first dosing day limited to only one of 12 males. Body weights were lowered by 8–17% from dosing day 7 through to the end of the dosing period in males and to recovery-maintenance day 14 in females given 200 mg/kg. At the scheduled sacrifice, there were no hematological changes at any dose, but blood biochemical examination of the 200 mg/kg group showed increases in γ-GTP in both sexes and total protein in males. In addition, significant increase in relative liver weights was noted in 9% of the females in the 60 mg/kg group and in 21–23% of both males and females in the 200 mg/kg group. On histopathological examination, slight hypertrophy of centrilobular hepatocytes was found in one female of the 60 mg/kg group, and in four males and three females from the 200 mg/kg group. During the recovery-maintenance period, no clinical signs were observed and the lowered body weights showed a tendency for recovery. In parameters for physical and sexual development and reflex ontogeny, no definitive changes were detected. At the end of the recovery-maintenance period, no chemical-related changes, also in urinalysis data, were found in any dose group.

The results of the newborn rat study of 2TBP are summarized in Table 2. Since clinical signs and histopathological changes in the liver were observed in the 60 mg/kg group, the NOAEL was concluded to be 20 mg/kg/day.

Young rat study

Ataxic gait were observed sporadically during the dosing period in nine males and 12 females, and decrease in locomotor activity in two females from the 500 mg/kg group. During the dosing period, there were no changes in body weight, food consumption, and urinalysis data. At the scheduled sacrifice, hematological and blood biochemical examination also showed no changes. Eighteen to 19% increases were found in relative liver weights of both sexes receiving 500 mg/kg, but no histopathological changes in liver were observed at any dose. No chemical-related changes were noted during and at the end of the recovery period.

Table 1 Clinical signs observed during the dosing period in the newborn rat study of 2-*tert*-butylphenol

	Dose (mg/kg/day)			
	0	20	60	200
No. animals (Male/Female)	12/12	12/12	12/12	12/12
No. animals with clinical signs				
Decrease in locomotor activity	0/0	0/0	1†/0	12/12
Ataxic gait	0/0	0/0	0/0	4/6
Deep respiration	0/0	0/0	0/0	12/12
Tremors	0/0	0/0	0/0	2/4
Muscle weakness	0/0	0/0	0/0	12/12
Emaciation	0/0	0/0	0/0	2/2
Pale skin	0/0	0/0	0/0	4/2

†Observed only on the first dosing day.

Table 2 Summary of the results of the newborn and young rat study of 2-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	20	60	200	
Clinical signs	–	M: Decrease in locomotor activity	Various†	
Body weight changes	–	–	8–17%↓	
Blood biochemical changes	–	–	GTP↑, M: TP↑	
Changes in relative organ weights	–	F: Liver 9%↑	Liver 21–23%↑	
Histopathological findings in liver				
– Slight centrilobular hypertrophy of hepatocytes	–	M: 0/6, F: 1/6	M: 4/6, F: 3/6	
Young rat study				
Dose (mg/kg/day)	4	20	100	500
Clinical signs	–	–	–	Ataxic gait F: Decrease in locomotor activity
Body weight changes	–	–	–	–
Blood biochemical changes	–	–	–	–
Changes in relative organ weights	–	–	–	Liver 18–19%↑
Histopathological findings	n.d.	n.d.	n.d.	–

Statistically significant increases ($P < 0.05$) in body weights, blood biochemical parameters and relative organ weights are shown as ↑, while decreases are shown as ↓. Data on histopathological findings are given as no. of animals with the findings/no. of animals examined, according to sex. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. No chemical-related changes were observed in developmental parameters (conducted only in newborn rat study), urinalysis (only in young rat study), and hematological parameters. †Decrease in locomotor activity, ataxic gait, deep respiration, tremors, muscle weakness, emaciation, and pale skin were observed, as shown in Table 1. GTP, γ-GTP; TP, total protein; –, no change; n.d., not determined.

A summary of the results of the young rat study of 2TBP is given in Table 2. The NOAEL was concluded to be 100 mg/kg/day, at which no changes were observed.

2,4-di-*tert*-butylphenol (DTBP)

Newborn rat study

Two males and one female of the 300 mg/kg group were found dead on dosing days 3, 4, and 7. In this group, decrease in locomotor activity (12 males and 12 females), bradypnea (10 males and 10 females), and hypothermia (one male) were observed from the first dosing day, but then the incidence decreased, with disappearance after dosing day 7. Body weights of the 300 mg/kg group were lowered by 15–25% in males and by 9–20% in females during the dosing period, compared with the control values. There were no definitive changes in parameters for physical development and reflex ontogeny in any dose group. At the scheduled sacrifice, blood biochemical examination showed an increase in total bilirubin and a decrease in the A/G ratio in both sexes, an increase in γ-GTP in males, and an increase in total protein and BUN in females of the 300 mg/kg group. In the 300 mg/kg group, there was a 39–51% increase in relative liver weights, a 37–41% increase in relative kidney weights in both sexes, and a 24% decrease in relative spleen weights in males. In the 40 mg/kg group, 14% increases in relative weight of liver were found in females. On histopathological examination, various changes were observed in livers and kidneys in the 300 mg/kg group, as shown in Table 3. Furthermore, periportal fatty degeneration of hepatocytes was evident in one female given 40 mg/kg, and basophilic tubules in kidneys in one animal of each sex receiving 40 mg/kg and one control group male. Regarding parameters of sexual development, a slight delay in preputial separation was noted in the 300 mg/kg group (the incidences were 0/5, compared with 2/6 in the control group at PND 42 [recovery-maintenance day 21]; 0/5, 3/6 at PND 43; 2/5, 5/6 at PND 44; 2/5, 6/6 at PND 46; 4/5, 6/6 at PND 47; and 5/5, 6/6 at PND 48). During this observation period, body weights were lowered by approximately 10% in males given 300 mg/kg than control levels, which was not statistically significant. In the last week of the recovery-maintenance period, there were no chemical-related changes on urinalysis in any dose group. At the end of the recovery period, changes noted in the scheduled-sacrifice group were not observed except for histopathological changes in the kidneys, significant in the 300 mg/kg group (Table 3).

