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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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REPRODUCTIVE TOXICOLOGY

Reversible aberration of neurogenesis targeting late-stage progenitor cells in the hippocampal dentate gyrus of rat offspring after maternal exposure to acrylamide

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Abstract We have recently shown that maternal exposure to acrylamide (AA) impaired neurogenesis in rat offspring measured by the increase in interneurons producing reelin, a molecule regulating migration and correct positioning of developing neurons, in the hippocampal dentate gyrus. To clarify the cellular target of AA on hippocampal neurogenesis and its reversibility after maternal exposure, pregnant Sprague–Dawley rats were given drinking water containing AA at 0, 4, 20, 100 ppm on day 10 of pregnancy through day 21 after delivery on weaning. Male offspring were examined immunohistochemically on postnatal day (PND) 21 and PND 77. For comparison, male pups of direct AA-injection control during lactation (50 mg/kg body weight, intraperitoneally, 3 times/week) were also examined. On PND 21, maternal AA-exposure decreased progenitor cell proliferation in the subgranular zone (SGZ) from 20 ppm accompanied with increased density of reelin-producing interneurons and NeuN-expressing mature neurons within the hilus at 100 ppm, similar to the direct AA-injection control. In the SGZ examined at 100 ppm, cellular populations immunoexpressing doublecortin or dihydropyrimidinase-like 3, suggesting postmitotic immature granule cells, were decreased. On PND 77, the SGZ cell proliferation and reelin-producing interneuron density recovered, while the hilar mature neurons sustained to increase from 20 ppm, similar to the direct AA-injection control. Thus, developmental exposure to AA reversibly affects hippocampal neurogenesis targeting the proliferation of type-3 progenitor cells resulting in a decrease in immature granule cells in rats. A sustained increase in hilar mature neurons could be the signature of the developmental effect of AA.

Keywords Acrylamide · Neuronal development · Hippocampal dentate gyrus · Impaired neurogenesis · Immunohistochemistry · Reversibility

Abbreviations

AA	Acrylamide
Actb	Beta actin
C_T	Threshold cycle
Dcx	Doublecortin
Dpysl3	Dihydropyrimidinase-like 3
GABA	Gamma-aminobutyric acid
GAD67	Glutamic acid decarboxylase 67
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GD	Gestational day
NeuroD1	Neurogenic differentiation 1
NeuN	Neuron-specific nuclear protein
Pax6	Paired box 6
PCNA	Proliferating cell nuclear antigen
PND	Postnatal day
SGZ	Subgranular zone
Tbr2	T-box brain protein 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Introduction

Acrylamide (AA) is a water-soluble vinyl monomer that is used primarily to produce polymers (polyacrylamides) that have broad applications in the chemical industry for water management, ore processing, and dye synthesis, as well as its use in experimental laboratories conducting molecular biology studies and/or biochemistry (LoPachin 2004; Lee et al. 2005). AA has been shown to be a neurotoxicant, reproductive toxicant, and genotoxic carcinogen in animal species (Exon 2006; WHO/IPCS 2006). Exposure to AA in foodstuffs has become a worldwide concern recently because of its generation in a variety of fried and oven-baked foods during cooking through Maillard reactions of sugars with asparagine residues (Mottram et al. 2002).

In the nervous system, it is well known that AA targets axon terminals in both the central and peripheral nervous systems as represented by distal axonopathy (LoPachin 2004). However, developmental exposure to AA by maternal transfer, at dose levels inducing maternal neurotoxicity, has shown no such neurotoxicity in offspring but results in a reduced body size by us and others (Friedman et al. 1999; Takahashi et al. 2009). On the other hand, direct injection of AA into neonatal rats throughout the lactation period resulted in neurotoxicity similar to that observed in adult animals (Takahashi et al. 2009), suggesting that neonates and adult animals have comparable sensitivity with AA. The lack of neurotoxicity of AA in maternally exposed pups was considered to be due to limited lactational transfer and perhaps to impairment in nursing/lactation activity as a consequence of maternal neurotoxicity (Takahashi et al. 2009), which might also explain the loss of offspring body weight.

Within the brain, the dentate gyrus in the hippocampal formation is a unique structure that can continue neurogenesis during postnatal life (Zhang et al. 2009). The postnatal neurogenesis (so-called "adult neurogenesis") occurs in the neuroblast-producing subgranular zone (SGZ) from the type-1 progenitor cells and produces intermediate generations in the order of type-2a, type-2b, and type-3 cells in the SGZ. Then, type-3 cells undergo final mitosis to differentiate to immature granule cells, and then to mature granule cells (Hodge et al. 2008). In addition, γ-aminobutyric acid (GABA)ergic interneurons in the hilus of the dentate gyrus produce reelin in the embryonic period and throughout adult life to regulate the migration and maturation of newborn granule cells in the granule cell layer (Lussier et al. 2009). In our laboratory, increase in reelin-producing interneurons in the hilus has recently been shown in a rat model of neuronal mismigration induced by developmental hypothyroidism (Saegusa et al. 2010). Recently, increasing numbers of chemicals have revealed to affect proliferation and differentiation of progenitor cells by exposure to mice or rats during postnatal life (Choi et al. 2011; Hwang et al. 2011; Nam et al. 2011; Yan et al. 2011; Yoo et al. 2011). Because neurogenesis in the dentate gyrus is rather active during the developmental stage than the adult stage, developmental exposure to such chemicals may risk stronger impact on neurogenesis. Therefore, it may be reasonable to analyze neuronal stage-defining markers in combination with reelin-producing interneurons for elucidation of the influence of exogenously administered chemicals on neurogenesis as well as the target cell affected.

