Table 2 Body and organ weights of young and adult rats given ACR in the drinking water for 4 weeks

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Young					
Body weight	(g)	287.4 ± 24.6[a]	273.5 ± 15.7	263.7 ± 14.4*	210.4 ± 24.3**
Brain	(g)	1.97 ± 0.05	1.94 ± 0.09	1.83 ± 0.09*	1.66 ± 0.04**
	(g%)	0.69 ± 0.06	0.71 ± 0.05	0.69 ± 0.03	0.80 ± 0.08**
Testes	(g)	2.57 ± 0.15	2.44 ± 0.22	2.39 ± 0.19	1.87 ± 0.36**
	(g%)	0.90 ± 0.07	0.90 ± 0.09	0.91 ± 0.07	0.89 ± 0.12
Epididymides	(g)	0.40 ± 0.04	0.35 ± 0.02**	0.37 ± 0.04	0.30 ± 0.02**
	(g%)	0.14 ± 0.02	0.13 ± 0.01	0.14 ± 0.02	0.15 ± 0.02
Adult					
Body weight	(g)	444.3 ± 38.0	433.0 ± 42.0	426.7 ± 42.1	409.2 ± 45.5
Brain	(g)	2.07 ± 0.06	2.08 ± 0.11	2.02 ± 0.09	1.99 ± 0.07
	(g%)	0.47 ± 0.03	0.48 ± 0.04	0.48 ± 0.04	0.49 ± 0.06
Testes	(g)	3.30 ± 0.26	3.39 ± 0.39	3.25 ± 0.20	3.19 ± 0.24
	(g%)	0.74 ± 0.07	0.78 ± 0.08	0.77 ± 0.08	0.79 ± 0.09
Epididymides	(g)	0.97 ± 0.05	1.04 ± 0.09	0.97 ± 0.07	0.84 ± 0.06**
	(g%)	0.22 ± 0.02	0.24 ± 0.02	0.23 ± 0.03	0.21 ± 0.02

10 animals per each group were examined

[a] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

Table 3 Histopathology and morphometry of lesions developing in the nervous system

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Young					
Trigeminal nerve					
No. of animals examined		10	10	10	10
Central chromatolysis (+/++/+++)[a]		0	3 (3/0/0)	10 (0/5/5)[##]	10(0/0/10)[##]
Sciatic nerve (distal portion)					
No. of animals examined		10	10	10	10
Density	(/100 μm^2)	2.56 ± 0.32[b]	2.73 ± 0.17	2.92 ± 0.25**	2.42 ± 0.25
Degenerated axons	(%)	0.28 ± 0.15	0.39 ± 0.14	0.82 ± 0.19**	7.51 ± 3.25**
Myelinated axons, <3 μm in diameter	(%)	18.01 ± 3.45	16.74 ± 2.79	18.80 ± 2.73	21.57 ± 4.07
Cerebellar cortex					
No. of animals examined		5	5	5	5
SYP-immunoreactive aberrant dots	(/mm cortex)	0.50 ± 0.20	0.41 ± 0.18	1.49 ± 0.59	6.09 ± 1.62*
Adult					
Trigeminal nerve					
No. of animals examined		10	10	10	10
Central chromatolysis (+/++/+++)[a]		0	3 (3/0/0)	10 (3/7/0)[##]	10 (0/3/7)[##]
Sciatic nerve (distal portion)					
No. of animals examined		10	10	10	10
Density	(/100 μm^2)	2.10 ± 0.23	2.03 ± 0.15	2.10 ± 0.24	2.15 ± 0.24
Degenerated axons	(%)	0.39 ± 0.16	0.65 ± 0.27	0.96 ± 0.37*	1.74 ± 0.77**
Myelinated axons, <3 μm in diameter	(%)	13.96 ± 2.75	12.30 ± 2.39	13.45 ± 2.68	14.16 ± 2.82
Cerebellar cortex					
No. of animals examined		5	5	5	5
SYP-immunoreactive aberrant dots	(/mm cortex)	0.54 ± 0.12	0.47 ± 0.09	1.71 ± 0.81	5.88 ± 2.61*

[a] *Grade of change* + mild, ++ moderate, +++ severe

[b] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

[##] $P < 0.01$ vs. 0 ppm group (Fisher's exact test)

SYP synaptophysin

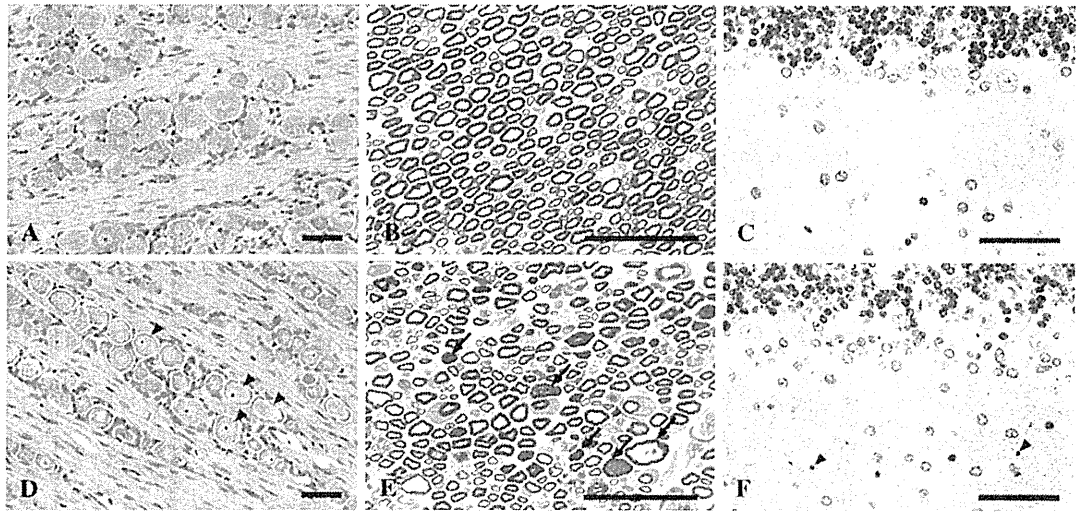

Fig. 3 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) in young rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of a young rat from the 0 ppm group. (**d–f**) At 200 ppm, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve was apparent. Increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were also found. **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

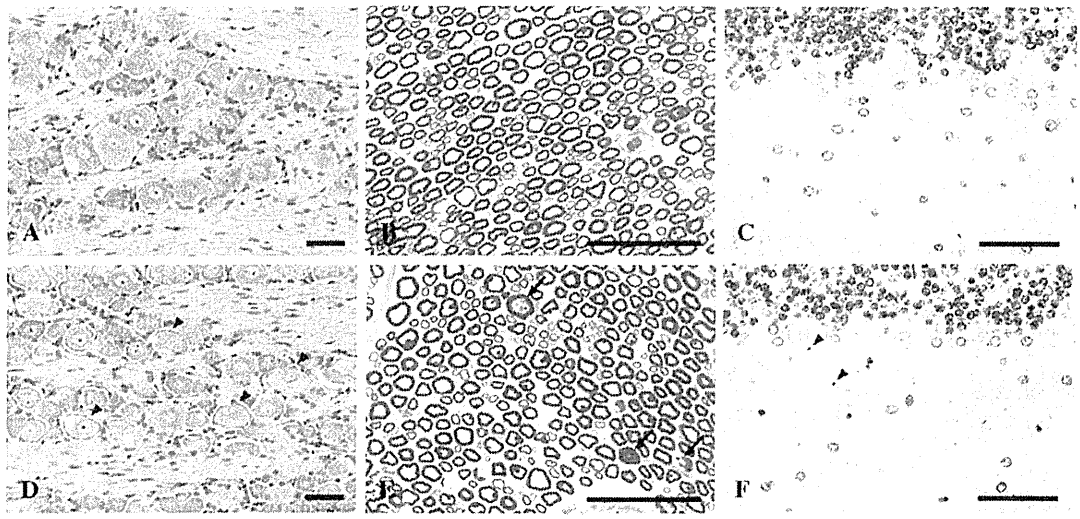

Fig. 4 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) of adult rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of an adult rat from 0 ppm group. (**d–f**) Similar to the young group, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve, increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were observed at 200 ppm **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

axons in the sciatic nerve was increased only at 100 ppm in the young group, but without dose dependence. In both young and adult groups, significant increase in degenerated axons in the sciatic nerve was observed from 100 ppm, and increase in dot-like SYP-immunoreactive structures in the cerebellar molecular layer was also found at 200 ppm. Although not statistically significant, myelinated nerves <3 μm in diameter showed a tendency for increase at 200 ppm in both young and adult groups. At 200 ppm, most parameters were higher in young groups compared to adult counterparts.

