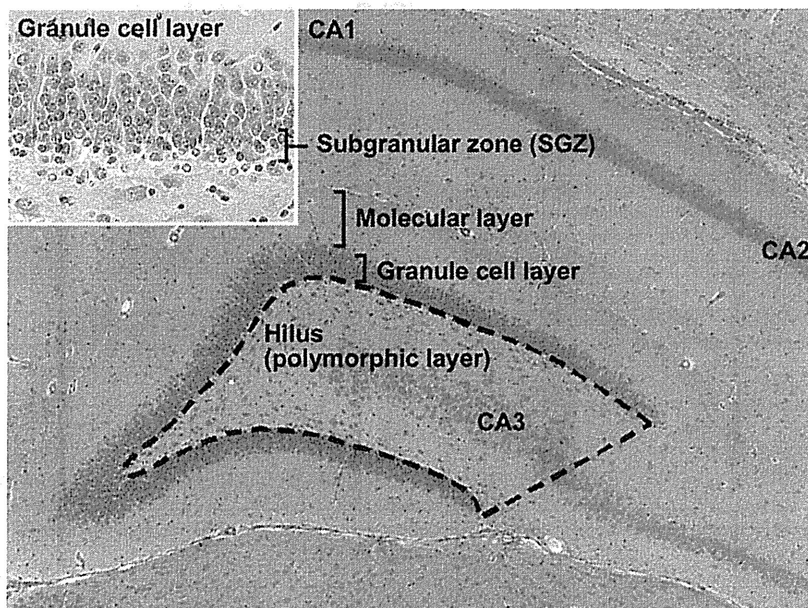

Fig. 1. Overview of the hippocampal formation of a male rat at PND 21 stained with hematoxylin and eosin. The numbers of cells in the hilus of the dentate gyrus (as demarcated by the dotted line) displaying immunoreactivity for reelin, GAD67, and NeuN were counted and normalized for the unit area. Positive immunoreactivity for these antigens was restricted to small-sized neurons in this area, as larger CA3 neurons were not immunoreactive. Magnification, ×40. (Inset) Higher magnification of the granular cell layer and SGZ. Distribution of immunoreactive cells for DCX, Tbr2 and GFAP as well as apoptotic cells and proliferating cells was measured in the SGZ. Magnification, ×400.

cellular component, digital photomicrographs at ×100 magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co.). Quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

2.8. Real-time RT-PCR analysis

For real-time RT-PCR analyses, the cerebrum of male offspring was removed at pre-pubertal necropsy on PND 21 (n = 5 or 6/group) and was fixed with methacarn solution for 8 h at 4 °C [25]. Then the hippocampus was removed and stored in ethanol at −80 °C. Analysis of mRNA levels for molecules shown in Supplementary Table 2 was performed with real-time RT-PCR in the hippocampal tissues. *Dcx*, *Neurod1*, *Pax6* and *Dpysl3* were genes encoding neuronal-stage defining marker molecules [21,26,27]. *Reln*, *Vldlr*, *Lrp8* and *Dab1* were genes encoding reelin and its receptors (*Vldlr*, *Lrp8*) and intracellular adaptor (*Dab1*) [28]. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA in the presence of dithiothreitol, deoxynucleoside triphosphate (dNTP), random primers, RNaseOUT and SuperScript™ III Reverse Transcriptase (Invitrogen Corp.) in a 20-μL total reaction mixture. Real-time PCR was performed using the SYBR®Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan Ltd.) according to the manufacturer's protocol. The PCR primers shown in Supplementary Table 2 were designed using Primer Express software (Version 3.0; Applied Biosystems Japan Ltd.). The relative differences in gene expression were calculated using threshold cycle (C_T) values that were first normalized to those of the beta-actin gene, the endogenous control in the same sample, and then relative to a control C_T value by the $2^{-\Delta\Delta C_T}$ method [29].

2.9. Statistical analysis

Maternal data regarding body weight, food consumption, Mn concentration in the brain and hormonal analysis were analyzed using the individual animal as the experimental unit. Data for offspring regarding body weight, food consumption, organ weight at necropsy, quantitative data of behavioral examinations, immunohistochemical data, TUNEL-assay data, real-time RT-PCR analysis, Mn concentration in the brain and hormonal analysis were analyzed using the litter as the experimental unit. Differences between the control and each treated group were evaluated using the following methods. Bartlett's test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using Dunnett's test to compare between the control and each treated groups. If a significant difference in variance was observed, Steel's test was used instead. Frequency data from behavioral examinations were analyzed using the individual animal as the experimental unit. The number of animals that showed normal reflexes was compared statistically using Fisher's exact test.

3. Results

3.1. Maternal parameters in Experiment 1

There were no statistically significant differences in the body weight during the gestational and lactation periods (Fig. 2). For food consumption, there were no statistically significant differences during the gestational period (Fig. 2). Although statistically significant increases were noted on PND 11 at 160 and 800 ppm of Mn exposure, PND 14 at 800 ppm and PND 17 at 160 ppm (Fig. 2) during the lactation period, these were minimal fluctuations.

The $MnCl_2 \cdot 4H_2O$ intakes of dams from GD 10 to PND 21 were 4.05 mg/kg body weight/day for 32 ppm, 20.62 mg/kg body weight/day for 160 ppm and 105.14 mg/kg body weight/day for 800 ppm. In addition, the $MnCl_2 \cdot 4H_2O$ intakes of dams from GD 10 to PND 21 in Experiment 2 were 100.84 mg/kg body weight/day for 800 ppm and 210.04 mg/kg body weight/day for 1600 ppm.

3.2. Body weight, food consumption, external differentiation and organ weights of offspring in Experiment 1

No statistically significant differences were observed between each treatment and the untreated controls in body weight (Fig. 2), food consumption (Supplementary Fig. 2), external differentiation with regard to pinna detachment, eruption of lower incisor, opening of eyelid, opening of vagina, and cleavage of the balanopreputial gland (Supplementary Table 3), and organ weights (Supplementary Table 4) of offspring after birth through to PND 77.

3.3. Brain Mn-concentration in dams and offspring in Experiment 1

In dams examined on the day 21 after delivery, there were no statistically significant differences in Mn concentration in the

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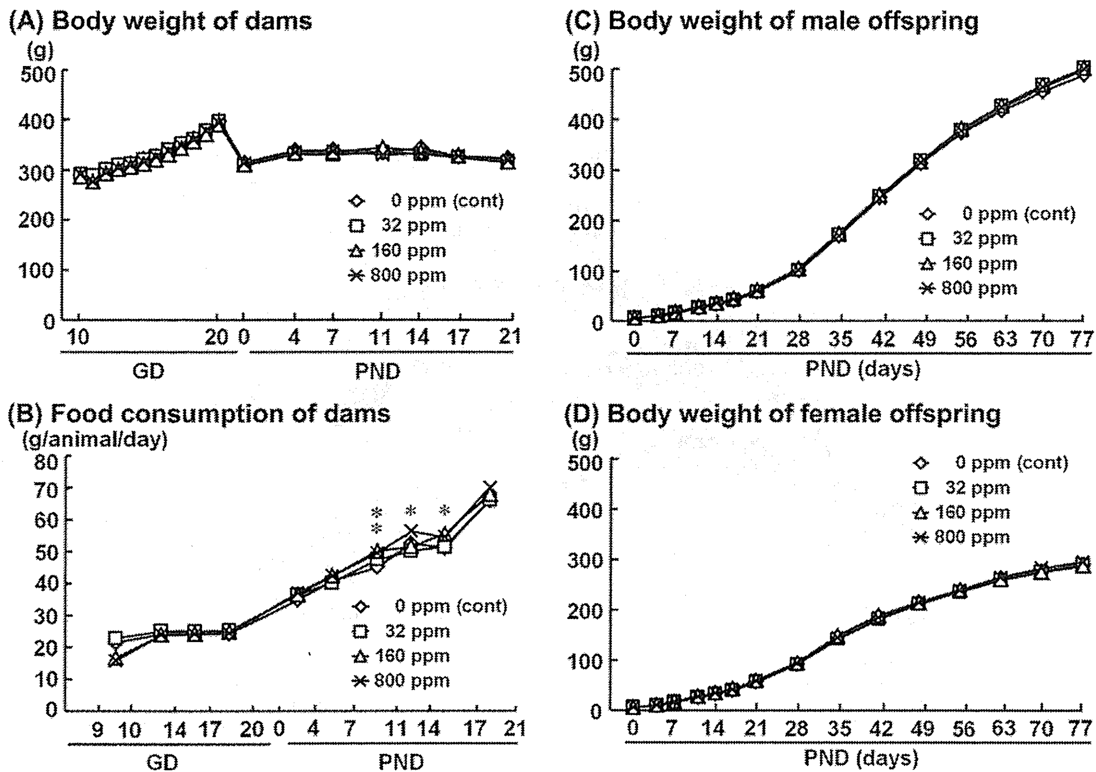

Fig. 2. Body weights and food consumption of dams and body weight of offspring exposed to $MnCl_2{\cdot}4H_2O$ from GD 10 through to day 21 after delivery. (A) Body weight of dams. (B) Food consumption of dams. (C) Body weight of male offspring. (D) Body weight of female offspring. *Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.05$).

cerebellum between the untreated controls and any treatment groups (Fig. 3). In offspring on PND 21, statistically significant increases in Mn concentrations in the cerebellum were observed at 160 and 800 ppm of $MnCl_2{\cdot}4H_2O$ exposure (Fig. 3). In offspring on PND 77, there were no statistically significant differences in Mn concentration in the cerebellum between the untreated controls and any of the treatment groups (Fig. 3).

3.4. Serum concentrations of thyroid-related hormones in Experiment 2

In dams, there were no statistically significant differences in serum concentrations of T_3, T_4 and TSH between the untreated controls and any of the treatment groups (Fig. 4). In offspring on PND 21, statistically significant decreases in serum concentrations of T_3 and T_4 were observed at 800 and 1600 ppm of $MnCl_2{\cdot}4H_2O$ exposure. Although a statistically significant increase was observed in serum concentration of TSH at 800 ppm, no significant change was observed in that at 1600 ppm (Fig. 4). In offspring on PND 77, there were no statistically significant differences in serum concentrations of T_3, T_4 and TSH between the untreated controls and any of the treatment groups (Fig. 4).