It is well established that the molecular mechanisms controlling neuronal migration during development have many similarities with those described for axon guidance (Nóbrega-Pereira and Marín 2009), suggesting that both migrating neuroblasts and immature axon terminals may be sensitive to AA. In our recent study, developmental exposure effect of AA on neurogenesis has been examined using samples verified as lacking any obvious axon terminal injury (Takahashi et al. 2009). The offspring of maternally exposed AA through drinking water (25, 50, 100 ppm) showed an AA dose-dependent increase in the density of reelin-immunoreactive GABAergic interneurons, suggesting an affection of neurogenesis and following neuronal migration (Ogawa et al. 2011). The lowest-observed adverse effect level of AA was determined to be 25 ppm by maternal exposure (3.72 mg/kg body weight/day). Although AA-exposed offspring had reduced body size, it has already been confirmed that there was no influence of systemic growth retardation during development using an intrauterine growth retardation model on the distribution of reelin-synthesizing interneurons in the hilus and the neurogenesis in the SGZ by us (Ohishi et al. 2010).

This study further investigates developmental exposure effect of AA on neuronal development in rat offspring. The reversibility of the aberration in neurogenesis was examined at the adult stage, as well as the target progenitor cell of AA in the SGZ employing a line of neuronal stage-defining markers. To measure the immunoreactive cell populations in the substructure of the dentate gyrus, a profile counting method was applied (Lussier et al. 2009).

Materials and methods

Chemicals and animals

Acrylamide was purchased from Sigma Chemical Co. (St. Louis, MO, USA; CAS #79-06-1) with a purity of more than 98%. Pregnant Sprague–Dawley rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) at gestational day (GD) 3 (the day when a vaginal plug was observed was designated as GD 0). They were housed individually in

polycarbonate cages with wood chip bedding, in an air-conditioned animal room on a 12 h light–dark cycle and conditioned at 23 ± 2°C with a relative humidity of 55 ± 15%. Animals were provided with pelleted basal diet (MF diet; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum during the 7-day acclimatization period.

Experimental design

Two experiments were carried out. In Experiment 1, dams received AA. Twenty-four dams were randomly divided into four groups of 6 dams each and given AA at either 0, 4, 20, 100 ppm in the drinking water from GD 10 to day 21 after delivery. The highest dose was selected as that inducing progressive neurotoxicity to dams in our previous study (Takahashi et al. 2009). In Experiment 2, AA was administered directly to delivered neonates. Sixteen dams were divided into two groups of 8 dams, all maintained untreated until delivery. Neonates received vehicle saline (control) or AA at 50 mg/kg BW by intraperitoneal (ip) injections 3 times a week from postnatal day (PND) 4 to 21 (where PND 0 is the day of delivery). This AA dosing regimen has been shown to induce degeneration of the sciatic nerve in adult rats within 3 weeks (Saita et al. 1996). Each dam was housed with her litter individually, and body weights and food and water consumption were measured regularly. Litter size, sex, and body weight of pups were checked at PND 2, and litters were culled to preserve eight male pups per dam at PND 4. Female pups were included to maintain a total of eight pups/litter if dams had fewer than eight male pups. Culled female pups were killed by rapid decapitation. Daily observation was conducted for clinical signs, including gait abnormalities and mortality of dams and offspring, throughout the experimental period.

To assess AA-induced neurological abnormalities, all animals in the AA-treated and untreated groups were scored with respect to the appearance of gait abnormalities as described previously (Lee et al. 2005). The degree of abnormality was classified into the following 4 categories: Grade 1 as normal gait; Grade 2 as slightly abnormal gait with slight degrees of ataxia, hopping gait, and foot splay; Grade 3 as moderately abnormal gait with moderate degrees of ataxia, foot splay, and limb abduction; Grade 4 as severely affected gait including inability to support the BW as well as foot splay.

On day 21 after delivery, necropsy was performed on all dams and 3–4 male pups/litter after deep anesthesia with ether to remove brains for immunohistochemical analysis (dams and pups) and real-time RT-PCR analysis (pups). The livers of dams were also removed for weight measurement. The remaining male pups were allocated to 4 rats per cage without any special treatments or administrations, and necropsy was performed to remove their brains after ether anesthesia on PND 77.