In the testis, marked degeneration and loss of or decrease in spermatids was observed from 100 ppm in young animals (Fig. 5 and Table 4). Elongate spermatids

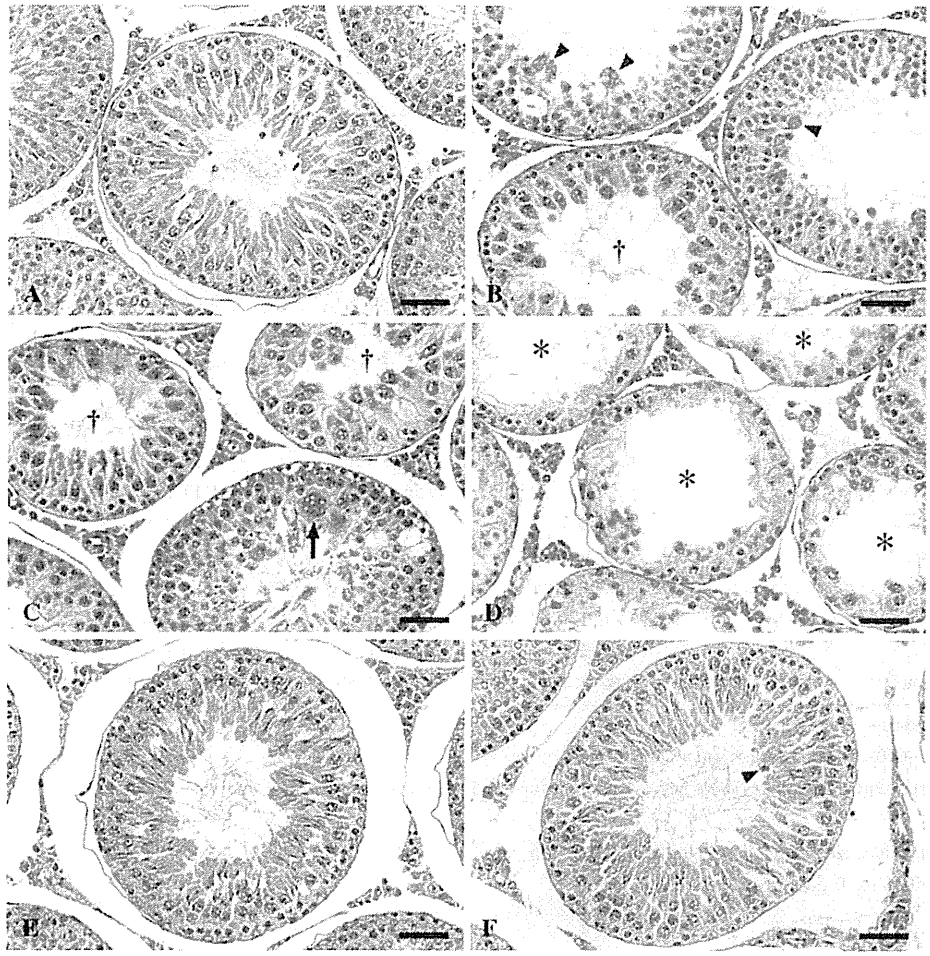

Fig. 5 Histopathology of the testis of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. **a** Normal seminiferous tubules of a young rat from the 0 ppm group. (**b–d**) Degeneration of spermatids (*arrowheads*), loss of or decreased in elongated spermatids (†), and multinucleated giant cells (*arrow*) are apparent in a young rat at 200 ppm. In severely affected cases, many seminiferous tubules showed marked germ cell depletion (*). **e** Normal seminiferous tubules of an adult rat from the 0 ppm group. **f** Only a small number of exfoliated germ cells (*arrowhead*) was found in the lumina of tubules in a case of the adult group at 200 ppm. HE stain. All *bars* = 50 μm

appeared to be most vulnerable to ACR, and in severely affected cases, many seminiferous tubules showed marked germ cell depletion. In addition, exfoliation of germ cells and appearance of multinucleated giant cells were also found. Many exfoliated germ cells were observed in the epididymal duct. In the adult groups, only small numbers of exfoliated germ cells was found in lumina of seminiferous tubules. Sertoli cells were morphologically unaffected in both young and adult animals. Similar histopathological changes were also observed in the testis of each young and adult animal at 200 ppm in the satellite groups used for measuring GST activity and GSH contents.

Data for relationships between ACR intake per kg body weight and neurotoxicity parameters, including the gait score at week 4, the number of degenerated axons in the sciatic nerves, and the number of SYP-immunoreactive structures in the cerebellar molecular layer are shown in Fig. 6a–c. All these parameters increased in proportion to ACR intake. For testicular toxicity, the relationship between ACR intake per kg body weight and the percentage of affected seminiferous tubules is shown in Fig. 6d. With increase in ACR dose, affected tubules profoundly increased in the young group, while the magnitude of increase was very small in the adult group.

Comet assays and MN tests

The comet assay revealed that ACR significantly induced DNA damage in a dose-dependent manner from 100 ppm

Table 4 Histopathological data for the testes of young and adult rats given ACR in the drinking water for 4 weeks

Findings (%)[a]	Acrylamide in the drinking water (ppm)			
	0	50	100	200
Young				
Affected tubules[b]	3.51 ± 1.68[c]	9.03 ± 18.81	16.93 ± 12.23*	66.59 ± 26.96**
Exfoliation of germ cells	3.39 ± 1.61	3.93 ± 3.07	9.80 ± 6.22*	10.44 ± 9.87
Multinucleated giant cells	0.02 ± 0.06	0.07 ± 0.14	0.57 ± 0.71	1.67 ± 3.06**
Degeneration of spermatids	0.10 ± 0.18	0.84 ± 2.54	3.95 ± 6.47	20.90 ± 13.37**
Loss of or decrease in elongated spermatids	0 ± 0	4.99 ± 15.74*	5.62 ± 8.87**	20.43 ± 14.61**
Loss of or decrease in round spermatids	0.02 ± 0.06	0 ± 0	1.51 ± 3.19	12.68 ± 10.97**
Atrophic tubules[d]	0 ± 0	0.17 ± 0.54**	0.12 ± 0.38**	24.03 ± 30.83**
Sertoli cell vacuolation	0.60 ± 0.57	1.07 ± 0.55	0.99 ± 0.62	1.06 ± 0.92
Adult				
Affected tubules[b]	0.47 ± 0.30	0.58 ± 0.23	1.17 ± 0.60*	1.53 ± 0.67**
Exfoliation of germ cells	0.45 ± 0.30	0.56 ± 0.23	1.17 ± 0.60*	1.46 ± 0.71**
Multinucleated giant cells	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.17
Degeneration of spermatids	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Loss of or decrease in elongated spermatids	0.02 ± 0.06	0.02 ± 0.07	0 ± 0	0 ± 0
Loss of or decrease in round spermatids	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Atrophic tubules[d]	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sertoli cell vacuolation	0.62 ± 0.45	0.53 ± 0.50	0.72 ± 0.43	0.81 ± 0.53

10 animals per each group were examined

[a] Approximately 400–650 tubules/rat were examined

[b] Affected tubules represent total tubules with findings, except for tubules showing only Sertoli cell vacuolation

[c] Mean ± SD

[d] Atrophic tubules are those showing marked germ cell depletion

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

in young and adult groups (Fig. 7a). Although the values did not greatly differ between the groups, the values in the young group were higher than those in the adult group at 200 ppm. On the other hand, MN was clearly induced only in young group in a dose-dependent manner (Fig. 7b) with statistical significance at both 100 and 200 ppm ($P < 0.05$). ACR slightly induced MN in the adult group at 200 ppm.