A summary of the results of the newborn rat study of DTBP is shown in Table 4. Since fatty degeneration of hepatocytes and increase in liver weight were demonstrated at 40 mg/kg, the NOAEL was concluded to be 5 mg/kg/day.

Young rat study

No chemical-related changes were found in general condition, body weight, and food consumption at any dose. On urinalysis at the fourth week of dosing, an increase in urine volume, and a decrease in specific gravity and osmotic pressure were noted in both sexes of the 300 mg/kg group. At the scheduled sacrifice, hematological examination showed a decrease in hemoglobin and hematocrit, an increase in segmented neutrophils in females, and prolongation of PT and APTT in males at 300 mg/kg. On blood biochemical examination, there was an increase in total bilirubin in males given 300 mg/kg, and an increase in total cholesterol and phospholipid in females given 75 mg/kg and above. For organ weights, there were

Table 3 Histopathological findings for the newborn rat study of 2,4-di-*tert*-butylphenol

		Scheduled-sacrifice group				Recovery-maintenance group†	
Dose (mg/kg/day)	Grade	0	5	40	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	5/6	6/6	5/5
Liver							
– Fatty degeneration of periportal hepatocytes	+	0/0	0/0	0/1	0/0	0/0	0/0
	++	0/0	0/0	0/0	3/4	0/0	0/0
	+++	0/0	0/0	0/0	2/2	0/0	0/0
Kidneys							
– Basophilic tubules	+	1/0	n.d.	1/1	4/4	0/0	3/0
– Granular casts	+	0/0	n.d.	0/0	4/2	0/0	0/0
– Cystic dilatation of collecting tubules	+	0/0	n.d.	0/0	0/0	0/0	5/4
	++	0/0	n.d.	0/0	3/4	0/0	0/0
	+++	0/0	n.d.	0/0	2/2	0/0	0/0
– Cellular infiltration of neutrophils	+	0/0	n.d.	0/0	2/1	0/0	1/0
	++	0/0	n.d.	0/0	1/1	0/0	1/0
	+++	0/0	n.d.	0/0	1/1	0/0	0/0

†No histopathological examination was conducted at 5 and 40 mg/kg in the recovery-maintenance group. +, mild; ++, moderate; +++, marked; n.d., not determined.

increases in relative liver weights by 40–43% in both sexes given 300 mg/kg, and by 13% in females receiving 75 mg/kg. On histopathological examination, mild to marked changes in livers and kidneys were observed in both sexes from the 300 mg/kg group, as shown in Table 5. At the end of the recovery period, the increase in total cholesterol and phospholipid and renal histopathological changes observed in the scheduled-sacrifice group remained significant in the highest-dose group (Table 5).

The results of the young rat study are summarized in Table 4. Based on increase in the relative liver weights with some changes in blood biochemical parameters in females given 75 mg/kg, the NOAEL was concluded to be 20 mg/kg/day.

DISCUSSION

During development, many rapid and complex biological changes occur, which can have profound consequences on sensitivity to the effects of exogenous chemicals (Scheuplein *et al.* 2002). Although the neonatal body at birth is reasonably well prepared for the abrupt changes associated with parturition, and most functional systems possess a significant portion of their adult capacity (Dourson *et al.* 2002), it is known that the various functions remain immature in early postnatal period and that some organs and tissues, especially in the nervous, immune and reproductive systems, continue to develop after birth (NAS 1993). Therefore, it is important to evaluate toxic effects by exposure to chemicals during the early postnatal period as well as the fetal period for comprehensive risk assessment. However, economic issues and lack of human resources, arising from practical difficulties regarding protocols, have hindered routine implementation of toxicity studies using newborn animals. Our series of comparative analyzes on susceptibility of the newborn are therefore of particular importance for risk assessment.

In the present study on 2TBP and DTBP, there were no clear differences in toxicity profiles between the newborn and young rats in either case. For 2TBP, clinical signs such as a decrease in locomotor activity and ataxic gait, and effects on liver such as an increase in organ weight were observed. In the DTBP case, hepatic and renal toxicity (histopathological changes, increase in organ weight, etc.) were noted. As a characteristic effect of DTBP on male sexual development, slight delay in preputial separation was also observed in the newborn rat study. Preputial separation, an androgen-dependent process which is an early marker of puberty, represents a reliable non-invasive indicator of chemical-induced perturbation of male pubertal development in the rat (Gaytan *et al.* 1988). However, it is known that decreased body weights can result in non-specific delay in puberty (Ashby & Lefevre 2000). Since DTBP lowered body weights in the period of observation of preputial separation and there were no DTBP-related changes in weights or histopathology of the testes and epididymides, well known to be essentially androgen-dependent, no specific effect on male sexual development could be concluded in the present study. As for NOAELs of both chemicals, clear differences were observed between newborn and young rats, with values of 20 and 5 mg/kg/day in newborn rats, and 100 and 20 mg/kg/day in young rats for 2TBP and DTBP, respectively. Therefore, the susceptibility was four- to five-fold higher in newborn than in young rats.