For immunohistochemical analysis, the brains of 10–12 male pups/group (1–2 pups/litter, mostly 2 pups/litter) were fixed at both PND 21 and PND 77 in Bouin's solution at room temperature overnight in both Experiment 1 and Experiment 2. Then, coronal brain slices were routinely processed approximately at the position of −2.9 mm from the bregma at PND 21 and −3.3 mm at PND 77 for paraffin embedding. Serial sections at 5 μm were prepared from the position of −3.1 mm from the bregma at PND 21 and −3.6 mm at PND 77. Immunohistochemistry was additionally performed on similarly fixed brains of female pups culled on PND 4 (9–10 pups/group; 1–2 pups/litter, mostly 2 pups/litter) in Experiment 1. For real-time RT-PCR analysis, brains of 6–12 male pups/group (1–2 pups/litter) removed on PND 21 were fixed in methacarn solution for 6 h and then dehydrated at 4°C overnight as described previously (Lee et al. 2006) in both Experiment 1 and Experiment 2. Then, coronal brain slices were made by cutting laterally at the positions of +1 mm and −5 mm from the bregma to dissect bilateral whole hippocampal tissues from slices using tweezers after removal of the cerebral cortical tissues. Dissected tissue samples were preserved in 99.5% ethanol at −80°C until use for real-time RT-PCR analysis.

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 of the Tokyo University of Agriculture and Technology.

Immunohistochemistry and apoptosis assays

Immunohistochemistry was performed on the brains of offspring and dams for the detection of reelin, proliferating cell nuclear antigen (PCNA), neuron-specific nuclear protein (NeuN), and neuronal stage-defining markers, dihydropyrimidinase-like 3 (Dpysl3, also known as TUC-4), doublecortin (Dcx), neurogenic differentiation 1 (NeuroD1), t box brain protein 2 (Tbr2, also known as Eomes), and paired box 6 (Pax6). Following primary antibodies were used for immunohistochemical analysis: reelin (mouse monoclonal, clone G10, 1:1,000; Novus Biologicals, Inc., Littleton, CO, USA), PCNA (mouse monoclonal, clone PC10, 1:200, Dako, Glostrup, Denmark), NeuN (mouse monoclonal, clone A60, 1:1,000, Chemicon, Billerica, MA, USA), Dpysl3 (rabbit polyclonal, 1:1,000, Chemicon), Dcx (rabbit polyclonal antibody, 1:2,000, Abcam Inc., Cambridge, UK), NeuroD1 (mouse monoclonal, 1:300, Abcam Inc.), Tbr2 (rabbit polyclonal, 1:500, Abcam Inc.), and Pax6 (mouse monoclonal, clone AD2.38, 1:500, Abcam Inc.). We applied a

horseradish peroxidase avidin–biotin complex method for immunodetection of antigens utilizing a VECTASTAIN® Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. Antigen retrieval was performed with PCNA antibody by autoclaving deparaffinized sections at 121°C for 10 min in 10 mM citrate buffer (pH 6.0). No antigen retrieval was applied for other antibodies. Immunostained sections were counterstained with hematoxylin for microscopic examination. The animal groups examined for immunohistochemistry on each antigen were listed in Table 1.

Apoptotic bodies were detected by Cresyl Violet-staining (Nuñez and McCarthy 2004) to evaluate apoptosis in the dentate SGZ of pups. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was carried out to confirm apoptosis using the ApopTag® Peroxidase In situ Apoptosis Detection kit (Millipore, Temecula, CA, USA). Animal groups examined are listed in Table 1.

Analysis of immunolocalization and apoptotic cells

Immunoreactive cells and apoptotic cells were counted by blind trial for the treatment conditions.

On both PND 21 and PND 77, positive cells showing reelin- or NeuN-immunoreactivity distributed in the dentate hilus were bilaterally counted and normalized for the number per unit area of the hilus as described previously in both Experiment 1 and Experiment 2 (Fig. 1; Saegusa et al. 2010; Ohishi et al. 2010). In the dentate SGZ, apoptotic bodies stained with Cresyl Violet were bilaterally counted, as well as cells showing nuclear immunoreactivity for PCNA, Dpysl3, NeuroD1, Tbr2, and Pax6, and TUNEL-positive cells and the number was normalized with the length of the granule cell layer measured. Dcx-positive cells showing cytoplasmic immunoreactivity was also counted and normalized similarly.

Reelin-positive cells distributed in the molecular layer or in the dentate hilus were bilaterally counted and the

Table 1 List of animal groups examined for immunohistochemistry and apoptosis assays

	Experiment 1				Experiment 2	
	AA in the drinking water (ppm)				Intraperitoneal injections	
	0 (control)	4	20	100	Saline (control)	AA
PND 21, male offspring						
Reelin	T	T	T	T	T	T
NeuN	T	T	T	T	T	T
PCNA	T	T	T	T	T	T
Dpysl3	T	NE	NE	T	NE	NE
Dcx	T	NE	NE	T	NE	NE
NeuroD1	T	NE	NE	T	NE	NE
Tbr2	T	NE	NE	T	NE	NE
Pax6	T	NE	NE	T	NE	NE
Cresyl Violet-staining	T	T	T	T	T	T
TUNEL-assay	O	O	O	O	O	O
PND 77, male offspring						
Reelin	T	T	T	T	T	T
NeuN	T	T	T	T	T	T
PCNA	T	T	T	T	T	T
Dpysl3	O	NE	NE	O	NE	NE
Dcx	O	NE	NE	O	NE	NE
Cresyl Violet-staining	T	T	T	T	T	T
PND 4, female offspring						
Reelin	O	O	O	O	O	O
Dams						
Reelin	T	NE	NE	T	NA	NA
PCNA	T	NE	NE	T	NA	NA