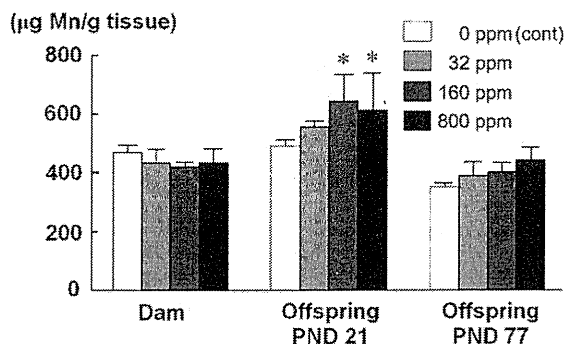

Fig. 3. Manganese concentrations in the cerebellum of dams and male offspring at PND 21 and 77 after maternal exposure to $MnCl_2{\cdot}4H_2O$ from GD 10 to PND 21. All eight dams and six male offspring (one animal per dam) were subjected to analysis in each group. *Significantly different from the untreated controls group by Dunnett's test ($P < 0.05$).

3.5. Behavioral examinations in offspring in Experiment 1

Results of sensory and reflex functional examinations are shown in Table 2. In the surface righting reflex, although a statistically significant lower value was recorded in males at 32 ppm of $MnCl_2{\cdot}4H_2O$ exposure, there were no changes in males at 160 and 800 ppm and in females at all doses. In the air righting reflex, statistically significant lower values were observed in males at 160 ppm or more and in females at 800 ppm. As for the pupillary reflex, Preyer's reflex and pain reflex, all animals showed normal responses in untreated control and all treatment groups.

Results of measurement of grip strength are shown in Table 3. In males, although a statistically significant lower value for the forelimb was recorded at 32 and 160 ppm of $MnCl_2{\cdot}4H_2O$ exposure, there were no changes at 800 ppm. In females, there were no statistically significant differences between the untreated controls and any of the treatment groups.

Results of water-filled multiple T-maze tests are shown in Fig. 5. A statistically significant shortening of the elapsed time was recorded at the 1st trial on the 2nd day in males at 800 ppm of $MnCl_2{\cdot}4H_2O$ exposure. In females, a statistically significant

Table 2
Functional examination of offspring exposed to $MnCl_2·4H_2O$ during the second half of gestation and lactation periods.

		0 ppm (control)	$MnCl_2·4H_2O$ in the diet		
			32 ppm	160 ppm	800 ppm
Males					
No. of offspring examined[a]		16	16	16	16
Surface righting reflex (PND 10, unit: s)		1.7 ± 0.7[b]	1.1 ± 0.3[*]	1.9 ± 1.0	1.3 ± 0.2
Air righting reflex (PND 15)	Normal	12	7	6[#]	4[##]
Pupillary reflex (PND 21)	Normal	16	16	16	16
Preyer's reflex (PND 21)	Normal	16	16	16	16
Pain reflex (PND 21)	Normal	16	16	16	16
Females					
No. of offspring examined[a]		16	16	16	16
Surface righting reflex (PND 10, unit: s)		1.8 ± 0.8	2.3 ± 2.0	1.7 ± 0.8	2.0 ± 1.4
Air righting reflex (PND 15)	Normal	10	8	5	3[#]
Pupillary reflex (PND 21)	Normal	16	16	16	16
Preyer's reflex (PND 21)	Normal	16	16	16	16
Pain reflex (PND 21)	Normal	16	16	16	16

Abbreviation: PND, postnatal day.
[a] Two male and female offspring per dam were subjected to examination.
[b] Mean ± SD.
[*] Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.05$).
[#] Significantly different from the control group by Fisher's exact test ($P < 0.05$).
[##] Significantly different from the control group by Fisher's exact test ($P < 0.01$).

prolongation of the elapsed time and increased counts of error were recorded at the 1st trial on the 2nd day at 800 ppm.

Results from the detailed clinical observations are shown in Supplementary Tables 5–7. There were no differences between the untreated controls and any of the treatment groups for any of these items. Results of manipulative tests are shown in Supplementary Table 8. In the auditory response, approach response, touch response, tail pinch response, pupillary reflex or aerial righting reflex, all animals showed normal responses in the control and all treated groups. In the landing foot splay, there were no statistically significant differences between the untreated controls and any of the treatment groups. Results of measurement of motor activity are shown in Supplementary Table 9. There were no statistically significant differences between the untreated controls and any of the treatment groups.

3.6. *Morphometry of immunolocalized cells in the SGZ in Experiment 1*

DCX expression was observed in the cytoplasm of many cells located within the SGZ (Fig. 6A). Tbr2 expression was observed in the nucleus of a small number of cells located within the SGZ (Fig. 6B). Immunoreactivity for GFAP was observed in the cytoplasm of a small number of cells located at a lower most portion of the SGZ (Fig. 6C). GFAP expression was also observed in the astrocytes.

On PND 21, a slight but statistically significant increase was observed in the number of DCX-positive cells in the SGZ at 800 ppm of Mn exposure (Fig. 6). As for the number of Tbr2- and GFAP-positive cells in the SGZ, there were no statistically significant differences between the untreated controls and any of the treatment groups (Fig. 6). On PND 77, no statistically significant differences were observed in the number of DCX-, Tbr2- and GFAP-positive cells in the SGZ (Fig. 6).

3.7. *Morphometry of immunolocalized cells in the dentate hilus in Experiment 1*

Reelin expression was observed in the cytoplasm of neurons located within the hilus of the dentate gyrus, similar to the previous reports [7] (Fig. 7). Immunoreactivity for NeuN was observed in the nucleus of neurons located within the dentate hilus. NeuN-positive neurons were also observed in the nucleus of granule cells, showing cytoplasmic immunoreactivity in addition to nuclear immunolocalization, as reported by others [30]. GAD67-expression was observed in the cytoplasm of the neurons located within the hilus and those sparsely distributed in the granule cell layer.

On PND 21, a slight but statistically significant increase was observed in the number of reelin-positive cells in the hilus at 800 ppm of Mn exposure (Fig. 7). As for the number of NeuN- and GAD67-positive cells in the hilus, there were no statistically significant differences in any of the treatment groups (Fig. 7). On PND 77,

Table 3
Grip strength of offspring exposed to $MnCl_2·4H_2O$ during the second half of gestation and lactation periods.

	0 ppm (control)	$MnCl_2·4H_2O$ in the diet		
		32 ppm	160 ppm	800 ppm
Males				
No. of offspring examined[a]	8	8	8	8
Forelimb (g)	1347 ± 157[b]	1170 ± 123[*]	1113 ± 149[**]	1216 ± 139
Hindlimb (g)	879 ± 205	810 ± 130	703 ± 248	755 ± 120
Females				
No. of offspring examined[a]	8	8	8	8
Forelimb (g)	1050 ± 98	1078 ± 49	1162 ± 160	1107 ± 75
Hindlimb (g)	741 ± 62	719 ± 137	822 ± 107	787 ± 71

[a] One male and female offspring per dam were subjected to examination.
[b] Mean ± SD.
[*] Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.05$).
[**] Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.01$).

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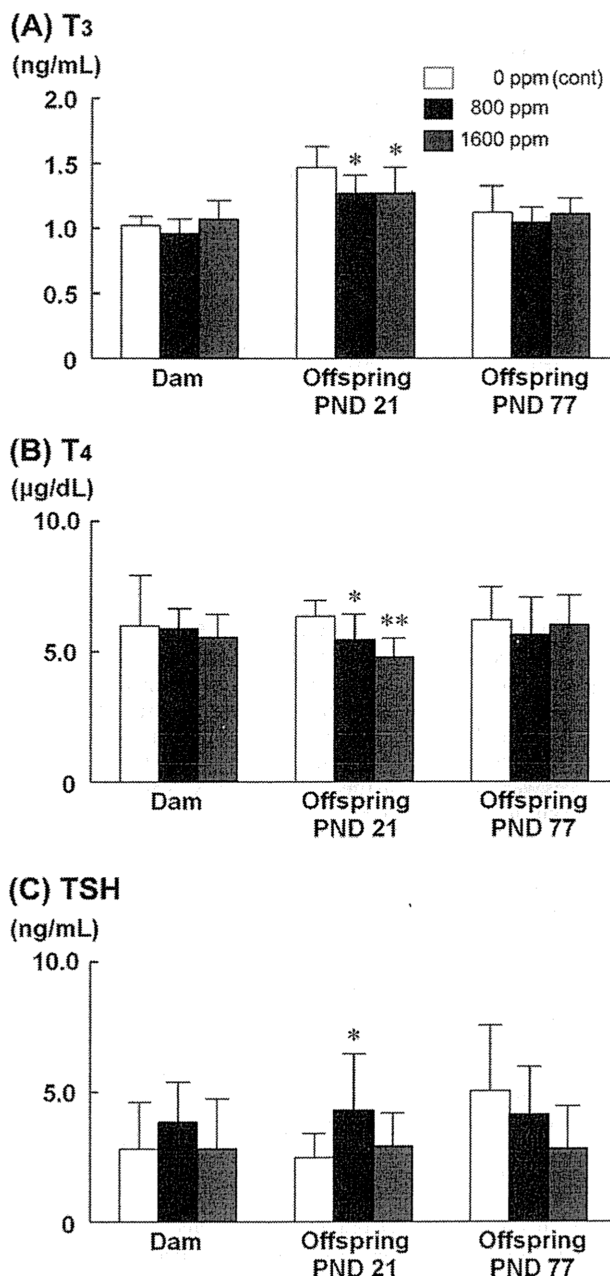

Fig. 4. Serum concentrations of thyroid-related hormones of dams and male offspring at PND 21 and 77 after maternal exposure to $MnCl_2{\cdot}4H_2O$ from GD 10 to PND 21. All six dams and 10 male offspring (one or two animals per dam) were subjected to hormone analysis in each group. Statistical analysis was performed using the litter as the experimental unit, and litter mean values were subjected to analysis on two offspring samples from the same dam. *Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.05$). **Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.01$).

no statistically significant differences were observed in the number of reelin-, NeuN- and GAD67-positive cells in the hilus (Fig. 7).

3.8. Apoptotic and proliferating cell indices in the dentate SGZ in Experiment 1

On PND 21 and 77, there were no statistically significant differences in the number of TUNEL-positive apoptotic cells and PCNA-positive cells in the SGZ between the untreated controls and any of the treatment groups (Fig. 8).