Our previous analysis of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005) showed dose–response curves to be very different between newborn and young rats. The same was recently reported for the widely used organophosphorus insecticide, chlorpyrifos (Zheng *et al.* 2000), as well as pyrethroid insecticides (Shafer *et al.* 2005). These data showed the importance of estimating unequivocally toxic levels (UETLs), defined for our comparative toxicity analysis as equivalent toxic doses inducing clear toxicity, including death, clinical toxic signs,

Table 4 Summary of the results of the newborn and young rat study of 2,4-di-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	5	40	300	
Death	–	–	M: 2/12, F: 1/12	
Clinical signs	–	–	Decrease in locomotor activity bradypnea, hypothermia	
Body weight changes	–	–	9–25%↓	
Urinalysis	n.d.	n.d.	n.d.	
Hematological changes	–	–	–	
Blood biochemical changes	–	–	Various†	
Changes in relative organ weights	–	F: Liver 14%↑	Liver 39–51%↑, Kidney 37–41%↑ M: Spleen 24%↓	
Histopathological findings	–	F: Fatty degeneration in liver	Various changes in liver and kidney‡	
Developmental parameters	–	–	Slight delay in preputial separation	
Young rat study				
Dose (mg/kg/day)	5	20	75	300
Death	–	–	–	–
Clinical signs	–	–	–	–
Body weight changes	–	–	–	–
Urinalysis	–	–	–	UV↑ SG↓ OP↓
Hematological changes	–	–	–	Various§
Blood biochemical changes	–	–	F: Tcho↑ Pho↑	M: TB↑ F: Tcho↑ Pho↑
Changes in relative organ weights	–	–	F: Liver 13%↑	Liver 40–43%↑
Histopathological findings	n.d.	n.d.	–	Various changes in liver and kidney¶

Data on death are shown as no. of dead animals/no. of animals examined, according to sex. Statistically significant increases ($P < 0.05$) in body weights, urinalysis and blood biochemical parameters, and relative organ weights are shown as ↑, while decreases are shown as ↓. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. †Increase in total bilirubin and decrease in the A/G ratio in both sexes, increase in γ-GTP in males, and increase in total protein and BUN in females were noted. ‡Various changes were observed as shown in Table 3. §Various hematological changes were noted such as decrease in hemoglobin and hematocrit and increase in segmented neutrophils in females and prolongation of PT and APTT in males. ¶Various changes were observed as shown in Table 5. OP: osmotic pressure; Pho: phospholipid; SG: specific gravity; TB: total bilirubin; Tcho: total cholesterol; UV: urine volume; –: no change; n.d.: not determined.

or critical histopathological damage (Koizumi *et al.* 2001). We here tried to apply this UETL approach to the present study. For 2TBP, clinical signs such as decrease in locomotor activity and ataxic gait were noted in most of the animals given 200 mg/kg (newborn rats) and 500 mg/kg (young rats) (Table 2). Furthermore, a 8–17% lowering of body weight was observed at 200 mg/kg in newborn rats, but not in the young rat study. Therefore, equivalent toxic effects to these observed at 500 mg/kg in young rats might be expected to appear at 100–150 mg/kg in newborn animals. The UETLs were concluded to be 100–150 and 500 mg/kg/day in newborn and young rats, respectively. In the case of DTBP, clear toxicity was observed at the top dose of 300 mg/kg in both newborn and young rat studies (Table 4), but the level of severity was very different, for example, deaths were only noted in the newborn cases. It was considered difficult to estimate the UETLs from the results of main studies only. However, the most critical endpoint for toxicity, mortality, was also noted at 100 mg/kg and more, and 500 mg/kg, in the dose-finding studies of newborn and young rats, respectively. Therefore, it would be possible to estimate the appropriate UETLs as the minimum lethal dose by taking the results of the dose-finding studies into consideration. The UETLs were concluded to be 100 mg/kg/day for the newborn, and 500 mg/kg/day for young rats, at which one out of eight rats was found dead in both cases. These analyzes of UETLs, considering equivalence in toxic degree, showed 3.3–5.0 times higher susceptibility of newborn rats to 2TBP and DTBP than young rats, consistent with our analytical results for NOAELs.

Higher susceptibility of newborn rats was also demonstrated in our previous analyzes of five phenols (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol and 2,4,6-trinitrophenol) (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004), considered mainly due to their poor metabolic and excretory capacity (Horster 1977; Cresteil *et al.* 1986). It has actually been reported that UDP-glucuronyltransferase and sulfotransferase activities, when 4-nitrophenol is used as the substrate, are lower in microsomes prepared from livers of newborn rats, and that the elimination rate of 2,4-dinitrophenol from serum of newborn rabbits is markedly slower than in young adults (Gehring & Buerge 1969; Matsui & Watanabe 1982). Unfortunately, there is no information on the toxicity mechanism and toxicokinetics of both 2TBP

Table 5 Histopathological findings for the young rat study of 2,4-di-*tert*-butylphenol

		Scheduled-sacrifice group†			Recovery group	
Dose (mg/kg/day)	Grade	0	75	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	6/6	6/6
Liver						
– Centrilobular hypertrophy of hepatocytes	+	0/0	0/0	4/4	0/0	0/0
Kidneys						
– Basophilic tubules	+	0/0	0/0	1/4	0/0	3/1
	++	0/0	0/0	4/0	0/0	2/0
	+++	0/0	0/0	1/1	0/0	1/0
– Granular casts	+	0/0	0/0	5/2	0/0	4/0
	++	0/0	0/0	1/1	0/0	0/0
– Proteinaceous casts	+	0/0	0/0	5/1	0/0	2/0
	++	0/0	0/0	1/0	0/0	0/0

†No histopathological examination was conducted for the 5 and 20 mg/kg scheduled-sacrifice groups. +, mild; ++, moderate; +++, marked.

and DTBP; however, the immature functions involved in the toxicokinetics in newborn rats would be implicated in the higher susceptibility, as in the case of five phenols previously analyzed. While there are very little data on toxicokinetics of environmental chemicals in the newborn, relatively plentiful information has been reported in humans for pharmaceuticals which are clinically applied during the early postnatal period. Recently, Ginsberg *et al.* (2002) conducted comparative analysis of pharmacokinetic parameters for 45 drugs in both children and adults, and showed half-lives in children aged two months or under to generally be two-fold longer than in adults.