T stained three different sections with approximately 250-μm interval in each brain, *O* stained one section in each brain, *NE* not examined, *NA* not applicable

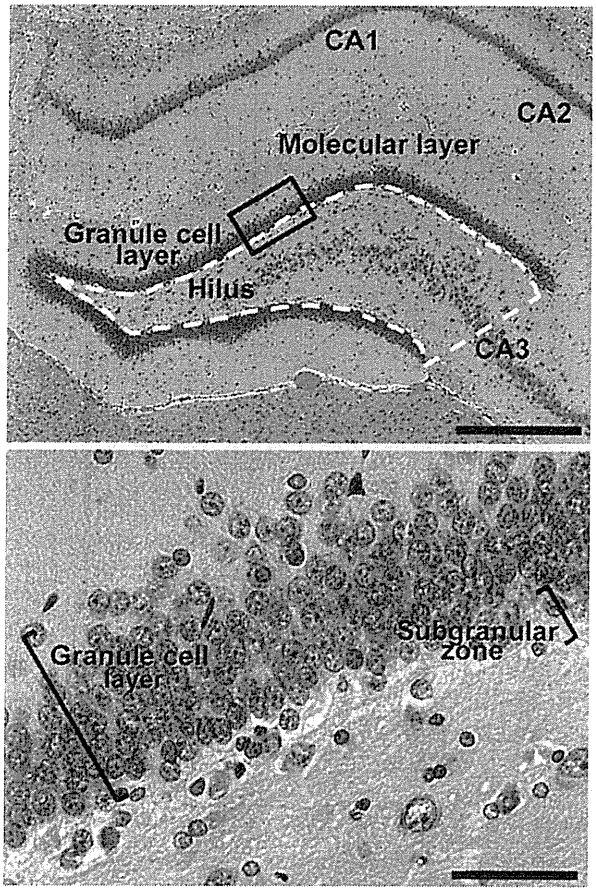

Fig. 1 Overview of the hippocampal formation of a male rat at PND 77 stained with hematoxylin and eosin. (*Upper panel*) Immunoreactive cells for reelin and NeuN were counted in the hilus of the dentate gyrus, as enclosed by the white dotted line. Magnification, ×40; *bar* 500 μm. (*Lower panel*) Higher magnification of the granule cell layer and SGZ as enclosed by the black line in the *upper panel*. Immunoreactive cells for PCNA, Pax6, Tbr2, NuroD1, Dcx, and Dpysl3, as well as apoptotic cells as detected by Cresyl Violet-staining and TUNEL-staining were measured in the SGZ. Magnification, ×400; *bar* 50 μm

number was normalized with the length of the SGZ measured in female pups culled on PND 4.

A profile counting method was applied using 3 brain sections at approximately 250-μm intervals for the measurement of cellular distribution of immunoreactive cells and apoptotic bodies in pups or dams with animal groups and items listed in Table 1. Positive cells were counted in one brain section per animal when TUNEL-assay was performed on pups at PND 21 and in those used for immunohistochemistry of neuronal stage-defining markers on pups at PND 77.

For quantitative measurement of cells positive for immunohistochemistry or TUNEL-assay and apoptotic bodies, digital photomicrographs at 200× or 400× magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co.), and quantitative measurements were performed using the WinROOF image analysis software package (Version 5.7, Mitani Corp., Fukui, Japan). Where the profile counting method was applied, the normalized number of positive cells or apoptotic bodies was averaged across the number of sections.

Real-time RT-PCR analysis

Transcript levels for genes listed in Online Resource 1 (Table s1) were analyzed with real-time RT-PCR in hippocampal tissues on PND 21. From total RNA samples extracted with RNeasy Mini Kit (QIAGEN, Hilden, Germany), first-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA), and then real-time PCR analysis was performed using the SYBR®Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc.). The PCR primers were designed for each gene using Primer Express software (Version 3.0; Applied Biosystems Inc.). The relative differences in gene expression was calculated using threshold cycle (C_T) values that were first normalized to those of the housekeeping gene, beta actin (*Actb*), or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), as a endogenous control in the same sample, and then relative to a control C_T value by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Statistical analysis

Maternal data regarding the body weight and food consumption during the animal study as well as immunoreactive cell counts in the Experiment 1 were analyzed using the individual animal as the experimental unit. Data for offspring regarding the body and organ weights at necropsy in both the Experiment 1 and Experiment 2 were analyzed using the litter as the experimental unit. With regard to the data for cell counts immunoreactive for each antigen or apoptotic cells and real-time RT-PCR of pups, the individual animal was examined as the experimental unit.