3.9. Real-time RT-PCR analysis in the hippocampus in Experiment 1

To examine the transcript levels of the molecules related to neuronal development, mRNA levels of *Dcx*, *Neurod1*, *Pax6* and *Dpysl3* in the hippocampus of offspring on PND 21 were analyzed by real-time RT-PCR. *Dcx*, *Neurod1*, *Pax6* and *Dpysl3* did not alter transcript levels in any of the treatment groups (Supplementary Table 10). Furthermore, to investigate the transcript levels of reelin and related molecules, mRNA levels of *Reln*, *Vldlr*, *Lrp8* and *Dab1* in the hippocampus of offspring on PND 21 were analyzed. *Reln*, *Vldlr*, *Lrp8* and *Dab1* did not alter transcript levels in any of the treatment groups (Supplementary Table 10).

4. Discussion

In the present study, supplemental Mn intakes of dams calculated from the $MnCl_2{\cdot}4H_2O$ intake were 1.13 mg/kg body weight/day for 32 ppm, 5.73 mg/kg body weight/day for 160 ppm, 29.21 mg/kg body weight/day for 800 ppm and 58.37 mg/kg body weight/day for 1600 ppm. However, the rodent basal diet contained high concentrations of Mn (7.27 mg/100 g CRF-1 basal diet). This dietary Mn level was equivalent to the Mn intake of 8.94 mg/kg body weight/day from GD 10 to PND 21 in dams of untreated controls. Therefore, total Mn intakes of dams were estimated 10.34 mg/kg body weight/day for 32 ppm, 15.10 mg/kg body weight/day for 160 ppm, 38.76 mg/kg body weight/day for 800 ppm and 67.91 mg/kg body weight/day for 1600 ppm. The ESADDI of Mn has been estimated to be approximately 0.6 mg/day at 7–12 months of age, 1.2 mg/day at 1–3 years of age, 1.5 mg/day at 4–8 years of age and 2–5 mg/day for adults. For newborns, the ESADDI has been estimated to be 0.003 mg/day, less than that for adults or children [1,3]. Therefore, rodent studies are performed with extremely high basal Mn-intake levels as compared with human counterparts. Even when adult value is converted into a measure of mg/kg of body weight (as 50 kg), the daily Mn intake is between 0.04 and 0.1 mg/kg body weight/day. At the lowest dose in the present study, total Mn intake was approximately 100 times higher than the ESADDI in adult humans. Mn-exposure resulted in no major changes in dams except for slight and sporadic increases in food consumption. Offspring also showed no changes in body weight, food consumption and organ weight through to PND 77. However, offspring revealed slight increases in the cellular distribution of DCX- and reelin-positive cells in the dentate gyrus of the hippocampus with Mn exposure at 800 ppm, as well as an increase in the brain Mn concentration at 160 ppm, changes in serum concentrations in thyroid hormones at 800 and 1600 ppm at the end of exposure on PND 21, and changes in reflex functional testing and maze tests at 160 ppm.

Maternal Mn exposure affected the cellular distribution of immunoreactivity for DCX in the SGZ on PND 21 at 800 ppm. Although DCX is expressed in the type-2b and type-3 progenitor cells and immature neurons [11], there were no changes in the numbers of Tbr2-positive cells, suggestive of the type-2 progenitor cells [21]. Therefore, it was considered that type-2b cells were not involved in the increased population of DCX-positive cells, suggestive of an increase in the type-3 progenitor cells or immature granule cells. On the other hand, an increase in the cellular distribution of immunoreactivity for reelin in the dentate hilus also was observed on PND 21 at 800 ppm. Reelin secreted from GABAergic interneurons plays a role for regulating postnatal neurogenesis in the hippocampal dentate gyrus, causing an increase

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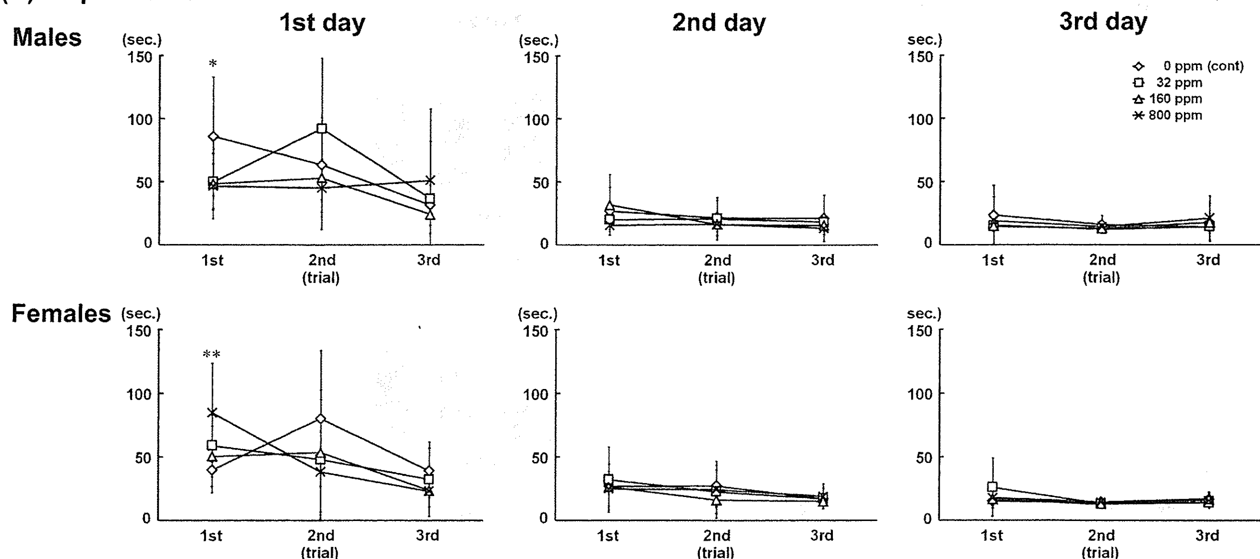

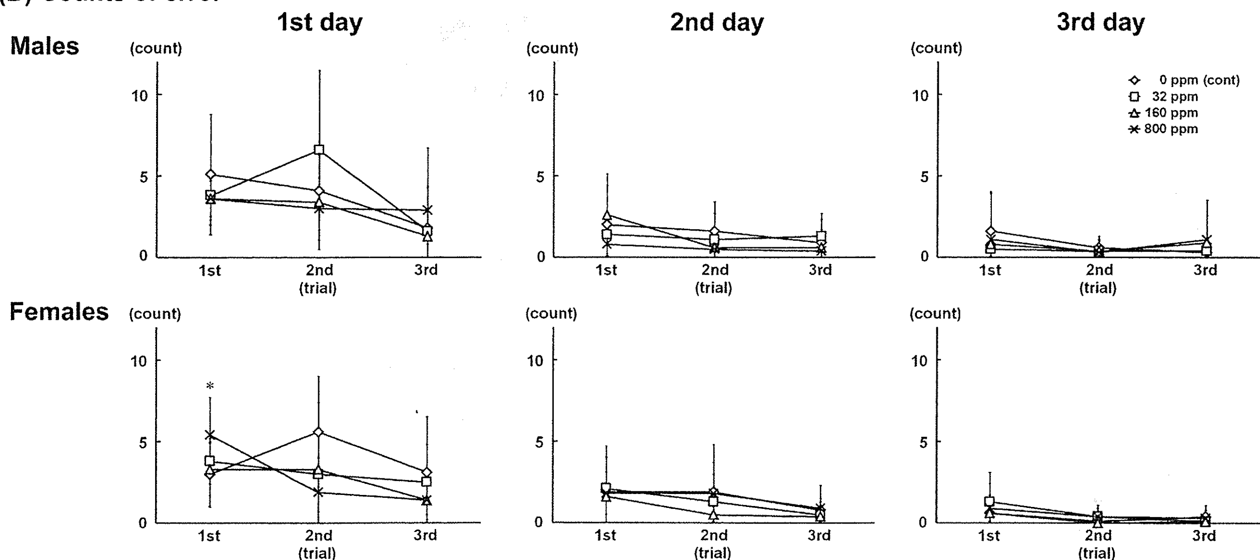

Fig. 5. Water-filled multiple T-maze test conducted on PND 55, 56 and 57 in offspring after maternal exposure to $MnCl_2 \cdot 4H_2O$ from GD 10 to PND 21. Eight male and eight female offspring (one male and one female animals per dam) were subjected to the examination in each group. (A) Elapsed time. (B) Counts of error. * Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.05$. ** Significantly different from the untreated controls by Dunnett's test or Steel's test ($P < 0.01$).

in DCX-positive immature granule cells [31]. Reelin can also be induced during the course of aberration in the postnatal neurogenesis by developmental exposure to methylazoxymethanol, anti-thyroid agents, or acrylamide in rats [7,18,32]. Therefore, increase of reelin-expressing cells by developmental Mn-exposure may be the reflection of an upregulation of reelin in the GABAergic interneurons causing increased DCX-positive cells, reflecting an aberration in the differentiation of the granule cells. Interestingly, an *in vitro* study using astrocyte–neuron co-cultures has shown that $MnCl_2$ inhibited the ability of astrocytes to promote the neurite outgrowth of hippocampal neuronal precursor cells [33]. This result might be related to the increase of DCX-positive cells in the SGZ found in the present study. Because immature granule cells already have dendritic growth cones and recurrent basal dendrites [34], it might be possible that type 3 progenitor cells is the target of Mn to suppress differentiation to immature granule cells. However, these changes were not observed on PND 77 and were reversible. While immunohistochemical analysis revealed increase of cells positive for DCX or reelin, real-time RT-PCR analysis did not show changes in the transcript levels of the molecules including *Dcx* and *Reln* in the present study. Considering the use of the whole hippocampal tissue including cornu ammonis in the real-time RT-PCR analysis, the changes observed in the substructures, such as the SGZ or dentate hilus, could not be detected.