As for the susceptibility of the newborn to toxicity of chemicals, although it is generally important to take the sensitivity of target organs and tissues themselves (toxicodynamics) into consideration besides toxicokinetics, there are insufficient data on differences between newborn and young/adult animals. For appearance of toxicity, which is the outcome of toxicokinetics and toxicodynamics, some comparative studies have relied on LD_{50} values (Goldenthal 1971; Sheehan & Gaylor 1990). However, it is not considered that information on acute toxicity at lethal dosage is appropriate when considering the susceptibility of newborn in risk assessment, because dose–response curves could differ, as mentioned above. With prolonged, subtoxic doses, which are basis for TDI or ADI, our series of comparative studies constitute the first systematic assessment, providing an important base for development of new methods of risk assessment of susceptibility of the newborn.

In conclusion, clinical signs and effects on the liver were observed for 2TBP, and hepatic and renal toxicity for DTBP. Although there were no clear differences in toxicity profiles between the newborn and young rats for both chemicals, the toxicity levels differed markedly. The susceptibility of the newborn to these chemicals appears to be 4–5 times higher than that of young animals.

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Locomotor hyperactivity following prenatal exposure to 5-bromo-2′-deoxyuridine: neurochemical and behavioral evidence of dopaminergic and serotonergic alterations

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Abstract

Prenatal exposure to 5-bromo-2′-deoxyuridine (BrdU) has been reported to induce abnormal behaviors in offspring, including marked hyperactivity. In this study, the contribution of the serotonin (5-HT) and dopamine (DA) systems to BrdU-induced developmental neurotoxicity was investigated. Sprague–Dawley rats were treated with BrdU on gestational days 9 through 15 (50 mg/kg, i.p.) and male offspring (BrdU-rats) were examined. The BrdU-rats exhibited a 3.5-fold increase in locomotor activity. The dopamine D_2 receptor antagonist sulpiride increased locomotor activity in the BrdU-rats, but decreased it in control rats. The BrdU-rats responded to the 5-HT_{1A} receptor antagonist NAN190 much more than the controls. The measurement of monoamines revealed significant decreases in DA, dihydroxyphenylacetic acid, and homovanilic acid, and significant increases in 5-HT and 5-hydroxy-3-indolacetic acid, with a decrease in the 5-HT turnover ratio in the striatum of BrdU-rats. Thus, prenatal exposure to BrdU induced alterations in both the DA and 5-HT systems.

Keywords: 5-Bromo-2′-deoxyuridine; Dopamine; Serotonin; Hyperactivity; ADHD; Developmental neurotoxicology

1. Introduction

5-Bromo-2′-deoxyuridine (BrdU), a thymidine analog, is incorporated into the DNA as 5-bromouracil during the synthesis (S) phase of the cell cycle of all cells, including embryonic neural cells (Schwartz and Kirsten, 1974; Yu, 1976, 1977; Biggers et al., 1987). This analog is well known to induce many biological

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responses which are of importance to the field of genetic toxicology (Morris, 1991). Prenatal treatment with BrdU results in a variety of fetal malformations, including those of the palate and face (Murphy, 1965; Skalko et al., 1971; Shah and MacKay, 1978) and the neural tube (Ruffolo and Ferm, 1965; Webster et al., 1973). We reported that male rats exposed prenatally to BrdU showed behavioral abnormalities such as impaired sexual behavior, impaired learning, and memory and hyperlocomotion (Nagao et al., 1997; Kuwagata and Nagao, 1998; Kuwagata et al., 2001). Other investigators have also reported that prenatal exposure to BrdU affected spatial learning and visual acuity, and disrupted skilled forelimb ability in rats (Kolb et al., 1999). However, no direct evidence of neuronal dysfunction related to the hyperactivity observed in prenatal BrdU-treated rats (BrdU-rats) has been reported.

Attention deficit hyperactivity disorder (ADHD) is a condition that is manifested by impulsivity, hyperactivity, and inattention. It has been reported that about 5% of United States elementary school-aged children are affected by this disorder (Hechtman, 1994; Gingerich et al., 1998; Sagvolden and Sergeant, 1998; Taylor, 1998). Several animal models of ADHD have been reported, e.g., prenatal methylazoxymethanol (MAM)-induced microencephalic rats (Ferguson et al., 1995; Watanabe et al., 1995; Kodama et al., 2000), spontaneously hypertensive rats (SHR) (Russell et al., 1995, 1998, 2000; Aspide et al., 2000; Russell, 2000) and neonatal 6-hydroxydopamine (6-OHDA)-lesioned rats (Heffner and Seiden, 1982; Luthman et al., 1989; Towle et al., 1989; Kostrzewa et al., 1994). In these animals, abnormalities in monoaminergic systems, including the DA system, have been reported. Recently, studies on dopamine transporter knockout (DAT-KO) mice have provided evidence to help understand the mechanisms associated with these ADHD models and the mechanisms of psychostimulants, which have been shown to control human ADHD (Giros et al., 1996; Gainetdinov et al., 1998, 1999; Jones et al., 1998). In a series of studies, Gainetdinov et al. (1999) reported that the marked increase in spontaneous locomotor activity seen in the DAT-KO mice was a direct consequence of the extended length of time that DA spends in the extracellular space following its release. They also demonstrated that 5-HT can compete with this DA, and that psychostimulants can attenuate the hyperactivity of DAT-KO mice by increasing extracellular levels of 5-HT.

In this study, we carried out biochemical measurements of the DA and 5-HT systems, as well as some pharmacological challenges using DA and 5-HT antagonists, to demonstrate dopaminergic and serotonergic abnormalities in the BrdU-rats, and discuss the possibility of BrdU-rats as an animal model of psychiatric disorders such as ADHD.