For comparison of numerical data between the 0 ppm (control) and AA-dosed groups in Experiment 1, data were analyzed by the Bartlett's test for the homogeneity of variance. If there is no significant difference in variance, Dunnett's test was performed for comparison between the groups. If a significant difference was found in variance, Steel's test was then performed.

In case of numerical data consisting of two sample groups, data were analyzed by the F test for the homogeneity of variance and Student's t test was applied when the variance was homogenous among the groups using a test for equal variance. If a significant difference was observed in variance, Aspin–Welch's t test was used instead.

As for gait score data, Mann–Whitney's U test was used to compare between the untreated controls and AA-dosed animals.

Results

Maternal and reproductive parameters (Experiment 1)

There was no observable gait abnormality of dams through to the day 21 after delivery. There was one animal that was judged to be non-pregnant in the 100 ppm group by the examination of uterine implantation sites, and therefore, this animal was excluded from the experiment. No significant changes were observed in food intake and water consumption during the whole exposure period as compared with the 0 ppm controls (Online Resource 1, Table s2). At the necropsy on day 21 after delivery, a slight reduction in the absolute liver weight was observed at 100 ppm as compared with the 0 ppm controls (Online Resource 1, Table s2). Maternal exposure to AA did not affect the duration of pregnancy, number of implantation sites, live birth ratio, or male pup ratio.

In life changes and necropsy data of offspring

In Experiment 1, deaths of offspring were sporadically found in 0, 4, 20 ppm groups during PND 7–15; however, no other apparent abnormalities were found on clinical observation until weaning on PND 21. At the necropsy on PND 21, statistically significant decreases were found in the body and absolute brain weights of offspring at 100 ppm that continued to PND 77 (Online Resource 2, Fig. s1); however, any gait abnormalities were not observed from PND 21 to PND 77.

In Experiment 2, gait abnormalities were detected on most pups given AA directly showing statistically significant difference in the score from PND 15 as compared to the saline controls (Online Resource 2, Fig. s2). From PND 28, gradual recovery from gait abnormalities was observed in AA-injected animals, and any statistically significant difference was not observed in the score from PND 63. The body and absolute brain weights of AA-injected pups were significantly lower than those of saline controls at both PND 21 and PND 77 (Online Resource 2, Fig. s1).

Reelin- or NeuN-positive neurons in the dentate hilus

Distribution patterns of reelin- or NeuN-positive neurons in the hippocampus on both PND 21 and PND 77 were described previously (Saegusa et al. 2010; Ohishi et al. 2010). On PND 4, reelin-positive cells within the dentate gyrus were predominantly distributed in the molecular layer rather than the hilar region.

In Experiment 1, an increased density of reelin-positive cells and NeuN-positive cells in the hilus was observed with dose relation to AA from 20 ppm showing a statistically significant difference at 100 ppm on PND 21 (Fig. 2). On PND 77, no changes were observed in the density of reelin-positive cells with AA-exposure at any dose, but a significant increase in NeuN-positive cells was observed with AA from 20 ppm. We additionally examined reelin-positive cells in culled female pups on PND 4; however, there were no changes in positive cell distribution in either the molecular layer or the hilus (Online Resource 2, Fig. s3).

In Experiment 2, a significantly increased density of reelin-positive cells and NeuN-positive cells was observed in the hilus of AA-injected pups on PND 21, as compared with the saline controls (Fig. 2). On PND 77, no changes in the density of reelin-positive cells were observed with AA-injections; however, a significantly increased density of NeuN-positive cells in AA-injected pups was observed as compared to saline controls.

Proliferating and apoptotic cell counts at the SGZ

On PND 21, both PCNA-positive proliferating cells and Cresyl Violet-positive apoptotic bodies were observed in the SGZ similarly to the previous report (Fig. 3; Saegusa et al. 2010). On PND 77, however, numbers of both PCNA-positive cells and apoptotic bodies were rather decreased, and apoptotic bodies were not detected in many animals.

In Experiment 1, dose-dependent decrease in PCNA-positive cells was observed with AA-exposure on PND 21 with statistically significant difference from 20 ppm as compared with 0 ppm control (Fig. 3). There were no significant changes in the number of apoptotic bodies after AA-exposure (Fig. 3). Similarly, no significant decrease in TUNEL-positive cells was found after AA-exposure (Online Resource 2, Fig. s4). On PND 77, no changes in the number of PCNA-positive cells and apoptotic bodies were observed with AA at any dose (Fig. 3).

As in Experiment 1, a significant decrease in the number of PCNA-positive cells was found with direct AA-injections as compared with saline controls on PND 21 in Experiment 2 (Fig. 3); however, the number of apoptotic bodies was unchanged with AA-injections. On PND 77, the number of PCNA-positive cells or apoptotic bodies was not changed following AA-injections.