GST activity and GSH contents in the liver and testis

In the liver, compared to the 0 ppm group, GST activity was significantly increased at 200 ppm in the young group (Fig. 8a). Although not statistically significant, GST activity in the adult animals at 200 ppm also showed a tendency for increase. GSH contents were unchanged in both groups. There were no differences in the level of GST activity and GSH contents in the liver between young and adult groups. In the testis, although GST activity and GSH contents in both groups were not changed by ACR treatment, the levels of GST activity in the young group were significantly lower than those in the adult group (Fig. 8b).

Discussion

In the present study, ACR dose-related suppression of body weight, and food and water consumption was observed only in young rats. When gait abnormalities progressed, animals became unable to support their body weights, and it was difficult to take food and water from containers set in the upper part of the cage. Therefore, in the housing conditions designed for adult animals, the suppressions might be due to immature body size of young animals causing difficulty in access to food and water associated with the development of neurotoxicity.

On clinical observation, although both young and adult animals exhibited similar symptoms from 100 ppm, earlier occurrence and faster progression of the symptoms were

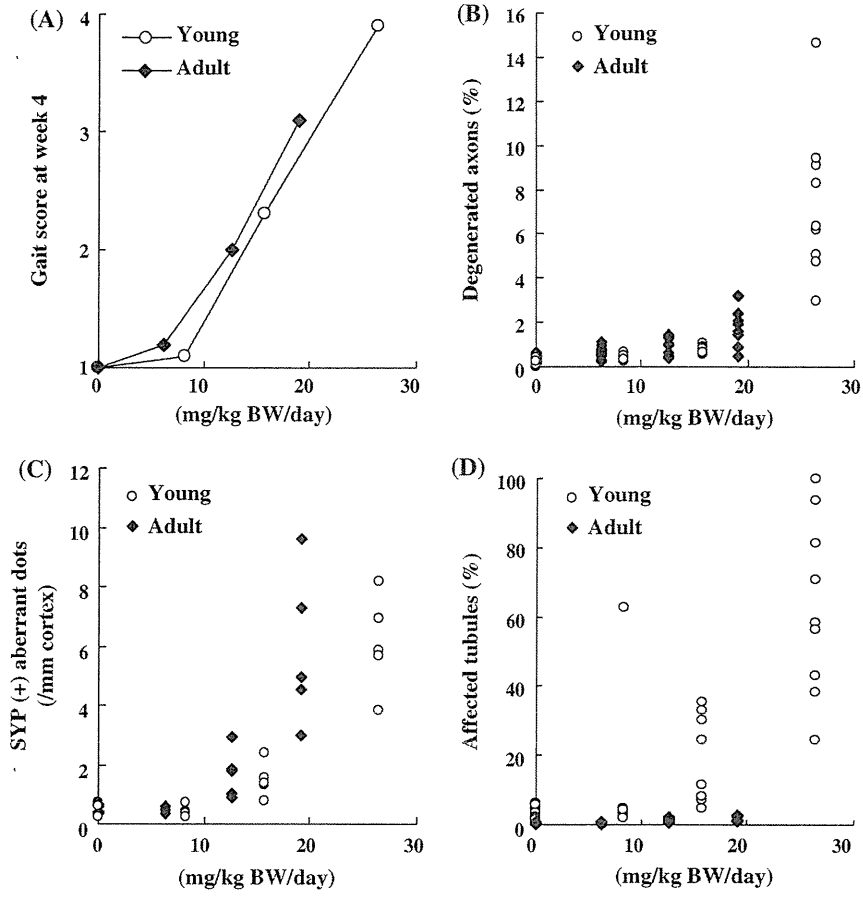

Fig. 6 Relationship between ACR intake per body weight and changes in neurotoxicity and testicular toxicity parameters in young and adult rats

here observed in the young group. Also, neurotoxic lesions such as central chromatolysis of ganglion cells in the trigeminal nerves, degenerated axons in the sciatic nerve and dot-like SYP-immunoreactive structures in the cerebellar molecular layer, were evident from 100 ppm in both young and adult groups. The magnitude of changes in these parameters was higher in the young group than in the adult group, especially at the highest dose, and neurotoxicity appeared stronger in young animals, though the types of lesions observed were similar between the young and adult groups. Compared to adult animals, intake of ACR per kg body weight was higher in young animals at each dose and the parameters indicating the neurotoxicity increased in proportion to ACR intake. Accordingly, the stronger neurotoxicity in the young animals can be considered to be a reflection of larger amount of ACR intake per body weight. These results suggest that the susceptibility to ACR-induced neurotoxicity in young and adult rats is qualitatively similar under the given experimental conditions. As mentioned in the Introduction section, a few studies have demonstrated life stage-related differences in susceptibility to ACR neurotoxicity, though the experimental conditions, such as age of animals, dosing methods, and parameters examined, were different. While Suzuki and Pfaff concluded that suckling rats were more susceptible (Suzuki and Pfaff 1973), it seems that there was not much difference in number of injections to cause apparent symptoms and myelin degeneration between suckling and adult rats. In the report by Ko et al., earlier occurrence and faster progression of neurological abnormalities in young animals were similar to those observed in our study (Ko et al. 1999). Although the authors stated that the daily intake was not significantly different between the young and adult groups, intake of ACR per body weight at the beginning of the experiment might have been higher in the young group, because younger animals usually take more water than older ones. Taken together, clear evidence of the susceptibility difference in neurotoxicity between young and adults animals is considered to be undetermined.

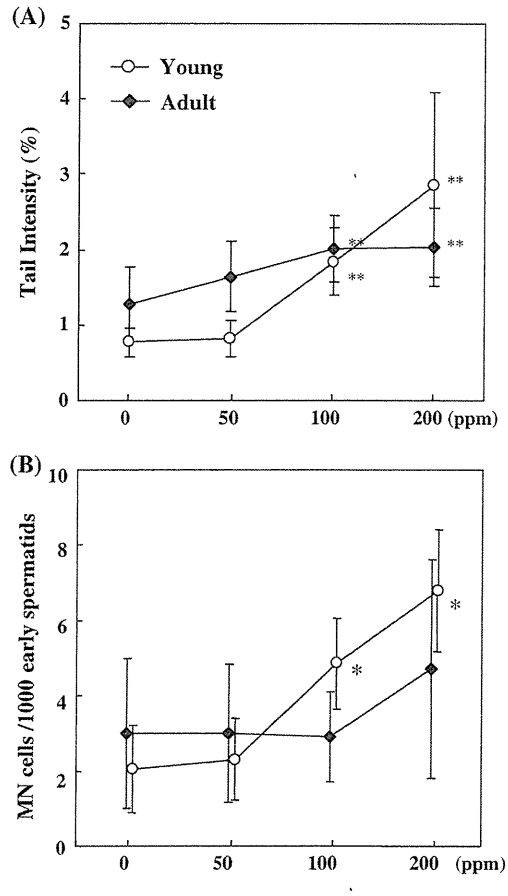

Fig. 7 Tail intensity of the comet image (**a**) and micronuclei frequency (**b**) obtained from young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. $^{**}P < 0.01$ vs. 0 ppm

Regarding the susceptibility to ACR testicular toxicity in the present study, young animals showed apparently diverse and more profound lesions exceeding the dose–effect relationship observed in adult animals. ACR is known to interfere with motor proteins such as kinesin found in the sperm flagellum and alkylate protein sulfhydryl groups in the sperm tail (Sickles et al. 2007; Friedman et al. 2008). Therefore, it is considered that elongate spermatids are highly susceptible to ACR. In the comet assay, although DNA damage in the young group was higher than that in the adult group at 200 ppm, the values were not greatly different. However, the MN test revealed that ACR clearly induced MN in the young group, but not in the adult group. These results well correspond with the observations on histopathological examination. Because the comet assay and MN test in the testis target spermatocytes and early spermatids, the late stage of spermatogenesis may be more susceptible to ACR-induced genotoxicity in young than in adult animals.