2. Materials and methods

2.1. Animals and BrdU treatment

Sprague–Dawley rats were purchased from Charles River Laboratories (Atsugi, Japan). They were housed in metal cages in a room in which the temperature and relative humidity were controlled at 24 ± 1 °C and 50 ± 5%, respectively. Lights were turned on from 07:00 to 19:00 h daily. Rats were given food and tap water ad libitum. At 11 weeks of age female rats were cohabited overnight with males of proven fertility of 12 weeks of age. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and the day was designated as gestational day 0 (GD 0). Once insemination was confirmed, the females were assigned to control and BrdU-treated groups using a computer-generated list of permutations. BrdU (Sigma, St. Louis, MO) was suspended in 0.5% sodium carboxymethyl cellulose (CMC Na) and administered immediately after preparation. On GD 9 through 15 at a defined time (13:00 h), BrdU was administered each day intraperitoneally to 17 inseminated rats at a dose of 50 mg/kg body weight. Fifteen controls received 0.5% CMC Na (5 ml/kg) on GD 9 through 15. The dosages were based on body weight on GD 9. Pregnant females were allowed to deliver naturally. Newborns were inspected externally on postnatal day 0 (PND 0, the day of birth). On PND 1, nurslings were sexed and each litter was reduced randomly to eight males. The number of nurslings in each litter during the lactation period was recorded, and viability on PND 21 was determined. On PND 21, all offspring were weaned.

2.2. Pharmacological challenge test

At 5–8 weeks of age, 57 male offspring from 15 dams in the control (three to four offspring from each dam) and 47 male offspring from 17 dams in the BrdU group (two to three offspring from each dam) were subjected to pharmacological challenge tests. Offspring were placed in a circular area (140 cm in diameter) surrounded by a wall (40 cm in height), and ambulation (moving distance) was recorded automatically by a computer (Unicom, Inc., Japan) during a 3-min trial between 13:00 and 16:00 h. The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the area. Under these conditions, offspring did not habituate after six to eight trials (ambulation did not decrease significantly). Each offspring used at each dose was selected from a different litter. Each individual rat was used for a total of one to two challenges with a minimum of 5–7 days between challenges. Antagonists were administered 3–4 days after a baseline session during which baseline data were obtained (prechallenge). One hour after the administration of antagonists, the ambulation in the open field was recorded. One hour was appropriate because significant drug effects were confirmed by a repeated measures *t*-test (pre- versus postchallenge). A selective dopamine D_1 receptor antagonist, SCH23390 (Sigma Chemical Co., St. Louis, MO), at 0.1, 0.25, and 0.5 mg/kg (n = 5–10) and a selective dopamine D_2 receptor antagonist, sulpiride (Sigma Chemical Co., St. Louis, MO), at 40 and 100 mg/kg (n = 5–12) were used. These doses were reported to suppress ambulatory behavior (Ågmo and Soria, 1999). A selective 5-HT_{1A} receptor antagonist, NAN190 (Sigma Chemical Co., St. Louis, MO), which has been shown to block 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT)-induced behavioral responses (Berendsen et al., 1990), was administered at 0.3 and 3.0 mg/kg (n = 6–11). A selective 5-HT_{2A} receptor antagonist, ketanserin (Sigma Chemical Co., St. Louis, MO), which has been shown to attenuate cocaine-induced hyperactivity (McMahon and Cunningham, 2001), was used at 0.25, 0.5, and 2.5 mg/kg (n = 5–12). All compounds were dissolved in 0.5% CMC Na and injected intraperitoneally at a volume of 1 ml/kg body weight for SCH23390, 2 ml/kg body weight for NAN190 and ketanserin, and 5 ml/kg body weight for sulpiride.

2.3. Biochemical analysis of neurotransmitters in the brain

At 10 weeks of age, seven male offspring from seven dams in both the control and BrdU groups were sacrificed randomly by decapitation and their brains were removed. Subsequently, the brains were dissected on ice into the frontal cortex, striatum, and midbrain, according to a method of Glowinski and Iversen (1966). All tissues were stored at −80 °C until being assayed. Levels of DA, 5-HT, and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), and 5-hydroxy-3-indolacetic acid (5-HIAA), were measured by reverse phase high-performance liquid chromatography with electrochemical detection (HPLC–ECD), as previously described (Muneoka et al., 1999). Tissue contents of monoamines were presented as ng/g tissue. As indices of DA and 5-HT turnover, (DOPAC + HVA)/DA and 5-HIAA/5-HT ratios were calculated, respectively.

2.4. Statistical analyses

Monoamine content and ambulation at 5 weeks of age were compared between the control and BrdU groups using the Student's *t*-test. For the pharmacological challenge tests, a two-factor repeated measures (vehicle versus BrdU and pre- versus postchallenge) was applied. When the interaction was detected, the paired *t*-test was performed for pre- versus postchallenge in each group (control and BrdU). The level of significance was set at $P < 0.05$ and 0.01.

3. Results

3.1. General conditions

No overt signs of maternal toxicity were apparent in the BrdU-rats during pregnancy or lactation. All of the pregnant rats delivered normally on GD 22. Body weights of offspring were lower for the BrdU-rats than for the controls throughout the study as reported in our previous studies (Nagao et al., 1997; Kuwagata and Nagao, 1998). Kinked tails were also observed in the BrdU-rats when inspected on PND21 with the same incidence (approximately 50% of all BrdU animals) as in our previous studies (Kuwagata and Nagao, 1998;

Nagao et al., 1997), whereas no externally malformed pups were detected when inspected on PND 0. The viability of BrdU juveniles on PND21 was comparable to that of the controls.