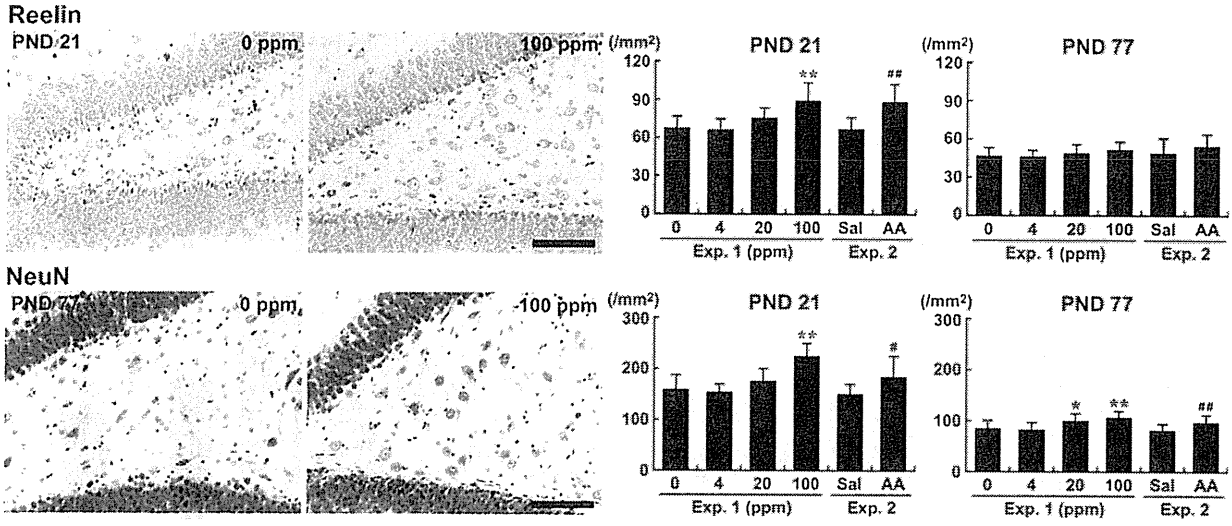

Fig. 2 Distribution of reelin- and NeuN-immunoreactive cells in the dentate hilus of male pups after AA-exposure. Photomicrographs are from Experiment 1. (*Left panels*) 0 ppm AA. (*Right panels*) 100 ppm AA. Magnification, ×200; *bar* 100 μm. The graphs show the density of reelin- or NeuN-positive cells in the hilus (bilateral) estimated applying a profile counting method. $N = 12$ (0 ppm controls), 11 (4 ppm), 11 (20 ppm), and 10 (100 ppm) in Experiment 1, and 12 (saline controls) and 12 (AA-injected animals) in Experiment 2 on PND 21. $N = 12$ (0 ppm controls), 12 (4 ppm), 12 (20 ppm), and 11 (100 ppm) in Experiment 1, and 12 (saline controls) and 12 (AA-injected animals) in Experiment 2 on PND 77. Values are expressed as mean + SD. $^{*}P < 0.05$; $^{**}P < 0.01$ versus 0 ppm control group (0 ppm) in Experiment 1 (Dunnett's or Steel's test). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus saline control group (vehicle saline) in Experiment 2 (Student's or Aspin–Welch's *t* test)