As reported by others (Yousef and El-Demerdash 2006), the basal level of testicular GST activity in our cases was much lower than that in the liver. Although there were no life stage differences in the liver levels of GST activity, testicular GST activity in the present study was significantly lower in the young groups, irrespective of the ACR treatment. The activity of GST is low at birth and then increases gradually, but it has been known that the developmental profiles of antioxidant enzymes including GST in the testis differ greatly from those in the liver (Peltola et al. 1992). A study of the immunolocalization of GST-Yo, a member of the mu class expressed at high levels in the testis and epididymides, revealed that this enzyme was not detectable until 39 days of age and then appeared mainly in the elongate spermatids, with expression reaching maturity by day 49 (Papp et al. 1994). Therefore, the detoxification capacity of the testis in young animals was considered to be much lower than that in the adult animals during the experimental period in the present study, and such a difference might reasonably account for the high susceptibility to ACR-induced testicular toxicity observed in our young animals. In the liver, although GST activity was increased at 200 ppm, there were no apparent life-stage differences. Considering that the liver is the main organ involved in detoxification of ACR, similar level of GST activity may have contributed to the lack of differences in susceptibility to neurotoxicity between young and adult rats. Increase in GST activity in ACR-treated rats has been reported and considered to be due to increased formation of S-conjugates between ACR and GSH (Yousef and El-Demerdash 2006). ACR is known to cause GSH depletion (Zhang et al. 2009); however, decrease in GSH contents was not found in the present study. Because recovery or rather increase in liver GSH contents after depletion by treatment animals with acetaminophen has been reported (Ishii et al. 2009), the level of GSH in the present study might possibly have recovered after repeated treatment with ACR during the experimental period.

In summary, our results suggest that susceptibility to ACR neurotoxicity in young animals might not be different from that in adult ones when exposure levels are adjusted for the body weight. Regarding testicular toxicity, young animals proved more vulnerable than adults, and this might be due to a low level of testicular GST activity.

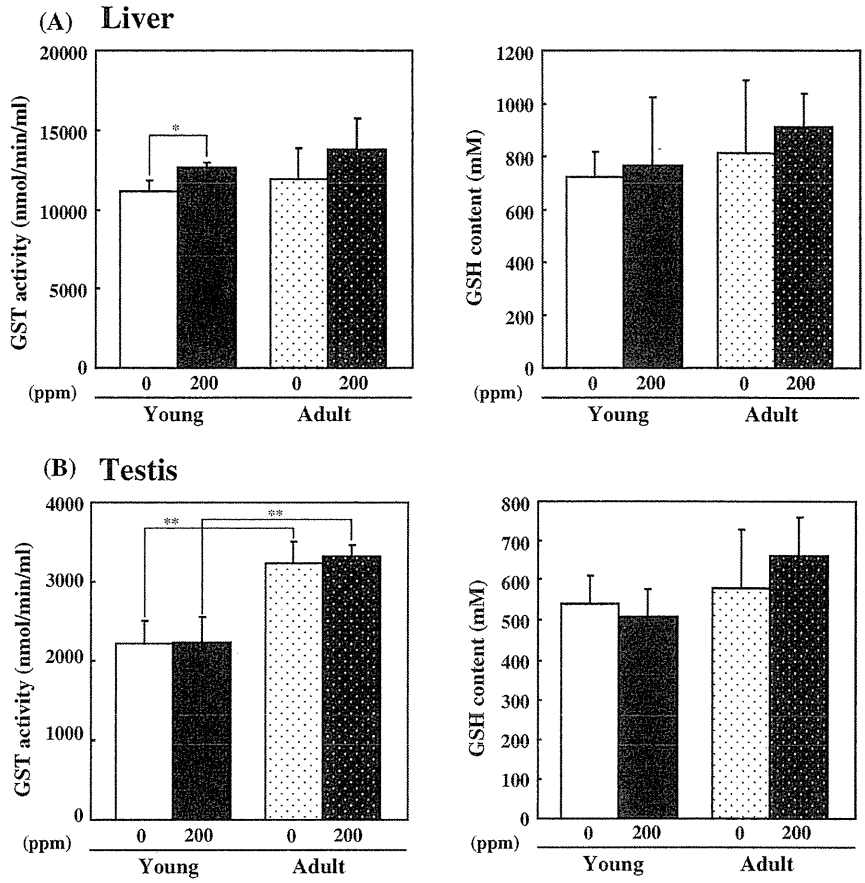

Fig. 8 GST activity and GSH contents in the liver (**a**) and testis (**b**) of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. Data are mean ± SD. *, **$P < 0.05$ and $P < 0.01$

Acknowledgments This work was supported by Health and Labour Sciences Research Grants (Research on Food Safety) from the Ministry of Health, Labour and Welfare of Japan. We thank Miss Ayako Kaneko for technical assistance in conducting the animal study.

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Reply to Letter to the Editor

Reply to Comment on "Impaired oligodendroglial development by decabromodiphenyl ether in rat offspring after maternal exposure from mid-gestation through lactation" [Reprod. Toxicol. 31(1) (2011) 86–94]

Keywords:
Decabromodiphenyl ether (DBDE)
Brominated flame retardants (BFRs)
Impaired brain development
Developmental neurotoxicity (DNT)
Maternal exposure
Hypothyroidism
White matter hypoplasia
Rat

We thank Biesemeier et al. for their interests in our recent report regarding the developmental toxicity study of decabromodiphenyl ether (DBDE), one of the representative brominated flame retardants (BFRs), indicating impaired oligodendroglial development in rat offspring after maternal exposure from mid-gestation through lactation [1]. Biesemeier and his colleague have also recently reported an oral developmental neurotoxicity (DNT) study on DBDE using rats as an industry study, and found no apparent DNT at doses up to 1000 mg/kg-day [2]. They addressed four points on our study in their comment: (1) failure to control for potential litter effects; (2) citation of study reports that failed to control for litter effect; (3) effect of data variability between studies conducted in our laboratory in relation with small sample size; and (4) lack of further studies regarding the brain changes in relation with functional abnormalities. We persevere in our main conclusion and give the following explanations and clarification.

First of all, we have to state that our DBDE study by Fujimoto et al. [1] has been performed in a series of our studies to establish a rapid screening system of DNT. As we have mentioned in the Introduction section, regular DNT studies require large numbers of animals for detection of subtle dose–response changes. For screening purposes of many new chemicals, smaller scale studies, preferably with short-term experiments, employing suitable and sensitive neurodevelopmental endpoints focusing on histopathological parameters need to be established. For histopathological analysis including immunohistochemistry, we, in our DBDE study, designed to use 10 pups or more/group/time point [1]. Similar number of animals (10 pups/sex/group/time point) was recommended to use to the neuropathology endpoint in a recent DNT testing guideline developed by Organisation for Economic Co-operation and Development (OECD) [3].

OECD testing guideline for DNT includes morphometric analysis on brain tissues as well as conventional neuropathological analysis; however, any efficient histopathological parameters based on the mechanism of DNT have remained optional [3]. Thus, our studies were focused on establishing a rapid and sensitive screening system of DNT based on the detection of functional abnormalities of the brain cellular components in a small scale animal study. As an experimental model to build up an evaluation system, we utilized a model of developmental hypothyroidism because of its unique influence on neuronal migration and oligodendroglial development, both of which are essential for brain development [4–6]. The outcome of this type of impairment is permanent and is accompanied by apparent structural and functional abnormalities. Also, some environmental chemicals, such as BFRs, are thought to potentiate a thyroid hormone-disrupting effect that may lead to abnormal brain development [7]. Therefore, there is a growing concern regarding the DNT of these chemicals [8,9].

With regard to the failure to control for potential litter effects, we understand the viewpoint by Biesemeier et al. that the DNT should be evaluated using sufficient number of animals avoiding possible litter effect (1 pup from 1 dam/group). In the study by Fujimoto et al. [1], 8 dams were used per group, and 10 male and 10–11 female offspring were examined at both PND 20 and PND 77 in every group. Therefore, 2 pups each were provided from 2 or 3 dams (mostly 2 pups each from 2 dams) in addition to 1 pup each from 6 dams in each group for examination. With regard to the data that have shown statistically significant difference between the untreated controls and treatment groups by analysis using the individual animal as the experimental unit, we recalculated them using the litter as the experimental unit. As a result, we could not find any difference in the items showing significant difference between the use of individual animal and the litter as the experimental unit (Supplementary data: Tables 1–3). In addition, inconsistency in the study design between the reports by Teshima et al. [10] and Fujimoto et al. [1] was due to the mistakes in the former study. Description in the latter study was correct, and therefore, speculation of the potential litter effects with $n=6$ dams/group by Biesemeier et al. is not the case.