3.2. Pharmacological challenge tests with DA and 5-HT receptor antagonists

Locomotor activity in the open field at 5 weeks of age (before challenge tests) was significantly greater in the BrdU-rats than in controls (Control 969 ± 66.4; BrdU 3493 ± 273.4, mean ± S.E.M., cm, $P < 0.01$). Locomotor activity for pre- and postpharmacological challenges are shown in Fig. 1. SCH23390 significantly decreased locomotor activity in both the control and BrdU groups, without an interaction (pre- versus postchallenge: 0.1 mg/kg, $F(1, 10) = 14.95$, $P < 0.01$; 0.25 mg/kg, $F(1, 16) = 15.73$, $P < 0.01$; 0.5 mg/kg, $F(1, 9) = 32.36$, $P < 0.01$). Sulpiride, at a dose of 40 mg/kg, affected the locomotor activity with a significant interaction (pre- versus postchallenge: $F(1, 22) = 7.07$, $P < 0.05$; interaction, $F(1, 22) =$

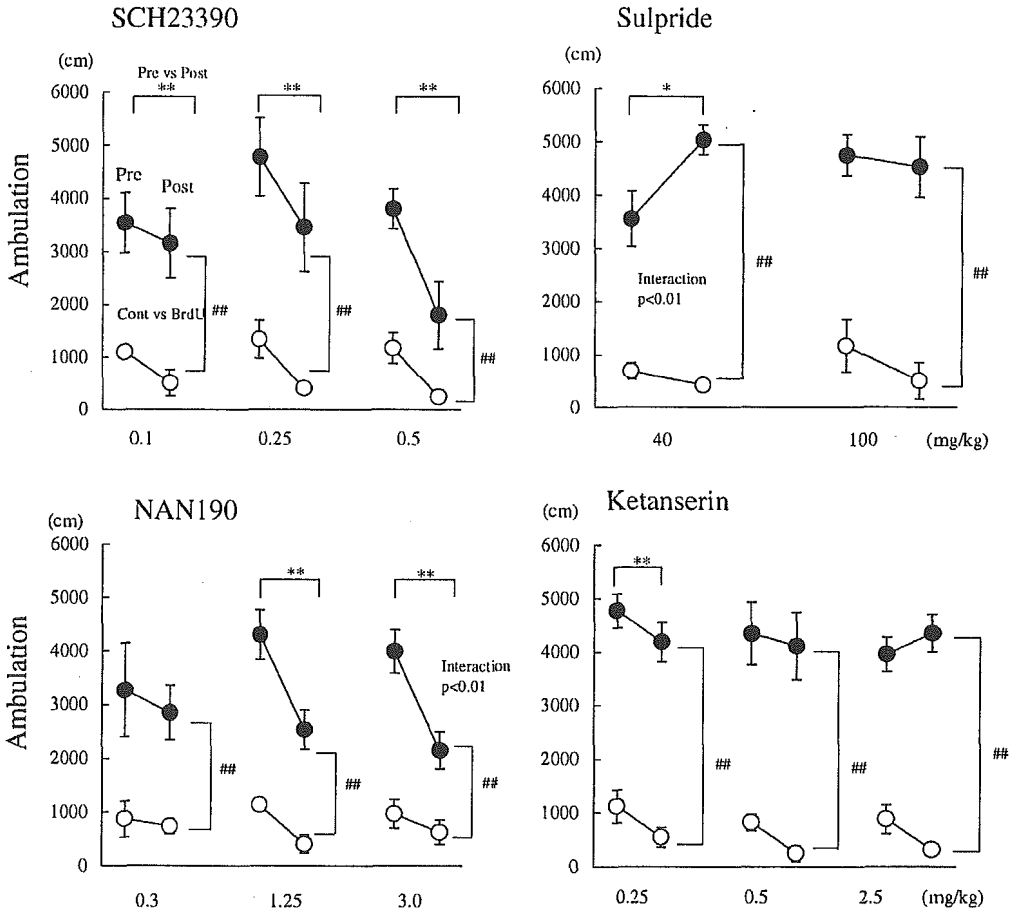

Fig. 1. Changes in locomotor activity after treatment with SCH23390, sulpiride, NAN190, and ketanserin. Data are expressed as mean±S.E.M. White and black circles indicate the control group (Cont) and BrdU group (Bu), respectively. Numbers of offspring tested for the SCH23390 challenge were Cont = 6, Bu = 6 (0.1 mg/kg); Cont = 8, Bu = 10 (0.25 mg/kg); and Cont = 5, Bu = 6 (0.5 mg/kg); for the sulpiride challenge were Cont = 12, Bu = 12 (40 mg/kg) and Cont = 5, Bu = 9 (100 mg/kg); for NAN190 challenge were Cont = 6, Bu = 6 (0.3 mg/kg) and Cont = 6, Bu = 6 (1.25 mg/kg) and Cont = 6, Bu = 6 (3.0 mg/kg); for the ketanserin challenge were Cont = 9, Bu = 12 (1.25 mg/kg); Cont = 4, Bu = 8 (0.5 mg/kg); and Cont = 5, Bu = 7 (2.5 mg/kg), respectively. Results from a two-factor repeated measure and a paired *t*-test analyses are described in the text. (*) and (**) indicate a significant difference between pre- and postpharmacological challenges at $P < 0.05$ and $P < 0.01$, respectively. (##) indicates a significant difference between control and BrdU groups at $P < 0.01$.

14.68, $P < 0.01$; control, $P < 0.05$; BrdU, $P < 0.05$ by paired t-test). Sulpiride decreased the locomotor activity in the control group while in the BrdU group, it actually increased. NAN190 decreased locomotor activity in both the control and BrdU groups (pre- versus postchallenge: 1.25 mg/kg, $F(1, 10) = 24.88$, $P < 0.01$; 3.0 mg/kg, $F(1, 10) = 67.40$, $P < 0.01$). At 3.0 mg/kg there was a significant interaction, suggesting a larger effect in the BrdU group ($F(1, 10) = 31.58$, $P < 0.01$; control, $P < 0.05$; BrdU, $P < 0.05$ by paired t-test). Ketanserin, at a dose of 0.25 mg/kg, decreased locomotor activity in both the control and BrdU groups (pre- versus postchallenge: $F(1, 19) = 4.61$, $P < 0.05$). However, at 0.5 mg/kg and 2.5 mg/kg, the two-factor repeated measures did not show any significant effect of challenge. There was consistently a significant difference between the vehicle and BrdU groups at each dose of each drug.