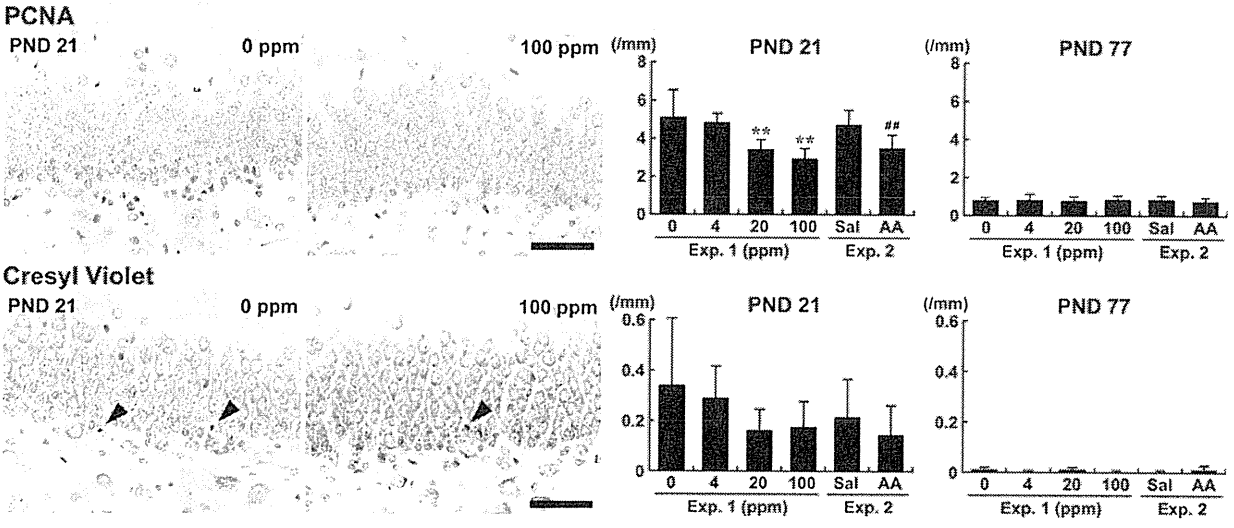

Fig. 3 Distribution of PCNA-immunoreactive proliferating cells and Cresyl Violet-positive apoptotic bodies (*arrowheads*) in the SGZ of male pups after AA-exposure. Photomicrographs are from Experiment 1. (*Left panels*) 0 ppm AA. (*Right panels*) 100 ppm AA. Magnification, ×200; *bar* 100 μm. The graphs show the number of PCNA-immunoreactive cells or apoptotic bodies in the SGZ (bilateral) estimated applying a profile counting method. Values are expressed as mean + SD. Numbers of animals examined in each group were identical to those in the analysis of reelin- or NeuN-immunoreactive cells shown in Fig. 2. $^{**}P < 0.01$ versus 0 ppm control group (0 ppm) in Experiment 1 (Dunnett's or Steel's test). $^{\#\#}P < 0.01$ versus saline control group (vehicle saline) in Experiment 2 (Student's or Aspin–Welch's *t* test)

Neuronal progenitor cells in the SGZ (Experiment 1)

On PND 21, statistically significant decreases in the number of Dpysl3- or Dcx-positive cells was found with AA-exposure as examined at 100 ppm as compared with 0 ppm controls (Fig. 4). However, there were no changes in the number of NeuroD1-, Tbr2- or Pax6-positive cells with AA at 100 ppm. By PND 77, no changes in the