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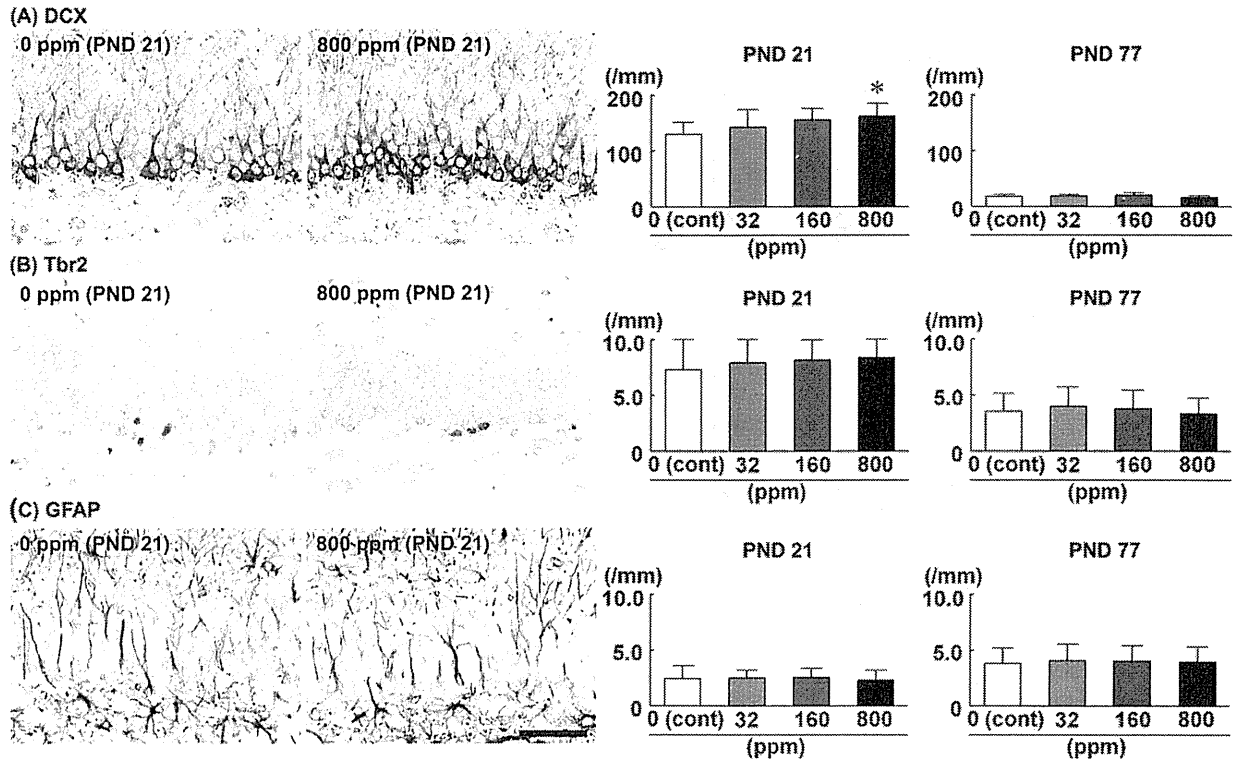

Fig. 6. Distribution of immunoreactive cells for DCX, Tbr2 and GFAP in the dentate subgranular zone of male offspring at PND 21 and 77 after maternal exposure to $MnCl_2{\cdot}4H_2O$ from GD 10 to PND 21. All identical 10 male offspring from eight dams (one or two animals per dam) were subjected to immunohistochemical analyses in each group. (A) DCX. (B) Tbr2. (C) GFAP. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 21 (bar = 50 μm). *Significantly different from the untreated controls by Dunnett's test ($P < 0.05$).

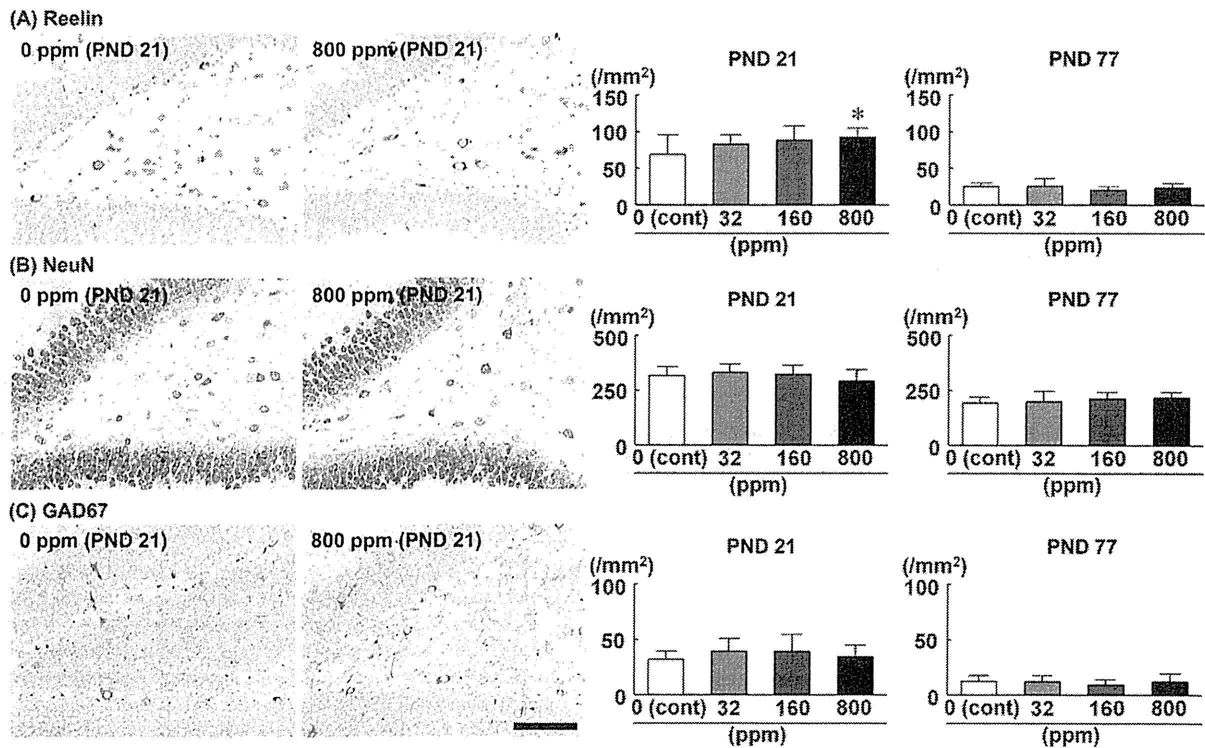

Fig. 7. Distribution of immunoreactive cells for reelin, NeuN and GAD67 in the hilus of the hippocampal dentate gyrus in male offspring at PND 21 and 77 after maternal exposure to $MnCl_2{\cdot}4H_2O$ from GD 10 to PND 21. All identical 10 male offspring from eight dams (one or two animals per dam) were subjected to immunohistochemical analyses in each group. (A) Reelin. (B) NeuN. (C) GAD67. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 21 (bar = 100 μm). * Significantly different from the untreated controls by Dunnett's test ($P < 0.05$).

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Air righting reflex indices in males at 160 ppm of Mn exposure or more and in females at 800 ppm were lower than those in the untreated controls. Tran et al. reported that neonatal rats exposed to high dietary Mn revealed prolongation in the surface righting test [35]. Therefore, a decreased index for the air righting reflex may be the effect of Mn exposure. However, we did not find any effects in other sensory and reflex functional examinations including the surface righting reflex. Therefore, we could not judge that reflex functions were affected by developmental Mn exposure. In addition, water-filled multiple T-maze testing at the 1st trial on the 2nd day (1st day for the T-maze course) revealed shortening of the elapsed time in males and prolongation of the elapsed time and increased counts of error in females at 800 ppm of Mn exposure. However, they were judged to be incidental fluctuations because the changes were almost within the range of three trials in the untreated controls; there were no changes at other trials on all days and there were opposite changes between males and females. Thus, we suggest no apparent effects of developmental Mn exposure on the behavioral examinations including those for learning and memory functions.

In the present study, Mn concentrations in the brain as represented by cerebellar tissue revealed increases in offspring at 160 and 800 ppm at the end of Mn exposure on PND 21, despite no changes in the dams. It is reported that exposure *in utero* and during lactation to inhaled $MnSO_4$ revealed increased Mn concentrations in the striatum and cerebellum of offspring at dose levels showing no changes of Mn concentrations in the dams [36]. In several studies of experimental exposure to $MnCl_2$, Mn concentrations in the brain in developing rats were higher than those concentrations in adult rats at the same dose levels [4,37]. Mn exposure *via* maternal milk from PND 4 to 21 caused Mn accumulation in the cerebellum, midbrain, striatum, cortex and hippocampus in the offspring [38]. Dorman et al. suggested that the increases in brain Mn concentrations might be related to increased Mn absorption from the juvenile gastrointestinal tract, as well as an incompletely formed neonatal blood–brain barrier and a virtual absence of excretory mechanisms until weaning [4]. Also, most Mn salts can penetrate readily the placenta and is toxic to the embryos [39]. These findings suggest that the both fetuses and neonates are rather unprotected against developmental Mn exposure, in contrast to adult animals that have protective functions against ingested Mn even at high doses. Therefore, only the offspring can be exposed to the risk of the neurotoxicity including impaired neuronal differentiation by maternal Mn exposure at high doses.

In the present study, we found decreases in serum concentrations in T_3 and T_4, while serum TSH concentrations increased only at 800 ppm of Mn in offspring at the end of exposure on PND 21. It is reported treatment of rats with a Mn-rich diet ($MnSO_4$) for 5 weeks resulted in decreases in serum T_3, T_4 and TSH concentrations [5]. Also, a 2-year study of Mn exposure in mice revealed thyroid follicular hyperplasia and dilatation, suggestive of the anti-thyroid action of administered Mn [5]. Thyroid hormones play a crucial role in brain development [6]. Experimentally, developmental hypothyroidism leads to neurological defects and impaired performance in a variety of behavioral learning tests [40,41]. The offspring of rats exposed to anti-thyroid agents such as 6-propyl-2-thiouracil show impaired brain development, with aberrant neuronal migration and white matter hypoplasia involving limited axonal myelination and reduced oligodendrocytic distribution [42,43]. In our recent study, the offspring of rats exposed to anti-thyroid agents during development revealed an increase in reelin-synthesizing GABAergic interneurons in the dentate hilus with an immature phenotype that was sustained into the later stage at PND 77 [7]. These results suggested a compensatory mechanism for the impaired neurogenesis and mismigration during neuronal development. Therefore, developmental Mn exposure at high doses might have affected the homeostasis of the thyroid hormones in the offspring, influencing neurogenesis. However, this effect was rather mild or essentially lacking because of the absence of the feature of developmental hypothyroidism such as sustained suppression of body growth and delayed physical development that have been observed in our previous study using anti-thyroid agents [6]. Also, it could not be sustained through to PND 77.