3.3. Biochemical analysis of the DA and 5-HT systems in the BrdU-rats (Fig. 2)

In the striatum, DA and DOPAC levels were significantly lower in the BrdU-rats compared with the

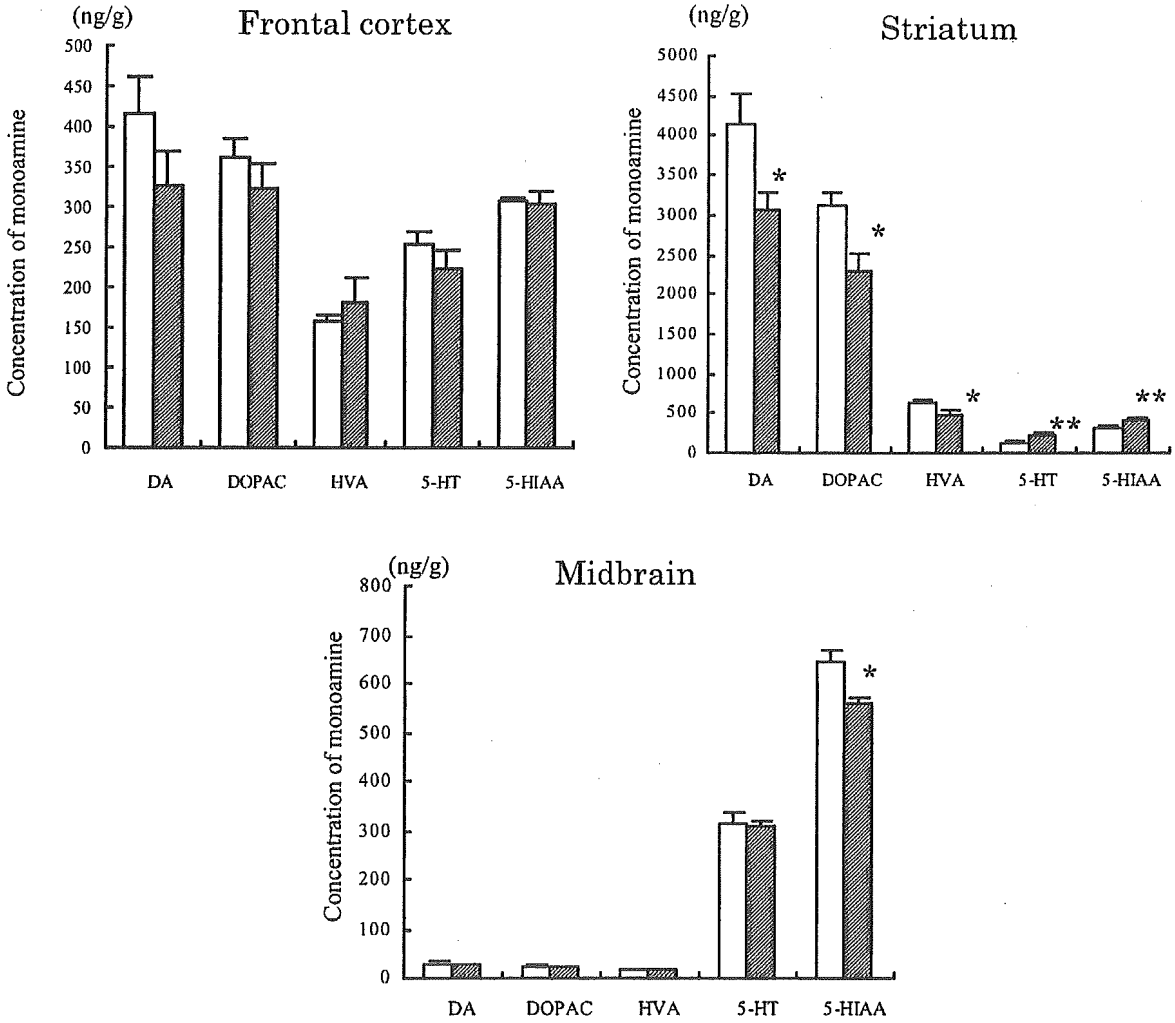

Fig. 2. Effects of prenatal exposure to BrdU on monoamine contents in the frontal cortex, striatum, and midbrain in male offspring. White and black columns indicate the control and BrdU groups, respectively. Data are expressed as mean ± S.E.M. (*) and (**) indicate a significant difference from the control at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

controls, whereas the (DOPAC + HVA)/DA ratio was comparable with the control levels (data not shown). In contrast, significant increases in 5-HT and 5-HIAA levels, as well as a significant decrease in the 5-HIAA/5-HT ratio (controls, 2.52 ± 0.16; BrdU-rats, 1.89 ± 0.15, mean $\pm$ S.E.M., $P < 0.05$) were found in the BrdU-rats compared with the controls. Although a trend toward decreases in DA levels was found in the frontal cortex, there were no significant changes in the monoamine contents or their turnover ratios in this area. A significant decrease in 5-HIAA level was found in the midbrain with no changes in other parameters.

4. Discussion

In the present study, prenatal exposure to BrdU induced marked hyperactivity (3.5-fold increase) in rat offspring, confirming previous studies (Kuwagata and Nagao, 1998; Kuwagata et al., 2001). In addition, neurochemical and behavioral pharmacologic investigations revealed alterations in both the dopaminergic and serotonergic systems in the BrdU-rats.