With regard to the citation of study reports that have been judged to fail to control for litter effect by others, a series of studies by Eriksson and/or Viberg [11–17] as pointed out by Biesemeier et al. have employed direct oral administration of test compound to delivered pups, and therefore, potential litter effect due to the difference in the prenatal environment among litters was considered to be rather low as compared with the exposure cases from prenatal periods. In experimental rodent studies using animals with identical (or mostly identical) genetic background, potential litter effect due to genetics as pointed out by Biesemeier et al. may not be the case. On the other hand, as pointed out by Johnson et al. [18], the study by Xing et al. have failed to control for litter effect [19]. We have cited the report by Viberg et al. [15] to discuss the possibility of the direct effect of DBDE on the brain to cause white matter hypoplasia in the study by Fujimoto et al. [1]. Viberg et al. [15] have

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shown increases of the amount of radioactivity after neonatal exposure to [U-^{14}C]DBDE in the brain during the first week after single administration; however, questions have been raised by others on the analytical methodology applied and data calculation [20,21]. As we have discussed [1], the biological relevance to our study results are unclear regarding the neurobehavioral effects in both mice and rats by Viberg et al. after single neonatal exposure to DBDE [15,16]. Although further study should be addressed regarding the role on developmental neurotoxic effects, Zhang et al. [22] recently reported tissue distribution including the brain of DBDE and its debrominated metabolites in sucking rat pups after prenatal and/or postnatal exposure, suggesting the possibility of direct effect of DBDE on the brain. In another recent study, Ibhazehiebo et al. [23] reported that DBDE disrupted thyroid hormone-mediated transcription in a reporter gene assay in fibroblast derived CV-1 cells, at concentrations as low as 0.01 nM. Such effect appeared to be due to interference with the thyroid receptor–DNA binding domain, rather than to competitive inhibition of T3-binding to the thyroid receptor or to alterations of thyroid receptor–cofactor binding. At similar low concentrations, they also reported that DBDE was found to inhibit thyroid hormone-induced dendrite arborization of Purkinje cells in vitro. However, as we have mentioned in the conclusion, mild hypoplastic changes of the white matter due to decreased number of oligodendrocytes observed in our DBDE study might be the hypothyroidism-related effects of DBDE because of sustained mild hypothyroidism at least at the high dose group [1].

The study conducted under the compliance of Good Laboratory Practice provides reliability in the data with regard to the parameters examined. It is said that DNT testing has been proven to be effective in identifying compounds with DNT potential [24]. However, it does not mean that current DNT testing guidelines [3,25] cannot be improved. Indeed, there is still controversy regarding the sensitivity; one has pointed out that they may be overly sensitive and produce a high rate of false positives [26], but another pointed out that they may be too insensitive and not enough comprehensive [27]. From neuropathology point of view, positive results obtained from simple morphometry as used in the DNT study conducted by Biesemeier et al. [2] may provide some evidence of subtle alterations of the brain components; however, it may sometimes be difficult to identify the target mechanism by this type of morphometry as well as by the conventional histopathological analysis. Therefore, interpretation of such morphometric changes in relation with cellular morphology and function becomes difficult, and toxicological relevance of such data may sometimes be judged to be low or negligible. Also it should be noted that the lack of changes just provides evidence of no effects on the parameters examined. Thus, there still is a room for improving the DNT testing guidelines by adding mechanism-based histopathological analysis, such as for thyroid hormone-disrupting chemicals.

On the other hand, Biesemeier et al., in their DNT study of DBDE [2], showed statistically significant decreases in the hemisphere height from the lowest dose (1 mg/kg body weight-day) in male offspring on PND 72 (Supplementary data: Table 4). At the dose of 1000 mg/kg body weight-day, male offspring also showed statistically significant decrease in the pons vertical thickness at PND 21 and cortex vertical thickness at PND 72 (Supplementary data: Table 4). Authors have concluded that these morphometric changes were not adverse, because all of statistically significant values were within the range of the laboratory's historical control data, and, in contrast, some of the control values were higher than the historical control values. Although this kind of morphometric data should have been discussed firstly in relation with cellular morphology and function of the brain area measured, such kind of discussion was lacking in their report. Moreover, they did not show historical control data as supplementary information. Although the morphometric method applied does not clarify the target cellular component(s), reduction in the height or thickness may be the reflection of the volume reduction of the cortex or white matter. It may be reasonable to apply morphometric analysis regarding oligodendroglial development in the brain tissue samples using immunohistochemistry with 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) that has been previously validated by us using a model of developmental hypothyroidism [28]. We have found reduction in the area of corpus callosum stained with CNPase as well as the number of CNPase-positive oligodendrocytes distributed in the cingulate deep cortex in our DBDE study [1], similarly to the hypothyroidism cases [28]. Moreover, because thyrotoxic effect is reported with DBDE [29] as observed in our DBDE study, Biesemeier et al. should have examined thyroid parameters in their study, such as weight measurement and histopathology of the thyroid and serum levels of thyroid-related hormones, in offspring; however, their study lacked such information.

With regard to the third comment on the effect of data variability between studies conducted in our laboratory in relation with small sample size, Biesemeier et al. pointed out variability in the control values of our already published studies regarding offspring (male) body and relative liver weights on postnatal day (PND) 20 at weaning; serum levels of thyroid-related hormones (triiodothyronine, thyroxine, and thyroid-stimulating hormone) in male offspring at both PND 20 and postnatal week 11; and corpus callosum area and CNPase-positive oligodendrocytes. There may be minor but unavoidable multifactorial influence operating on these differences. We think variability of data between studies may preferentially arise in the absolute values, such as in absolute body weight, levels in thyroid-related hormones, area of corpus callosum in our studies, and use of different animal lots between studies may be operated on such variability. With regard to serum levels of thyroid-related hormones, measurement at different time points may also cause variability between the studies. In terms of relative liver weights, we do not think there is variability between the studies. With regard to the counts of CNPase-positive oligodendrocytes, in which values were normalized by the area measured, difference in the immunohistochemistry conditions, especially of the visualization of immunoreactivity, may cause variability between the studies. As Biesemeier et al. pointed out, litter effects may arise in the offspring values of our data; however, as shown in Supplementary data (Tables 1–3), we found that the litter effect was negligible in numerical values, even with small sample size (i.e., $n = 8$ dams/group).

With regard to the fourth comment on the lack of further studies regarding the brain changes in relation with functional abnormalities, we could say our study purpose was different from that of large scale DNT study. As mentioned above, the purpose of our studies was to establish a small scale efficient screening system of environmental chemicals, based on the pathological mechanism of DNT. As one approach for this scope, we focused on the responses in parameters related to neuronal migration and oligodendroglial development utilizing a model of developmental hypothyroidism [1,28,30–32]. In addition, using the same animal samples of the study by Fujimoto et al. [1], we found distribution changes of the subpopulation of interneurons in the hilus of the dentate gyrus by DBDE, suggestive of aberration of neuronal migration probably due to aberration of neurogenesis in the subgranular zone of the dentate gyrus, at doses of 100 ppm and higher (Y. Saegusa et al., data submitted), similarly to the development of mild white matter hypoplasia as reported by Fujimoto et al. [1]. However, the effect on neuronal development itself was found to be reversible, while a sustained increase of mature neurons was detected in the dentate hilus.

In summary, as Biesemeier et al. pointed out, litter effects could have been operated on the observed changes in the study by Fujimoto et al. [1]; however, recalculation of the data using the litter as the experimental unit revealed no changes in items showing statistically significant difference. With regard to the citation of study reports that have been judged to fail to control for litter effect by others, the study by Xing et al. [19] has actually failed to control for potential litter effects; however, a potential litter effect derived from prenatal environment in a series of postnatal exposure studies by Eriksson and/or Viberg was considered to be rather low as compared with the exposure cases from prenatal periods. We have cited them as an example of the direct brain effect of DBDE; however, our results supported the possibility of hypothyroidism-related effect as described in the conclusion of the study by Fujimoto et al. [1], despite the recent study results suggestive of the direct effect on the brain. With regard to the possible effect of variability of data between studies conducted in our laboratory in relation with the use of small number of animals, we think that minor but unavoidable multifactorial influence may be operated. We here judge that the litter effects were negligible in our experimental system even with small sample size. With regard to the lack of further studies regarding the brain changes in relation with functional abnormalities, we could say that our study was aimed at different endpoint from that of large-scale DNT study. The purpose of our studies was to establish a new screening system in a small scale animal study applying morphometrically measurable parameters selected based on the mechanism of DNT.