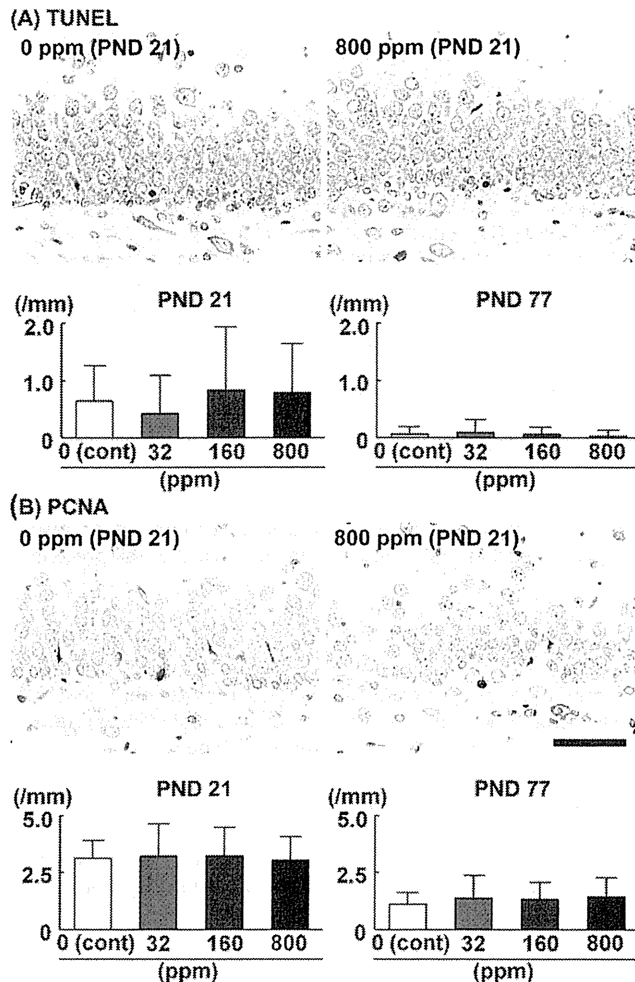

Fig. 8. Distribution of apoptotic cells and proliferating cells in the dentate subgranular zone of male offspring at PND 21 and 77 after maternal exposure to $MnCl_2 \cdot 4H_2O$ from GD 10 to PND 21. All identical 10 male offspring from eight dams (one or two animals per dam) as used in the immunohistochemical analysis were subjected to TUNEL-assay and PCNA-immunohistochemistry in each group. Statistical analysis was performed using the litter as the experimental unit and litter mean values were subjected to analysis on two offspring samples from the same dam. (A) TUNEL. (B) PCNA. Representative images from 0 ppm group (left) and from 800 ppm group (right) at PND 21 (bar = 50 μm).

As described above, although the behavioral examinations could not clarify the obvious effects of developmental Mn exposure, monitoring of the cellular distribution in the SGZ and dentate hilus revealed changes suggestive of effects on neuronal development. Hippocampal neurogenesis in the SGZ continues through the life-span period and decreases in depression, posttraumatic stress disorder and Parkinson's disease, and increases in epileptic seizure, ischemia, Alzheimer's disease and Huntington's disease [44,45]. If developmental exposure to xenobiotics affects neurogenesis and risks the brain diseases mentioned above, analysis of distribution changes in neuronal progenitor cells and interneurons in the dentate gyrus in experimental animals may provide a valuable tool

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for detection of developmental neurotoxicants affecting neurogenesis.

5. Conclusion

In conclusion, we revealed that maternal Mn exposure mildly and reversibly affects neurogenesis targeting late-stage differentiation in the hippocampal dentate gyrus of rat offspring at 800 ppm $MnCl_2{\cdot}4H_2O$ in diet, a level translating to 38.76 mg/kg body weight/day as Mn. This level is 380–970 times larger than the ESADDI in adult humans (0.04 and 0.1 mg/kg body weight/day with 50 kg body weight). Direct effects of accumulated Mn in the developing brain might be implicated in the mechanism of the development of aberrations in neurogenesis; however, indirect effects through thyroid hormone fluctuations might be rather minor.

Conflict of interest statement

All of the authors disclose that there are no conflicts of interest that could inappropriately influence the outcomes of the present study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.04.009.

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Bull, Natl. Inst. Health Sci., **129**, 105-110（2011）　**Note**

有機リン系化合物クロルピリホスの経胎盤・経母乳暴露が発達期のマウス免疫系に及ぼす影響について

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Effects of transplacental and trans-breast milk exposure to the organophosphate compound chlorpyrifos on the developing immune system of mice

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Navarro et al (2001) have reported that neonatal exposure of rat to the organophosphate compound chlorpyrifos (CPF) resulted in long-term deficits in T lymphocyte mitogenic response, although the mechanism has been unclear. In this study, pregnant BALB/c mice were exposed to 0, 2.8, 14, 70ppm CPF via diet from gestational day 10 to postnatal day (PND) 21, and subpopulational changes in T lymphocytes of offspring were analyzed at PND21. The irreversibility of the effects was also investigated at PND77 after ceasing exposure by weaning at PND21. Serum cholinesterase activity was significantly reduced after exposure to CPF at PND21. An increase in the proportion of CD4 positive splenocytes was observed after exposure to CPF, which remained until PND77. We found that regulatory T cells were the only one CD4 positive subset which increased in the spleen of CPF-exposed mice at PND77.

Keywords: organophosphates, chlorpyrifos, developing immune system, CD4 T cells

1．緒　言

免疫系は，神経系および内分泌系との密接な相互作用を通じ，生体防御に重要な役割を果たしている．これら3つのシステムは発達期における外部環境の影響を受けやすいことが知られており，この感受性の高い時期はしばしば「critical window」とよばれている[1,2]．しかし，従来の免疫毒性試験評価では発達期への影響を検討していない．

シロアリ駆除剤として居室を含有する建築物への使用は禁止されたものの，殺虫剤として使用されるクロルピリホス（CPF）は，有機リン系化合物の一種で，アセチルコリンエステラーゼを阻害して神経伝達物質のアセチルコリン濃度を高めることにより作用を発揮する（Fig. 1）．Navarroらは，新生児ラットに出産後4日間CPFを皮下投与すると，成熟後におけるT細胞のconcanavalin A刺激に対する増殖能が有意に低下したと報告しており[3]，CPFの発達期免疫への影響が疑われているが，そのメカニズムの詳細は明らかになっていない．

細胞性免疫や液性免疫など，適応免疫の型を決定づける上で最も重要な役割を担っているのは細胞表面マーカーCD4を発現するT細胞集団（ヘルパーT細胞；Th）であるが，これは少なくとも4種の機能的に異なるサブセットから構成されており，そのバランスにより免疫反応の型が制御されていることが知られている．すなわち，インターフェロンγ（IFNγ）等を発現し，細胞性免疫に関与するTh1[4]，インターロイキン（IL）4，5

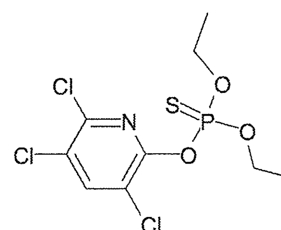

Fig. 1　Structure of chlorpyrifos (CAS#2921-88-2)

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等を発現し，アレルギーや液性免疫に関与するTh2[4]，IL-17を発現し，多くの自己免疫疾患に関与するTh17[5]，そしてIL-10等を発現し，免疫反応の抑制に中心的な役割を果たしている制御性T細胞（regulatory T cell；Treg）[6]の4種である．本研究では，Navarroらがラットにおいて発見したCPFの発達期免疫への影響をより詳細に解析するため，リンパ球のサブポピュレーション解析（CD4$^+$ T細胞，CD8$^+$ T細胞，NK細胞）およびこれら4種のCD4$^+$ T細胞サブセット解析を行い，特に免疫反応の抑制に関与するTregの増加が誘導されている可能性について追究した．

暴露系としては，ラット[7-11]およびマウス[12]を用いて抗甲状腺作用が疑われる化学物質への暴露が発達期の神経系および免疫系に及ぼす影響を簡便にスクリーニングすることに成功した先行研究が存在するため，このプロトコルに則った．具体的には，妊娠マウスを妊娠10日（GD10）から出産後21日（PND21）までCPFに混餌（0，2.8，14，70ppm）投与にて暴露し，暴露終了時での児マウスの解析を行った．また，PND21までの暴露終了後，PND77まで通常飼料による飼育を行い，PND21の時点で認められた変化が11週齢で回復するかどうかを調べた．

2．実験方法

2．1．被験物質

CPFの構造式を図1に示した．CPF（DURSBAN™ XP；M.W.350.6，CAS No.2921-88-2，純度99.8%，通常の保存条件下で安定．弱酸・弱アルカリで安定）はDow AgroSciences社の厚意により分与された．

2．2．試薬

粉末CRF-1は日本チャールスリバー（株）より購入した．次の蛍光標識抗体はBioLegend社より購入した：APC/Cy7標識抗マウスCD3抗体（145-2C11），FITC標識抗マウスCD4抗体（RM4-5），APC標識抗マウスCD8a抗体（53-6.7），PerCP/Cy5.5標識抗マウスIFNγ抗体（XMG1.2），PE標識抗マウスIL-4抗体（11B11），APC標識抗マウスIL-17A抗体（TC11-18H10.1），PerCP/Cy5.5標識（PEおよびAPCも同様）ラットIgG1κアイソタイプコントロール抗体（RTK2071）．PE標識抗マウスCD25抗体（PC61）はBD Pharmingen社より購入した．PE標識抗マウスCD49b抗体（DX5）およびAPC標識抗マウスFoxp3抗体（FJK-16s）はeBioscience社より購入した．Leukocyte Activation Coktail，with BD GolgiPlugおよびBD GolgiPlugはBD Pharmingen社より購入した．

2．3．使用動物

9-11週齢の妊娠2日（GD2；プラグがついた日を妊娠1日と起算）のBALB/cマウスを日本チャールスリバー（株）より購入し，7日間予備飼育後，実験に供した．動物は，GD9までは群飼い（1ケージあたり3匹），GD10からは個別飼育とし，照明12時間，温度24±1℃，湿度55±5%に保たれたバリアシステムの飼育室（SPF）で飼育した．動物実験は国立医薬品食品衛生研究所の規定に準拠し，動物実験委員会の承認に基づき実施した．