Reductions in DA, DOPAC, and HVA in the striatum were induced by prenatal treatment with BrdU. An index of DA turnover, the (DOPAC + HVA)/DA ratio, was not altered. The lack of change in DA turnover, along with the reduction in DA and its metabolites, suggests a treatment-induced decrease in the density of DA neuron terminals in the striatum. In addition, a moderate but not significant reduction in DA was found in the frontal cortex. These results suggest that dopaminergic projections to terminal regions were reduced in the BrdU-rats. In contrast, increases in 5-HT and 5-HIAA were found in the striatum. The alterations in 5-HT and 5-HIAA levels, accompanied by a decrease in 5-HT turnover in the striatum, suggest a functional disturbance in the 5-HT system in this area. Hyperinnervation of 5-HT fibers in the striatum is known to occur when DA neurons are denervated by 6-OHDA treatment during the neonatal period (Towle et al., 1989; Descarries et al., 1992; Kostrzewa et al., 1994). This evidence suggests that prenatal exposure to BrdU disrupts development of both DA and 5-HT systems in their terminal areas, mainly the striatum. Alternatively, the significant change in the striatum, but not in the frontal cortex, suggests that BrdU may affect the DA and 5-HT systems in the striatum indirectly through other transmitters from other brain areas or through other neurons in the striatum.

In the pharmacological challenge tests we examined behavioral responses to D_1, D_2, 5-HT_{1A}, and 5-HT_{2A} receptor antagonists. It has been reported that a marked increase in spontaneous locomotor activity seen in DAT-KO mice is a direct consequence of the extended length of time that DA spends in the extracellular space following release, and that the serotonin can antagonize this effect (Gainetdinov et al., 1998, 1999; Jones et al., 1998). The D_1 receptor antagonist SCH23390 decreased locomotor activity in both the control and BrdU-rats, suggesting that the D_1 receptor was not affected by BrdU. However, the D_2 receptor antagonist sulpiride increased locomotor activity in the BrdU-rats, whereas it significantly decreased activity in the control rats. This result suggests that prenatal exposure to BrdU altered the dopaminergic function through the D_2 receptor. The presynaptic D_2 receptor on nigrostriatal neurons is known to work as an autoreceptor, inhibiting the release of DA from presynaptic terminals (Tepper et al., 1984; Boyar and Altar, 1987; Lacey et al., 1987). Therefore, it is possible that sulpiride at 40 mg/kg preferentially blocked this presynaptic receptor more than the postsynaptic receptor in the BrdU-rats, resulting in a greater release of dopamine and increased locomotor activity. The 5-HT_{1A} receptor antagonist NAN190 decreased the locomotor activity in both the control and BrdU groups with a greater effect in the latter group. This result suggests that hyperfunction of the autoreceptor in the Raphe nuclei was induced in the BrdU-rats, which resulted in the hypofunction of the terminal area in the striatum and increased locomotor activity (Hjorth and Magnusson, 1988; Elliott et al., 1990). The result from monoamine measurement also supports this hypofunction in the striatum. The higher dose of ketanserin did not seem to affect the BrdU-rats, while a significant decrease was observed at 0.25 mg/kg. However, the reason for this was not clear. Further studies, including agonist challenges, are needed to interpret the mechanism(s) underlying the BrdU-induced hyperactivity.

Several animal models exhibiting hyperactivity have been reported, e.g., prenatal methyazoxymethanol-induced microencephalic rats (Ferguson et al.,

1995; Watanabe et al., 1995; Kodama et al., 2000), spontaneous hypertensive rats (Okamoto and Aoki, 1963; Moser et al., 1988; Russell et al., 1995, 1998, 2000; Aspide et al., 2000; Russell, 2000), and neonatal 6-OHDA-lesioned rats (Heffner and Seiden, 1982; Luthman et al., 1989; Towle et al., 1989; Kostrzewa et al., 1994). In these animals, abnormalities in monoaminergic systems, including the DA system, have been reported. However, the neurochemical characteristics of MAM-induced microencephalic rats are somewhat different from those of the BrdU-rats. Increases in DA content and DA transporter density in the striatum, as well as increases in 5-HT content and 5-HT transporter density in the cerebral cortex, were observed in MAM-treated rats, indicating hyperinnervation of the forebrain with DA and 5-HT neurons (Jonsson and Hallman, 1981; Kodama et al., 2000; Watanabe et al., 1995). A decrease in DA and an increase in 5-HT in the striatum, as well as hyperlocomotion, are common characteristics in BrdU-rats and in neonatal 6-OHDA-treated rats (Heffner and Seiden, 1982; Luthman et al., 1989; Towle et al., 1989; Kostrzewa et al., 1994), suggesting the importance of a specific balance between 5-HT and DA systems for normal motor activity. However, simply measuring monoamine tissue levels does not appear sufficient to fully understand the mechanism(s) of prenatal chemical-induced abnormal behaviors. An analysis of extracellular dopamine and 5-HT levels by voltametry and microdialysis in BrdU-rats would definitely be helpful.

Impulsiveness, inattentiveness, and overactivity are regarded as the main symptoms of ADHD (Gingerich et al., 1998; Hechtman, 1994; Taylor, 1998). Clinical as well as molecular studies indicate that ADHD is a polygenic or oligogenic disorder and that many genes are likely to be involved in the development of ADHD. BrdU is known to induce mutagenesis in mammalian cells (Morris, 1991); the noted alternations in the DA and 5-HT systems seen in the BrdU-rats should encourage further studies using this animal model, especially with reference to molecular investigations. Considering that BrdU is one of the several genotoxic compounds, and that exposure to these chemicals can occur in humans, the BrdU model may prove useful in the study of the cause and prevention of clinically observed psychiatric disorders such as ADHD.

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