In closing, comments raised by Biesemeier et al. on our recently published study regarding developmental toxicity of DBDE were revealed to be rather minor ones or on the point out of the scope of our study. We rather recommend them to perform additional assessment on their recently published DNT study of DBDE regarding histopathological parameters on neuronal migration and oligodendroglial development that have been proven to fluctuate in relation with developmental hypothyroidism [28,32].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2011.06.074.

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Original Article

Adolescent hyperactivity of offspring after maternal protein restriction during the second half of gestation and lactation periods in rats

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ABSTRACT — To clarify the effect of systemic growth retardation on behavior, pregnant rats were fed a synthetic diet with either a normal (20% casein) or low (10% casein) protein concentration from gestational day 10 to postnatal day (PND) 21 at weaning. Offspring were examined for sensory and reflex functions, detailed clinical observations, manipulative test, grip strength, motor activity and water-filled multiple T-maze test. Lowering trend in the air righting reflex index during lactation period and a decrease in grip strength on PND 72 were observed in the low protein diet group showing suppression of systemic growth. However, they were simply the reflection of delayed systemic growth, because parameters on impaired reflex function, disturbance of motor function and paralysis were unaffected. On the other hand, low protein diet resulted in increased motor activity in female offspring. Thus, malnutrition due to maternal protein restriction may cause adolescent hyperactivity.

Key words: Systemic growth retardation, Maternal protein restriction, Behavior

INTRODUCTION

Malnutrition at perinatal period is the major concern for various neurobehavioral disorders after adolescence. A number of experimental studies and epidemiological researches were conducted to investigate the relationship between perinatal malnutrition and neurobehavioral disorders. Lahti *et al.* (2006) reported that small body size at birth may increase the behavioral symptoms of attention deficit hyperactivity disorder (ADHD). Prenatal exposure to famine was reported to increase the risk of schizophrenia (St Clair *et al.*, 2005; Susser *et al.*, 2008), affective disorder (Brown *et al.*, 2000), antisocial personality disorder (Neugebauer *et al.*, 1999) and addiction (Franzek *et al.*, 2008). Experimental evidence should be accumulated to clarify the relationship between perinatal malnutrition and neurological dysfunction including behavioral abnormalities.

In order to establish an *in vivo* evaluation system for developmental neurotoxicity, we focused on neurogenesis and neuronal migration in the hippocampal dentate gyrus in rats exposed maternally to various xenobiotics. An increase in reelin-synthesizing γ-aminobutyric acid (GABA)-ergic interneurons with immature phenotype to sustain into the later adult stage was observed in the dentate hilus in the offspring of maternal rats exposed to anti-thyroid agents (Saegusa *et al.*, 2010). Considering the role of reelin in regulating migration and correct positioning of developing neurons (Rice and Curran, 2001), this result suggested a compensatory mechanism for the impaired neurogenesis and mismigration caused by exposure to thyroid hormone-disrupting chemicals during neuronal development. However, developmental hypothyroidism also causes a suppression of systemic growth (Shibutani *et al.*, 2009). Systemic growth suppression causes delayed brain growth that may result in affection of neurogenesis. In our previous study, we have found no effects on the distribution of reelin-synthesizing GABAergic interneurons in the dentate hilus and postnatal neurogenesis in the subgranular zone (SGZ) in rats until adult stage by maternal protein restriction during the 2nd half of gestation and lactation causing systemic growth retar-

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dation sustained through to the adult stage and retarded brain growth at weaning (Ohishi *et al.*, 2010). However, functional evidence was not obtained with regard to behavioral endpoints involving dentate gyrus function.

The present study was conducted to clarify whether systemic growth retardation affects brain function by analysis of behavioral endpoints in offspring of maternal rats fed a synthetic low-protein diet from the mid-gestation to the end of the lactation period to cause growth restriction in the offspring utilizing an intrauterine growth restriction model (Haugaard and Bauer, 2001; Zambrano *et al.*, 2005).

MATERIALS AND METHODS

Animals, diets and experimental design

The animal experiment was identical to that previously reported (Ohishi *et al.*, 2010). In brief, pregnant Crl:CD®(SD) rats were purchased from Charles River Japan Inc. (Kanagawa, Japan) at gestational day (GD) 1 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in mesh cages in an air-conditioned animal room (temperature: 23 ± 2°C; relative humidity: 45 ± 10%) with a 12-hr light/dark cycle and were allowed *ad libitum* access to food and tap water. Animals were housed individually with their litter in plastic cages with wood chip bedding from GD 17 to postnatal day (PND) 21.

Pregnant rats were fed a CRF-1 basal diet (Oriental Yeast Co. Ltd., Tokyo, Japan) from GD 1 to GD 10. Eight dams per group were then randomly divided into two groups and fed a synthetic diet with either a normal (20% casein) or a low (10% casein) protein concentration from GD 10 to PND 21 (where PND 0 is the day of delivery).

On PND 4, the litters were culled randomly, leaving 4 male and 4 female offspring per dam. On PND 21, 2 male and 2 female offspring per dam were subjected to prepubertal necropsy. The remaining 2 male and 2 female offspring were weaned on PND 21 and kept through PND 77. All offspring consumed the CRF-1 basal diet and tap water *ad libitum* from PND 21 onwards.

All procedures of this study were conducted in compliance with the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee at BOZO Research Center Inc. All efforts were made to minimize animal suffering.

Sensory and reflex functional examinations

During the lactation period, sensory and reflex functional examination was conducted for 2 male and 2 female offspring in each litter.

Surface righting reflex was examined on PND 10 by measuring the time required to return to a normal position.

Air righting reflex was examined on PND15 and pupillary reflex, Preyer's reflex and pain reflex were examined on PND 21. The air righting reflex was assessed to examine the normal landing response of an animal to right itself from an inverted position in free fall of about 300 mm height. The pupillary reflex was assessed to examine the normal miotic response to the light. The Preyer's reflex was assessed to examine the normal pinna or startle response to the sound of the Galton's whistle. The pain reflex was assessed to examine the normal response such as avoiding and vocalization to the pinching stimuli of the tail.

Detailed clinical observations

Detailed clinical observations were conducted for all remaining animals (2 male and 2 female offspring in each litter) on PND 30, 44 and 72.

In home cage observations, animals were observed for posture, convulsion and abnormal behavior. During observation, animals were observed for ease of removal from cage, fur condition, skin, secretions of the eyes and nose, exophthalmos, palpebral closure, visible mucosal membranes, autonomic nervous functions (lacrimation, piloerection, pupil size, salivation, abnormal respiration), and vocalization and reactivity to handling.

Manipulative testing

Following the detailed clinical observations on PND 72, all animals were examined for auditory response, visual approach response, touch response, tail pinch response, pupillary reflex (light reflex) and air righting reflex and measured landing foot splay (hind foot).

The auditory response was assessed to examine the normal startle response to clackety-clack stimuli. The visual approach response was assessed to examine the normal response such as sniffing or avoiding to the pen approaching the nose. The touch response was assessed to examine the normal response such as avoiding or soft vocalization to the pen touching the abdomen. The tail pinch response was assessed to examine the normal response such as quick avoiding and vocalization. The pupillary reflex and air righting reflex were assessed as mentioned above.

Grip strength

Following the manipulative testing on PND 72, grip strengths of the forelimbs and hind limbs in all animals were measured using a CPU gauge MODEL-RX-5 (Aikoh Engineering Co., Ltd., Osaka, Japan).

Motor activity

Following the measurement of grip strength on PND 72, motor activity was measured using an experimental animal motor activity sensor NS-AS01 (NeuroScience Inc., Tokyo, Japan). The length of measurement was 1 hour. Values of 10-minute intervals and the 0-60 min value were recorded.

Water-filled multiple T-maze testing

Examination was conducted using Biel's water maze (Biel, 1940) from PND 55 to 58. Elapsed time to reach the goal and error count (number of times the whole body entered into the error area of T-maze course) were measured in three trials each day in the straight course on the 1st day (PND 55) and in the water T-maze course on the 2nd, 3rd and 4th days (PND 56, 57 and 58). In the water-filled multiple T-maze test, the maximum elapsed time for each trial was set at 3 min and the trials where animals did not reach the goal within 3 min were excluded from statistical analysis.