2．4．CPFへの暴露

CPFは，粉末CRF-1飼料に0，2.8，14，70ppm（公比5）にて乳鉢，ビニル袋，ポリプロピレン容器を用いて混合し，混餌飼料を調製した．検体は各段階で十分混合しつつ，3段階の段階希釈により得た．検体の濃度は，ラットにおける生殖毒性を検討した過去の報告[13]および血清中コリンエステラーゼ活性の抑制を指標とした用量設定試験により決定した．妊娠2日目（GD2）のBALB/cマウス（9～11週齢）を購入し，1ケージあたり3匹でGD9まで飼育し，GD10より単独飼育にすると同時に粉末CRF-1への混餌投与にてCPFの暴露を開始した（1群12匹）．最終的に，12匹中コントロール群は3匹，暴露群はすべて6匹のマウスが出産に至った．出産後3週目（PND21）まで暴露を継続し，その間体重および摂餌量を計測した．また，出産した児マウスの体重も同様に計測した．児マウスの飼育数は，栄養状態が均等となるよう，9匹以上生まれたケージからは1ケージあたり8匹となるように無作為に間引いて8匹未満の出産ケージに移動し，総数を8匹に揃えた．PND21に同腹から体重が中央値に近い児マウスを選び解剖（雌雄それぞれn＝4）を行った．さらに，雄の児動物については，暴露終了後PND77まで飼育し，被験物質による影響の回復性を調べた．一般状態および体重測定は7日ごとに行った．

2．5．血液学的検査

エーテル深麻酔下のマウス眼底から末梢血30 μlを採取し，120 μlの0.5%EDTA/CELLPAKに懸濁し，多項目自動血球計数装置（M-2000，Sysmex corp.）に供した．解析項目は次の通り：赤血球数（RBC），白血球数（WBC），ヘモグロビン濃度（HGB），ヘマトクリット値（HCT），平均赤血球容積（MCV），平均赤血球血色素量（MCH），平均赤血球血色素濃度（MCHC），および血小板（PLT）．

2．6．血液生化学的検査

母動物および児動物について，末梢血より血清200 μlを採取し，SRL社に委託して次の項目の検査を行った：アルブミン/グロブリン（A/G）比，アスパラギン酸アミノトランスフェラーゼ（AST），アラニンアミノトランスフェラーゼ（ALT），および血清中コリンエス

テラーゼ活性（ChE）．ただし，PNW 3 の児動物から採取できる血清はわずかであったため，2 匹分の血清試料を 1 検体にまとめて測定した．その際，PND21群においては雌児の数が十分確保できなかったため，雌 2 検体に 3 または 4 検体の雄を合わせて n＝5 または 6 として検定を行った．

2．7．病理組織学的解析

児マウスについては，肝臓，脾臓，胸腺，および骨髄（大腿骨）における病理組織学的解析を行った．採取組織を定法に従って中性緩衝ホルマリン液で固定し，薄切切片をヘマトキシリン・エオジン染色した．

2．8．フローサイトメトリー

リンパ球のポピュレーション解析を行うため，児マウスの脾臓および胸腺を冷温下でシリンジにより破砕して口径70 µm のメッシュに通し，10 ml の10%FCS（Gibco）を添加した RPMI1640培地に懸濁した．トリパンブルー染色の後，自動細胞数計測装置（Countess；Invitrogen 社）により細胞数を計測し，以下に述べる 3 種の条件により染色を行った．測定には BectonDickinson 社の FACSAria を用い，データ解析には FlowJo（トミーデジタルバイオロジー社）を用いた．フローサイトメトリーでは total event 数として10万個の細胞を計測し，各サブセットの存在比率は，特に断らない限り，定法通り前方散乱および側方散乱により定義したリンパ球ゲート内の総リンパ球数に対するパーセンテージとして表した[7,8,10]．

2．8．1．CD 3 /CD 4 /CD 8 a/CD49b を抗原とするリンパ球サブポピュレーション解析

T 細胞および NK 細胞のサブポピュレーションを解析するため，セルストレイナー付き丸底ポリスチレンチューブに細胞を 2 ×10^6cells 分注し，成熟 T 細胞マーカーである CD3，ヘルパー T 細胞のマーカーである CD4，細胞傷害性 T 細胞のマーカーである CD8a，および NK 細胞のマーカーである CD49b を2.1.に示した抗体により氷上で30分間染色した．

2．8．2．CD 4 /IFNγ/IL 4 /IL17A を抗原とする CD 4 陽性 T 細胞サブセット解析

ヘルパー T 細胞のサブセットである Th1/Th2/Th17 を染色するため，表面抗原である CD4とともに，それぞれのマーカーとなる細胞内サイトカイン IFNγ/IL-4/IL-17A を染色した．採取後の細胞を24well プレートに 4 ×10^6cells/ 1 ml/well ずつ分注し，PMA とイオノマイシンを含むリンパ球活性化試薬（Leukocyte Activation Coktail，with BD GolgiPlug）により CO_2インキュベータ中で37℃，4 時間刺激し，セルストレイナー付きチューブに回収した．FOXP3Fix/Perm buffer（BioLegend）により室温で30分固定後，CD4を染色，洗浄後に FOXP 3Perm buffer で細胞を可溶化し，細胞内サイトカイン抗体で染色した．アイソタイプコントロールとしては，同じ蛍光色素で標識された非特異的ラット IgG1κ を用いた．また，未刺激のネガティブコントロールには，BD GolgiPlug のみを用いた．

2．8．3．CD 4 /CD25/Foxp 3 を抗原とする Treg 解析

Treg は，CD4とともに表面マーカーの CD25または核内抗原である Foxp3により同時染色し解析した．セルストレイナー付きチューブに細胞を 2 ×10^6cells 分注し，氷上で CD4/CD25を染色後，FOXP3Fix/Perm buffer セットにより固定・可溶化し，Foxp3を染色した．

2．9．統計処理

有意差の有無に関する統計計算は，Dunnett の方法（n＝4）により，$p<0.05$を有意とした．なお，病理組織学的解析の判定には Fisher の直接確率検定によった．

3．結果および考察

3．1．一般毒性学的影響

母マウスおよび児マウスについて，体重・臓器重量・血液学的検査・血液生化学的検査および病理組織学的解析を行った．なお，CPF 暴露群において摂餌量に有意な変化はなく，性比への影響も認められなかった．CPF 暴露群においては，一部の母マウスで体重または脾臓重量の増大が認められたが，児については70ppm 暴露群の雄において，PND21の胸腺比重量の有意な増大が認められた（Table 1）．しかし，PND77には回復していたことから，その影響はごく軽微にとどまっているものと考えられた．血液学的影響としては，70ppm 暴露群の母マウスにおいて白血球の増加と赤血球および各種赤血球関連パラメータの減少が認められたが，児マウスにおいては用量依存性のない軽微な変化のみが認められた（data not shown）．血液生化学的検査のうち，肝機能関連のパラメータ（A/G 比，AST，ALT）には有意差は認められなかった（data not shown）．一方，中用量以上の CPF 暴露は母親および児に対し，PND21における顕著な血清中コリンエステラーゼ（ブチリルコリンエステラーゼ）阻害を誘導した．このことは，CPF の経胎盤・経乳的暴露が正しく成立していたことを示している．しかし，血清中のコリンエステラーゼ活性の多くはブチリルコリンエステラーゼによるものであり，CPF の神経作用の本態であるアセチルコリンエステラーゼ阻害とは必ずしも対応しないことには注意を要する．実際，立毛や痙攣，運動失調などの所見は認められなかった．PND77の児動物においては活性は回復している．また，児動物の肝臓・脾臓・胸腺・骨髄の病理組織学的

Table 1 General toxicity of perinatal exposure of chlorpyrifos on dams and offspring.

Dam	0	2.8	14	70	CPF（ppm）
Number of delivery	3	6	6	6	
Body weight（g）	23.8 ± 0.1	24.0 ± 0.9	26.4 ± 1.0**	25.2 ± 0.7	
Liver/BW（%）	7.70 ± 0.36	7.50 ± 0.40	7.63 ± 0.63	8.21 ± 0.41	
Spleen/BW（%）	0.43 ± 0.05	0.47 ± 0.03	0.44 ± 0.02	0.50 ± 0.04	
Thymus/BW（%）	0.17 ± 0.02	0.17 ± 0.04	0.14 ± 0.02	0.14 ± 0.03	
ChE（IU/L）	52.33 ± 6.81	45.75 ± 3.77	31.50 ± 4.43**	18.25 ± 1.50**	
Female offspring（PND21）	**0**	**2.8**	**14**	**70**	**CPF（ppm）**
Body weight（g）	9.9 ± 0.5	10.0 ± 0.8	11.0 ± 0.3	10.0 ± 0.8	
Liver/BW（%）	5.57 ± 0.35	5.32 ± 0.69	5.25 ± 0.49	5.56 ± 0.73	
Spleen/BW（%）	0.86 ± 0.14	0.86 ± 0.04	0.80 ± 0.11	0.71 ± 0.13	
Thymus/BW（%）	0.71 ± 0.17	0.67 ± 0.07	0.71 ± 0.04	0.66 ± 0.11	
[a]ChE（IU/L）	31.00 ± 1.87	29.83 ± 2.71	19.17 ± 2.48**	11.50 ± 2.35**	
Male offspring（PND21）	**0**	**2.8**	**14**	**70**	**CPF（ppm）**
Body weight（g）	11.0 ± 0.7	10.3 ± 0.7	11.4 ± 0.5	10.2 ± 0.6	
Liver/BW（%）	5.77 ± 0.57	5.45 ± 0.52	5.45 ± 0.28	5.83 ± 0.75	
Spleen/BW（%）	0.84 ± 0.08	0.84 ± 0.07	0.85 ± 0.08	0.77 ± 0.14	
Thymus/BW（%）	0.54 ± 0.11	0.60 ± 0.04	0.57 ± 0.06	0.68 ± 0.06*	
[a]ChE（IU/L）	31.00 ± 1.87	29.83 ± 2.71	19.17 ± 2.48**	11.50 ± 2.35**	
Male offspring（PND77）	**0**	**2.8**	**14**	**70**	**CPF（ppm）**
Body weight（g）	23.8 ± 0.2	24.1 ± 0.4	24.1 ± 1.9	24.9 ± 0.9	
Liver/BW（%）	4.70 ± 0.24	4.67 ± 0.52	4.32 ± 0.41	4.5.0 ± 0.24	
Spleen/BW（%）	0.38 ± 0.02	0.38 ± 0.04	0.37 ± 0.04	0.41 ± 0.03	
Thymus/BW（%）	0.14 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	
ChE（IU/L）	35.50 ± 2.08	36.00 ± 2.58	37.00 ± 2.16	34.75 ± 3.95	

Pregnant BALB/c mice（12per group）were exposed to chlorpyrifos（CPF；0, 2.8, 14, and 70ppm）in diet, from gestational day 10 to postnatal day（PND）21. Exposure was ceased by weaning. At PND21 and PND77, mice were sacrificed to determine effects of the compound on body weights（BW）, organ weights, and cholinesterase（ChE）activities. [a] Specimens from 2 females and 3-4 males were lumped together for statistics（see Materials and Methods）. Values are mean ± SD（n=4）. *$p<0.05$, **$p<0.01$（Dunnett's test）.