Statistical analysis

Numerical data were analyzed using the litter as the experimental unit. The homogeneity of variance between the normal and low protein diet groups was analyzed by F-test. If the variance was homogenous, numerical data were assessed using Student's *t*-test to compare between the normal and low protein diet groups. If a significant difference in variance was observed, the Aspin-Welch's *t*-test was used instead.

Frequency data were analyzed using the individual animal as the experimental unit and compared statistically using Fisher's exact probability test.

RESULTS

Sensory and reflex functional examinations

There were no statistically significant differences between the normal and low protein diet groups in either sex. However, the air righting reflex index in male offspring in the low protein diet group decreased as compared with that in the normal protein diet group, although statistically significant difference was not attained (Table 1).

Detailed clinical observations

There were no statistically significant differences in any items between the normal and low protein diet groups in either sex (Table 2).

Manipulative test

There were no statistically significant differences in any items between the normal and low protein diet groups in either sex (Table 3).

Grip strength

In male offspring, grip strength of the fore and hind limbs in the low protein diet group was significantly lower than that in the normal protein diet group. In female

Table 1. Functional examination of offspring after maternal protein restriction during the second half of gestation and lactation

		Males		Females	
		Normal protein 20%[c]	Low protein 10%[c]	Normal protein 20%	Low protein 10%
No. of offspring examined[a]		16	16	16	16
Surface righting reflex (PND 10, unit: sec.)		1.6 ± 0.8[b]	1.7 ± 0.9	1.9 ± 1.2	2.3 ± 2.0
Air righting reflex (PND 15)	Normal	8	4	4	5
Pupillary reflex (PND 21)	Normal	16	15	16	14
Preyer's reflex (PND 21)	Normal	16	16	16	16
Pain reflex (PND 21)	Normal	16	16	16	16

No significant differences between the normal and low protein groups.

[a] Two male and two female offspring per dam were subjected to examination.

[b] Mean ± S.D. The values were obtained using the litter mean.

[c] Casein level.

Table 2. Detailed clinical signs of offspring after maternal protein restriction during the second half of gestation and lactation

		Males		Females	
		Normal protein 20%[b]	Low protein 10%[b]	Normal protein 20%	Low protein 10%
PND 30					
No. of offspring examined[a]		16	16	16	16
Home cage observation					
Posture	Normal	16	16	16	16
Convulsion	None	16	16	16	16
Abnormal behavior	None	16	16	16	16
In-the-hand observation					
Ease of removal from cage	Easy	16	16	16	16
Fur condition	Normal	16	16	16	16
Skin	Normal	16	16	16	16
Secretions-eye, nose	Absent	16	16	16	16
Exophthalmos	Absent	16	16	16	16
Palpebral closure	Normal	16	16	16	16
Mucosal membranes	Normal	16	16	16	16
Lacrimation	Normal	16	16	16	16
Piloerection	Absent	16	16	16	16
Pupil size	Normal	16	16	16	16
Salivation	None	16	16	16	16
Abnormal respiration	Absent	16	16	16	16
Vocalization	None	16	16	15	16
	Soft	0	0	1	0
Reactivity to handling	Easy	16	16	16	16
PND 44					
No. of offspring examined[a]		16	16	16	16
Home cage observation					
Posture	Normal	16	16	16	16
Convulsion	None	16	16	16	16
Abnormal behavior	None	16	16	16	16
In-the-hand observation					
Ease of removal from cage	Easy	16	16	16	16
Fur condition	Normal	16	16	16	16
Skin	Normal	16	16	16	16
Secretions-eye, nose	Absent	16	16	16	16
Exophthalmos	Absent	16	16	16	16
Palpebral closure	Normal	16	16	16	16
Mucosal membranes	Normal	16	16	16	16
Lacrimation	Normal	16	16	16	16
Piloerection	Absent	16	16	16	16
Pupil size	Normal	16	16	16	16
Salivation	None	16	16	16	16
Abnormal respiration	Absent	16	16	16	16
Vocalization	None	15	16	14	15
	Soft	1	0	2	1
Reactivity to handling	Easy	16	16	16	16
PND 72					
No. of offspring examined[a]		16	16	16	16
Home cage observation					
Posture	Normal	16	16	16	16
Convulsion	None	16	16	16	16
Abnormal behavior	None	16	16	16	16
In-the-hand observation					
Ease of removal from cage	Easy	16	16	16	16
Fur condition	Normal	16	16	16	16
Skin	Normal	16	16	16	16
Secretions-eye, nose	Absent	16	16	16	16
Exophthalmos	Absent	16	16	16	16
Palpebral closure	Normal	16	16	16	16
Mucosal membranes	Normal	16	16	16	16
Lacrimation	Normal	16	16	16	16
Piloerection	Absent	16	16	16	16
Pupil size	Normal	16	16	16	16
Salivation	None	16	16	16	16
Abnormal respiration	Absent	16	16	16	16
Vocalization	None	16	16	16	16
Reactivity to handling	Easy	16	16	16	16

No significant differences between the normal and low protein groups.

[a] Two male and two female offspring per dam were subjected to examination. [b] Casein level.

Table 3. Manipulative test of offspring after maternal protein restriction during the second half of gestation and lactation

		Males		Females	
		Normal protein 20%[c]	Low protein 10%[c]	Normal protein 20%	Low protein 10%
No. of offspring examined[a]		16	16	16	16
Auditory response	Normal	16	16	16	16
Approach response	Normal	16	16	16	16
Touch response	Normal	16	16	16	16
Tail pinch response	Normal	16	16	16	16
Pupillary reflex	Normal	16	16	16	16
Air righting reflex	Normal	16	16	16	16
Landing foot splay (mm)		86 ± 13[b]	91 ± 15	66 ± 23	65 ± 9

No significant differences between the normal and low protein groups.
[a] Two male and two female offspring per dam were subjected to examination.
[b] Mean ± S.D. The values were obtained using the litter mean.
[c] Casein level.

offspring, there were no statistically significant differences between the normal and low protein diet groups (Table 4).

Motor activity

In male offspring, there were no statistically significant differences between the normal and low protein diet groups. In female offspring, statistically significant high values were recorded in the motor activities at 0-10 and 30-40 min and the 0-60 min total motor activities in the low protein diet group (Table 5).

Water-filled multiple T-maze test

There were no statistically significant differences in any trials between the normal and low protein diet groups in either sex (Table 6).

DISCUSSION

We previously reported that maternal protein restriction resulted in systemic growth retardation in offspring from birth to PND 77 (Ohishi *et al.*, 2010). Throughout the lactation period and also after weaning, body weights of the offspring of both sexes were significantly lower in the low protein diet group than in the normal protein diet group (Supplementary Table 1). Maternal food restriction was reported to cause decreases in surface righting reflex, negative geotaxis reflex and cliff avoidance reflex indices (Zhang *et al.*, 2010). In the present study, sensory and reflex functional examinations showed a tendency to suppress the air righting reflex index on PND 15 in male offspring in the low protein diet group. However, we found no obvious abnormalities in other reflex parameters of sensory and reflex function in males. Females were unaffected with these parameters. Furthermore, manipulative test including air righting reflex on PND 72 revealed no changes. Considering the severe growth suppression by maternal protein restriction (Ohishi *et al.*, 2010), showing approximately 30% suppression in the body weight on PND 14 in males as compared with normal protein diet group, delayed growth may be responsible for this non significant fluctuation.

In the present study, grip strength was examined on PND 72 and revealed a decrease in male offspring in the low protein diet group. On the other hand, detailed clinical observation such as ease of removal from cage, manipulative test such as landing foot splay, motor activity and water-filled multiple T-maze test revealed no disturbance of motor function or paralysis. Considering that the body weight on PND 70 in male offspring in this group was 16% lower than that of the normal protein diet group (Ohishi *et al.*, 2010), lowered grip strength was considered to be due to the difference of body growth including gripping ability between the normal and low protein diet groups.