解析を行ったが，統計学的に有意な変化は認められなかった（data not shown）．

3．2．フローサイトメトリー

前述のように，免疫系は各種のリンパ球サブポピュレーションやCD4陽性T細胞サブセットのバランスにより，反応の型が制御されている．そこで，フローサイトメトリーにより一次リンパ器官である胸腺，および二次リンパ器官である脾臓における各種リンパ球サブセットの存在比率への影響を解析した．しかし，CPF暴露は全般的に大きな影響は与えず，NK細胞，CD8陽性T細胞の各サブポピュレーション比率，Th1，Th2の各サブセット比率については有意な影響が認められなかった(data not shown)．

Table 2に，少なくともPND21またはPND77いずれかにおいて有意な変化を示したサブセットの存在比率を挙げた．なお，総細胞数に占めるリンパ球ゲート画分の細胞数には顕著な変化はなかった（data not shown）．

雌については，PND21の脾臓における$CD3^+CD4^+$細胞の増加および胸腺における$CD4^+Foxp3^+$細胞（Treg）の増加が観察された．

雄については，PND21の脾臓における$CD4^+$シングルポジティブ細胞が増加していた．この変化は，PND77でも回復せず持続していた．PND77の脾臓で増加していたのは，$CD4^+CD25^+$細胞（Treg）のみであった．なお，PND21の胸腺で$CD4^+IL\text{-}17A^+$細胞（Th17）の減少が認められたが，用量依存性はなく，軽微な影響と考えられた．

CPFの周産期暴露が成熟後のBALB/cマウス脾臓において免疫反応の抑制に関与するTregの存在比率を増加させるという知見は本研究によって初めてもたらされたものである．Tregはほぼ全ての免疫反応に抑制的に働くため，Navarroら[3]がラットにおいて発見したCPFの脾臓T細胞への抑制的影響と考え合わせると，腫瘍免疫などに代表される全身の免疫応答への影響の解析が今後望まれる．

なお，Tregには，胸腺内で分化するFoxp3陽性の内在性Treg（nTreg）と，ナイーブT細胞が抗原提示を受け分化する過程でTGF-β依存的に誘導される誘導性Treg（iTreg）とが存在する[14]．本研究では抗原特異的なiTregの増加は調べていなかったため，現在胸腺依存性抗原（KLH）の免疫実験を遂行中である．

Table 2 Flow cytometry analysis of the effect of chlorpyrifos on lymphocyte subpopulations

Female offspring (PND21)	0	2.8	14	70	CPF (ppm)
Spl: $CD4^+$/Lymph	4.0 ± 0.9	4.0 ± 0.9	4.2 ± 1.1	5.1 ± 0.4	
[a]Spl: $CD4^+$/$CD3^+$	44.3 ± 1.0	45.8 ± 2.3	48.1 ± 2.9	49.9 ± 3.2*	
Spl: $CD4^+CD25^+$/Lymph (Treg)	0.71 ± 0.02	0.69 ± 0.09	0.82 ± 0.08	0.84 ± 0.11	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.42 ± 0.02	0.42 ± 0.04	0.44 ± 0.30	0.85 ± 0.17*	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.14 ± 0.03	0.17 ± 0.05	0.14 ± 0.09	0.09 ± 0.03	
Male offspring (PND21)	**0**	**2.8**	**14**	**70**	**CPF (ppm)**
Spl: $CD4^+$/Lymph	4.5 ± 0.4	4.2 ± 0.7	4.0 ± 0.9	5.8 ± 0.2*	
[a]Spl: $CD4^+$/$CD3^+$	48.3 ± 2.0	49.4 ± 2.6	48.9 ± 1.0	52.2 ± 2.6	
Spl: $CD4^+CD25^+$/Lymph (Treg)	0.76 ± 0.08	0.79 ± 0.06	0.82 ± 0.05	0.79 ± 0.17	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.41 ± 0.07	0.71 ± 0.45	0.83 ± 0.26	0.63 ± 0.25	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.14 ± 0.05	0.10 ± 0.03	0.07 ± 0.02*	0.10 ± 0.03	
Male offspring (PND77)	**0**	**2.8**	**14**	**70**	**CPF (ppm)**
Spl: $CD4^+$/Lymph	16.3 ± 1.4	16.9 ± 1.8	17.8 ± 0.6	20.0 ± 1.3**	
[a]Spl: $CD4^+$/$CD3^+$	57.4 ± 2.3	57.7 ± 0.9	58.2 ± 3.3	60.5 ± 1.0	
Spl: $CD4^+CD25^+$/Lymph (Treg)	1.93 ± 0.19	2.14 ± 0.21	2.39 ± 0.26*	2.41 ± 0.24*	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.60 ± 0.09	0.61 ± 0.08	0.66 ± 0.23	0.55 ± 0.09	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.38 ± 0.09	0.55 ± 0.19	0.38 ± 0.10	0.35 ± 0.10	

Pregnant BALB/c mice were exposed to chlorpyrifos (CPF; 0, 2.8, 14, and 70ppm) in diet from gestational day 10 to postnatal day (PND) 21. Exposure was ceased by weaning. At PND21 and PND77, mice were sacrificed to determine the effects of the compound on the relative proportions of lymphocyte subsets in the spleen (Spl) and thymus (Thy). The percentage of lymphocytes (Lymph) are shown ([a] percent CD3 positive cells). Values are mean ± SD (n=4). $^*p<0.05$, $^{**}p<0.01$ (Dunnett's test).

4. 結　語

本研究では，新生児期における CPF への暴露が成長後の胸腺細胞のレクチン刺激による細胞増殖応答の低下を招くという Navarro らの報告[3]を受け，我々が以前より開発している化学物質の発達期影響の簡便なスクリーニング系[7,8,10]にフローサイトメトリーによるＴ細胞のサブセット解析を追加し，CPF の潜在的な発達期免疫影響をより詳細に解析することを目指した．

その結果，CPF 暴露後の PND21時点で児の血中コリンエステラーゼ活性の抑制が認められたが，PND77には回復した．それにも関わらず，二次リンパ器官である脾臓における CD4陽性Ｔ細胞の増加が PND21および PND77で観察され，その中でも PND77の CPF 暴露群において増加していた唯一の CD4陽性サブセットは制御性Ｔ細胞（$CD4^+CD25^+$）であることを発見した．

なお，緒言で述べた通り免疫系と神経系・内分泌系との間には密接な相互作用があるため，今回観察された *in vivo* での免疫影響が免疫系への直接影響なのか，それとも神経系等を介する間接影響なのかについては明らかでなく，今後の研究が待たれる．

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Antiviral Activities of Diarylheptanoids Isolated from *Alpinia officinarum* against Respiratory Syncytial Virus, Poliovirus, Measles Virus, and Herpes Simplex Virus Type 1 *in vitro*

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Alpinia officinarum has been used as a folk medicine and contains diarylheptanoids that have various biological activities. However, their antiviral activities are less elucidated. We examined the antiviral activities of nine diarylheptanoids isolated from *A. officinarum* against respiratory syncytial virus (RSV), poliovirus, measles virus, and herpes simplex virus type 1 (HSV-1) using a plaque reduction assay. The 50% inhibitory concentrations of seven of the nine diarylheptanoids for RSV were moderately but significantly lower than their 50% cytotoxic concentrations, as determined by a trypan blue exclusion assay. Four diarylheptanoids with anti-RSV activity also showed anti-poliovirus and anti-measles virus activities and three of the four exhibited anti-HSV-1 activity. Thus, seven of the nine diarylheptanoids examined exhibited potential antiviral activity against RSV, and most of the diarylheptanoids with anti-RSV activity, including two diarylheptanoids without anti-RSV activity, were effective against poliovirus, measles virus, and/or HSV-1 *in vitro*. Diarylheptanoids were suggested to have a broad spectrum of antiviral activity.

Keywords: diarylheptanoids, antiviral activity, RSV, HSV-1, measles virus, poliovirus.

Alpinia officinarum (*A. officinarum*), family Zingiberaceae, is known as lesser galangal. This rhizome has been used in various Asian cuisines and as a traditional medicine, such as an antiemetic, stomachic, and analgesic in Asia from ancient times.

Respiratory syncytial virus (RSV) infection is very common in children less than 2 years old and sometimes causes serious bronchilitis and pneumonia [1]. In elderly and high-risk adults, RSV infection is an important illness [2]. Ribavirin, palivizumab, and motavizumab are used for the treatment and prevention of RSV infection [3–6], but there are few clinically specific and effective anti-RSV drugs.

In a series of studies on the development of bioactive components from natural sources, we found that a methanol extract from the rhizome of *A. officinarum* is effective in inhibiting tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in mouse skin [7]. An extract of *A. officinarum* was previously shown to exhibit therapeutic efficacy against herpes simplex virus type 1 (HSV-1) infection in mice [8]. Diarylheptanoids isolated from *A. officinarum* have been shown to exhibit cytotoxic activity [9], suppressive activity of inducible nitric oxide synthase expression [10], inhibitory activity of biosynthesis of prostaglandin and leukotrienes [11,12], and inhibitory activity of proinflammatory mediators [13]. Although a variety of biological activities associated with diarylheptanoids have been demonstrated, antiviral activity of diarylheptanoids has been reported only against influenza virus [14,15]. In the present study, we examined the potential anti-RSV activity of diarylheptanoids *in vitro*. Their anti-RSV activities were compared with antiviral activities against poliovirus, measles virus, and HSV-1 to characterize the anti-RSV activity.

Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were examined for their anti-RSV activity and cytotoxicity *in vitro*. As shown in Table 1, the EC_{50} values of seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7-9**) were significantly lower than their CC_{50} values. DMSO at 1%, the maximum concentration used to dissolve diarylheptanoids in the culture medium, was not cytotoxic. The therapeutic indexes (CC_{50}/EC_{50}) of 7-(4"-hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-2**) and (5*S*)-5-methoxy-1,7-diphenyl-3-heptanone (**AO-7**) were 4.6 and more than 6.1, respectively, and RSV was more

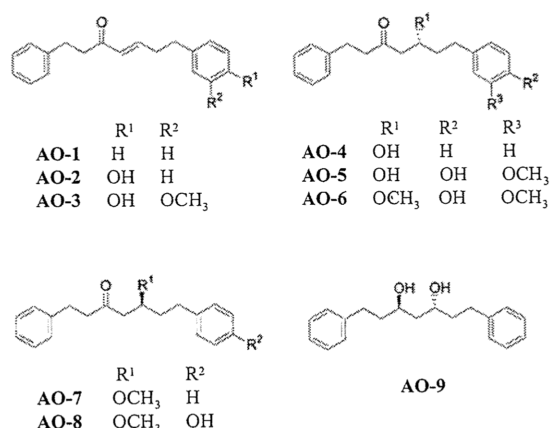

Figure 1: Structures of diarylheptanoids (**AO-1** to **9**) from *A. officinarum*.

susceptible to **AO-2** and **AO-7** than to the other diarylheptanoids examined. In this assay, the EC_{50} value of ribavirin, used as a control, was similar to the results reported previously [16,17]. Thus, seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7-9**) were demonstrated to show moderate, but potential antiviral activity against RSV *in vitro*. This is the first evidence demonstrating the anti-RSV activity of diarylheptanoids *in vitro*.

To evaluate the antiviral spectrum of diarylheptanoids with anti-RSV activity, four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) with anti-RSV activity were examined for anti-poliovirus, -measles virus, and -HSV-1 activities. However, three diarylheptanoids (**AO-2**, **8**, and **9**) with anti-RSV activity were not used, because there were not sufficient amounts to perform a plaque reduction assay. As shown in Table 2, the EC_{50} values of four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) for poliovirus and measles virus were significantly lower than their CC_{50} values. The EC_{50} values of three (**AO-4**, **5**, and **7**) of the four for HSV-1 were also significantly lower than their CC_{50} values. Therefore, among the four diarylheptanoids, three (**AO-4**, **5**, and **7**) with anti-RSV activity exhibited anti-poliovirus, -measles virus, and -HSV-1 activities. **AO-1** exhibited anti-poliovirus and -measles virus activities, but not anti-HSV-1 activity. Because the three diarylheptanoids (**AO-4**, **5**, and **7**) showed antiviral activity against all viruses used in this study, they were suggested to have broad spectrum antiviral activity.

We also examined anti-poliovirus, -measles virus, and -HSV-1 activities of **AO-3** and **6** that did not exhibit anti-RSV activity *in vitro*. As shown in Table 2, **AO-3** was significantly effective for measles virus, but not for poliovirus and HSV-1. However, all three viruses examined were significantly susceptible to **AO-6**. Only measles virus was susceptible to all of the six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) without relation to anti-RSV activity. Diarylheptanoids without anti-RSV activity were also effective against poliovirus, measles virus, and/or HSV-1 *in vitro* and the broad spectrum of antiviral activity was confirmed.

RSV has a different virus structure and replication cycle from poliovirus and HSV-1. However, it has a similar virus structure and replication cycle to measles virus as some paramyxoviruses. In Table 2, six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) exhibited anti-measles virus activity. However, two (**AO-3** and **6**) of them had no anti-RSV activity (Table 1). It is possible that **AO-3** and **6** interfered with a replication step specific to measles virus but not RSV in paramyxoviruses. Although we focused on diarylheptanoids with anti-RSV activity in this study, **AO-3** and **6** were suggested to be potent candidates as anti-measles virus compounds. In our screening of anti-RSV activity *in vitro*, the CC_{50}/EC_{50} value (>6.1) of **AO-7** was highest (Table 1). **AO-7** also exhibited anti-poliovirus, -measles virus, and -HSV-1 activities (Table 2) and may be characterized as a candidate for an anti-RSV compound with a broad antiviral spectrum. Studies of the structure-antiviral activity relationships of many diarylheptanoids isolated from *A. officinarum* [18–20] against various kinds of viruses may be worthwhile to analyze the antiviral actions and to obtain more effective antiviral diarylheptanoids.

RSV was significantly susceptible to seven of the nine diarylheptanoids isolated from *A. officinarum*. Of the nine, six (**AO-1**, **3**, **4**, **5**, **6**, and **7**) with or without anti-RSV activity were effective against poliovirus, measles virus, and/or HSV-1.Thus, diarylheptanoids were suggested to possess a broad spectrum of antiviral activity.

Table 1: Anti-RSV activity and cytotoxicity of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)	CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}
1,7-Diphenyl-4*E*-hepten-3-one(**AO-1**)	36.3 ± 4.2[c]	47.3 ± 1.3	1.3
7-(4"-Hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one(**AO-2**)	5.0 ± 0.0[c]	22.8 ± 2.5	4.6
7-(4"-Hydroxy-3"-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-3**)	42.7 ± 3.5	39.3 ± 6.4	0.9
(5*R*)-5-Hydroxy-1,7-diphenyl-3-heptanone (**AO-4**)	21.7 ± 0.6[c]	38.3 ± 3.4	1.8
(5*R*)-5-Hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone(**AO-5**)	37.0 ± 7.2[c]	84.8 ± 3.8	2.3
(5*R*)-5-Methoxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone (**AO-6**)	13.3 ± 3.8	17.0 ± 0.8	1.3
(5*S*)-5-Methoxy-1,7-diphenyl-3-heptanone (**AO-7**)	16.3 ± 3.5[c]	>100	>6.1
(5*S*)-5-Methoxy-7-(4"-hydroxyphenyl)-1-phenyl-3-heptanone (**AO-8**)	21.7 ± 0.6[c]	31.5 ± 6.6	1.5
(3*R*,5*R*)-1,7-Diphenylheptan-3,5-diol (**AO-9**)	22.3 ± 0.6[c]	56.3 ± 3.1	2.5
Ribavirin	0.67 ± 0.08	NT[d]	NT[d]

The structures of these diaryheptanoids are shown in Figure 1.
[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]P<0.05 vs. CC_{50}; [d]Not tested.

Table 2: Anti- poliovirus, -measles virus, and -HSV-1 activities and cytotoxicities of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)			CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}		
	Poliovirus	Measles virus	HSV-1		Poliovirus	Measles virus	HSV-1
AO-1	8.3±2.3[c]	17.3±1.2[c]	53.7±4.7	45.8±1.7	5.5	2.6	0.9
AO-3	64.3±4.9	47.0±4.6[c]	59.7±0.6	63.0±10.4	1.0	1.3	1.1
AO-4	22.7±1.5[c]	17.0±2.0[c]	54.0±5.6[c]	69.5±5.2	3.1	4.1	1.3
AO-5	44.3±4.0[c]	18.3±1.2[c]	58.7±1.5[c]	>100	2.3	5.5	1.7
AO-6	3.7±0.6[c]	6.3±0.6[c]	5.7±0.6[c]	10.8±1.3	2.9	1.7	1.9
AO-7	16.7±2.1[c]	18.0±1.0[c]	18.3±0.6[c]	40.5±5.4	2.4	2.3	2.2
Acyclovir	NT[d]	NT[d]	0.23±0.04	NT[d]	NT[d]	NT[d]	NT[d]

[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]$P<0.05$ vs. CC_{50}; [d]Not tested.

Experimental

Chemicals: Dimethyl sulfoxide (DMSO) and ribavirin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Acyclovir was purchased from GlaxoSmithKline, Tokyo, Japan. Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were isolated from the rhizome of *A. officinarum*, as described previously [9,20].

Cells and viruses: Human epidermoid carcinoma (HEp-2) cells (American Type Culture Collection CCL-23) were purchased from Dainippon Pharmaceutical, Osaka, Japan, and grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% and 2% heat-inactivated fetal calf serum, respectively. Vero E6 cells were provided by Dr K. Shiraki (Toyama University, Japan) and grown and maintained in EMEM supplemented with 8% and 2% heat-inactivated calf serum, respectively. The A2 strain of RSV was obtained from American Type Culture Collection (Rockville, MD) and grown in HEp-2 cell cultures. Poliovirus type 1 (Sabin strain), measles virus (Tanabe strain), and HSV-1 (7401H strain) were provided by Dr K. Shiraki (Toyama University, Japan) and propagated in Vero cells [8].

Antiviral and cytotoxic assays: The anti-RSV activities of 9 diarylheptanoids were examined by a plaque reduction assay using HEp-2 cells [21,22]. Briefly, HEp-2 cells grown in 24-well plates were infected with 100 plaque-forming units (PFU)/0.2 mL of RSV at 37°C for 1 h. The cells were overlaid with 1 mL of maintenance EMEM containing 0.8% methylcellulose and various concentrations of either diarylheptanoids or ribavirin and maintained in a humidified atmosphere containing 5% CO_2 for 4–5 days.

The anti-poliovirus, -measles virus, and -HSV-1 activities were also examined by a plaque reduction assay using Vero cells [8]. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU/0.2 mL of poliovirus, measles virus, or HSV-1 for 1 h. Then the cells were overlaid with 5 mL of nutrient 0.8% methylcellulose medium containing various concentrations of either diarylheptanoids or acyclovir. The virus-infected cultures were incubated for 2–5 days at 37°C. The infected cells were fixed and stained, and the plaques were counted [8]. All diarylheptanoids were dissolved in DMSO and diluted with culture medium to make the various final concentrations. The concentration of DMSO in each medium was less than 1%. Ribavirin and acyclovir were dissolved in distilled water and DMSO, respectively, and used as controls. The 50% effective antiviral concentration (EC_{50}) was the concentration that reduced virus-induced cell destruction by 50%, as described previously [8,21].

The cytotoxicity of diarylheptanoids was assessed by trypan blue exclusion assays using mock-infected HEp-2 or Vero cells. The cells were seeded at a concentration of 5 x 10^4 cells/mL in 24-well plates. After incubation at 37°C for 24 h, the culture medium was replaced with fresh medium containing one of the diarylheptanoids at various concentrations and the cells were further incubated for 48 h. After 48 h, the cells were trypsinized and the number of viable cells was determined by a trypan blue exclusion assay. The 50% cytotoxic concentration (CC_{50}) was determined as the concentration that reduced cell destruction by 50% [21].

Statistical analysis: Statistical significances of differences between the EC_{50} and CC_{50} values were evaluated using Student's *t*-test. A *P* value of 0.05 or less was considered to be significant statistically.

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