With regard to the motor activity, high values in the low protein diet group were observed in female offspring. Increase in exploratory activity has been reported in rat offspring after maternal protein restriction during lactation period (Franková and Barnes, 1968). Pre- and/or postnatal protein restriction in rats increased open arm entries, time and distance in elevated plus maze test, suggesting decreased avoidance (less anxiety) and increased

Table 4. Grip strength of offspring after maternal protein restriction during the second half of gestation and lactation

	Males		Females	
	Normal protein 20%[c]	Low protein 10%[c]	Normal protein 20%	Low protein 10%
No. of offspring examined[a]	16	16	16	16
Fore limb (g)	1297 ± 167[b]	1107 ± 159*	932 ± 172	992 ± 142
Hind limb (g)	899 ± 69	778 ± 122*	673 ± 55	612 ± 104

Significantly different from the normal protein group by Student's or Aspin-Welch's t-test ($P < 0.05$).
[a] Two male and two female offspring per dam were subjected to examination.
[b] Mean ± S.D. The values were obtained using the litter mean.
[c] Casein level.

Table 5. Motor activity of offspring after maternal protein restriction during the second half of gestation and lactation

	Males		Females	
	Normal protein 20%[c]	Low protein 10%[c]	Normal protein 20%	Low protein 10%
No. of offspring examined[a]	16	16	16	16
Total (0-60 minutes)	2319 ± 125[b]	2410 ± 188	1960 ± 168	2241 ± 171**
0-10 min	452 ± 23	446 ± 27	379 ± 35	434 ± 26**
10-20 min	404 ± 18	424 ± 41	344 ± 49	389 ± 40
20-30 min	386 ± 42	415 ± 45	330 ± 35	366 ± 41
30-40 min	359 ± 28	379 ± 41	310 ± 51	398 ± 61**
40-50 min	374 ± 38	371 ± 64	303 ± 47	328 ± 50
50-60 min	344 ± 47	375 ± 39	295 ± 45	326 ± 70

Significantly different from the normal protein group by Student's or Aspin-Welch's t-test ($P < 0.01$).
[a] Two male and two female offspring per dam were subjected to examination.
[b] Mean ± S.D. The values were obtained using the litter mean.
[c] Casein level.

exploration (Reyes-Castro *et al.*, 2011). In another study using the similar elevated plus maze test, growth restriction through the maternal deficiency of methyl donors, i.e., choline, folate and methionine, showed an increase in anxiety-related behaviors in rats (Konycheva *et al.*, 2011). In mice, maternal protein restriction resulted in persistent alterations in dopamine circuitry and increases in dopamine-dependent behaviors (Vucetic *et al.*, 2010). In human, small body size at birth might increase the susceptibility to behavioral symptoms of ADHD (Lahti *et al.*, 2006). Therefore, hyperactivity in female rat offspring in the present study may be related to malnutrition due to maternal protein restriction. Motor activity is regulated by synaptic circuitry of multiple brain areas. Brain imaging of ADHD cases suggested decreases in total brain size and the volume of the multiple brain substructures (Giedd *et al.*, 2001). However, we did not find the change in the absolute brain weight on PND 77 in our present cases (Ohishi *et al.*, 2010), suggesting the effect of female hyperactivity was functional one that do not alter the brain substructures. Although we did not find the effects on the motor activity in male offspring, there are many experimental examples that show gender difference in the response of locomotor activity with high reactivity in females (Becker *et al.*, 1982; Lukas and Wetherington, 2005; Mandt *et al.*, 2009).

With regard to the water-filled multiple T-maze test, there were no effects of maternal protein restriction. In contrast to our results, impaired learning and memory behavior was observed in a study using Morris water maze by maternal protein restriction using 6% protein diet (Wang and Xu, 2007). In the present study, we used 10% casein diet for maternal protein restriction. In addition, maze test was conducted between postnatal week 4 and 5 in the study by Wang and Xu, while it was postnatal week 7and 8 (from PND 55 to 58) in our case. These differences might be responsible for the difference in the result between the two studies. We, using the identi-

Table 6. Water-filled multiple T-maze test of offspring after maternal protein restriction during the second half of gestation and lactation

		Males		Females	
		Normal protein 20%[c]	Low protein 10%[c]	Normal protein 20%	Low protein 10%
No. of offspring examined[a]					
1st day (Straight maze)					
1st trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	14.7 ± 3.4[b]	12.9 ± 3.3	15.7 ± 2.4	17.9 ± 3.8
2nd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	6.6 ± 0.8	8.6 ± 2.9	7.6 ± 1.9	9.1 ± 4.6
3rd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	7.3 ± 3.2	6.8 ± 2.3	6.9 ± 2.7	5.3 ± 0.5
2nd day (T-maze)					
1st trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	51.1 ± 22.2	44.5 ± 13.8	57.1 ± 19.4	61.8 ± 22.7
	Counts of error	3.7 ± 1.5	2.8 ± 0.6	4.5 ± 1.0	4.6 ± 1.9
2nd trial	No. of animals that reached the goal	16	15	16	16
	Elapsed time (sec.)	45.9 ± 9.1	61.9 ± 32.2	45.5 ± 13.8	41.8 ± 22.8
	Counts of error	3.3 ± 0.8	4.4 ± 2.8	3.4 ± 1.4	3.1 ± 2.1
3rd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	28.6 ± 10.0	34.3 ± 23.2	21.9 ± 8.5	26.6 ± 13.8
	Counts of error	1.8 ± 0.8	1.9 ± 1.5	0.9 ± 1.0	1.7 ± 1.5
3rd day (T-maze)					
1st trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	32.3 ± 22.8	26.0 ± 9.5	23.9 ± 6.9	23.6 ± 8.5
	Counts of error	2.3 ± 2.2	1.9 ± 1.2	2.1 ± 0.9	2.0 ± 1.4
2nd trial	No. of animals that reached the goal	16	15	16	16
	Elapsed time (sec.)	16.5 ± 2.7	18.3 ± 3.1	20.6 ± 6.1	15.6 ± 5.7
	Counts of error	0.8 ± 0.5	0.8 ± 0.3	1.3 ± 0.7	0.6 ± 0.6
3rd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	14.3 ± 1.8	15.1 ± 1.8	19.0 ± 5.5	14.6 ± 3.1
	Counts of error	0.3 ± 0.4	0.5 ± 0.5	0.9 ± 0.6	0.4 ± 0.4
4th day (T-maze)					
1st trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	13.8 ± 4.0	24.1 ± 19.4	20.9 ± 13.4	16.5 ± 4.1
	Counts of error	0.7 ± 0.7	1.8 ± 2.6	1.3 ± 2.1	0.8 ± 0.7
2nd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	14.3 ± 2.8	17.1 ± 5.5	22.3 ± 9.9	19.5 ± 6.6
	Counts of error	0.5 ± 0.4	0.7 ± 1.2	1.1 ± 1.2	0.8 ± 0.8
3rd trial	No. of animals that reached the goal	16	16	16	16
	Elapsed time (sec.)	14.5 ± 3.8	14.6 ± 1.6	16.5 ± 3.2	15.4 ± 4.2
	Counts of error	0.5 ± 0.5	0.3 ± 0.3	0.3 ± 0.4	0.3 ± 0.4

No significant differences between the normal and low protein groups.

[a] Two male and two female offspring per dam were subjected to examination.

[b] Mean ± S.D. The values were obtained using the litter mean.

[c] Casein level.

cal study samples, previously reported no effect of maternal protein restriction on the distribution of reelin-synthesizing GABAergic interneurons in the dentate hilus and postnatal neurogenesis in the SGZ through PND 77 by immunohistochemical analysis (Ohishi *et al.*, 2010), suggesting no functional affection of the hippocampal dentate gyrus. The hippocampus is known to play important roles in memory, learning and spatial navigation. There-

fore, our immunohistopathological results on hippocampal neurogenesis support the absence of any abnormalities in the water-filled multiple T-maze test in the present study.

In summary, maternal protein restriction caused lowering trend in the air righting reflex index during lactation period and a decrease in grip strength. However, they were unlikely to be the reflection of impaired sensory and reflex function, disturbance of motor function or paralysis. Delayed growth may rather be responsible for these fluctuations. While learning and memory function was unaffected, maternal protein restriction may cause adolescent hyperactivity because motor activity increased in female offspring in the low protein diet group.

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