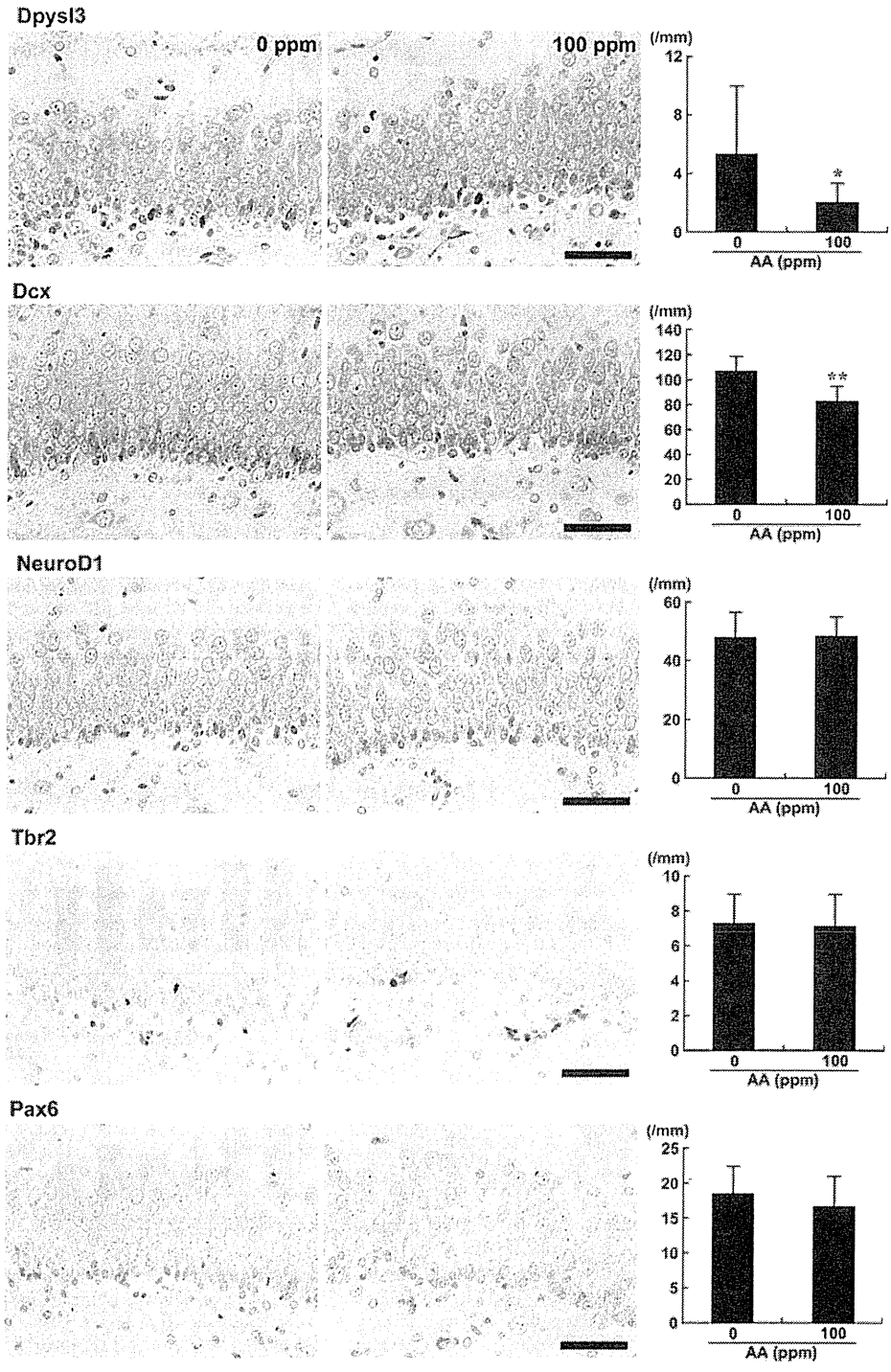

Fig. 4 Distribution of immunoreactive cells for neuronal stage-defining markers in the SGZ of male pups after AA-exposure examined on PND 21 in Experiment 1. (*Left panels*) 0 ppm AA. (*Right panels*) 100 ppm AA. Magnification, ×400; *bar* 50 μm. The graphs show the number of immunoreactive cells for each marker in the SGZ (bilateral) estimated applying a profile counting method. Values are expressed as mean + SD. $N = 12$ (0 ppm controls) and 10 (100 ppm). $^{*}P < 0.05$; $^{**}P < 0.01$ versus 0 ppm control group (0 ppm) (Student's or Aspin–Welch's *t* test)

number of Dpysl3- or Dcx-positive cells was observed with AA at 100 ppm (Online Resource 2, Fig. s5), while the numbers of immunoreactive cells were rather fewer than those at PND 21.

Dentate hilar area and length of the SGZ

In Experiment 1, a statistically significant decrease in the hilar area was found at 100 ppm as compared with 0 ppm

controls on PND 21 (Online Resource 2, Fig. s6). By PND 77, hilar area did not change after AA-exposure. There were no dose-related fluctuations in the length of SGZ at both PND 21 and PND 77, but a statistically significant increase was found at 4 and 20 ppm as compared with 0 ppm controls.

In Experiment 2, a statistically significant reduction was found in the hilar area by AA-injections as compared with the saline controls at both PND 21 and PND 77; however, there were no statistically significant difference in the SGZ-length at either PND 21 or PND 77 (Online Resource 2, Fig. s6).

Real-time RT-PCR data

There were no genes showing statistically significant up- or down-regulation more than one and half times the transcript levels between the AA-dosed animals and the corresponding controls commonly in both Experiment 1 and Experiment 2 (Online Resource 2, Fig. s7, s8). Also, there were no genes showing statistically significant fluctuations commonly in the values with the *Actb* and *Gapdh* normalizations in both Experiment 1 and Experiment 2 except for a slight upregulation of *Neurod1* transcript levels by both normalizations in Experiment 2.

Reelin- or PCNA-positive cells of dams

In dams of Experiment 1, there were no changes in the density of reelin-immunoreactive cells in the hilus or in the distribution of PCNA-positive cells in the SGZ with AA at 100 ppm (Fig. 5).

Discussion

In the present study, we detected an increased density of reelin-expressing interneurons in the hilus at 100 ppm and a decrease in PCNA-positive (i.e., proliferating) progenitor cells in the SGZ from 20 ppm in male offspring examined on PND 21, similar to the directly AA-injected cases. These results reproduce those in our recent study on weaning as found in male offspring (Ogawa et al. 2011). In the present study, we also observed a decrease in the hilar area at 100 ppm on PND 21, suggesting that the high density of reelin-producing interneurons in this group may be due to the reduction in the hilar volume. Because reelin is a large glycoprotein secreted to function as a ligand locally for the correct positioning of neurons (Rice and Curran 2001), cellular density rather than total cell number may be critical for exerting the local function of reelin. We have already shown decreased apoptotic bodies in the SGZ with AA at 100 ppm on PND 21 (Ogawa et al. 2011) but we did not detect apparent decrease in apoptotic bodies and TUNEL-positive cells after AA-exposure in the present study. We think that the shorter exposure period (GD 10–PND 21) in this study compared with that of the previous study (GD 6–PND 21) could explain the difference.

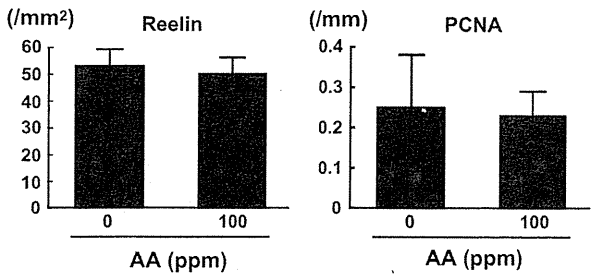

Fig. 5 Density of immunoreactive cells for reelin in the hilus and PCNA in the SGZ of dams in Experiment 1. The graphs show the density of reelin-immunoreactive cells in the hilus (bilateral) or the number of PCNA-positive cells in the SGZ (bilateral) estimated applying a profile counting method. Values are expressed as mean + SD. $N = 6$ (0 ppm controls) and 5 (100 ppm)

In the developmental exposure study of anti-thyroid agents in rats, we have previously observed sustained increase in reelin-positive and NeuN-negative immature interneurons in the dentate hilus on PND 77, as well as the increase in reelin-negative and NeuN-positive mature neurons (Saegusa et al. 2010). This result suggests that developmental hypothyroidism caused sustained impairment of neurogenesis and neuronal mismigration. In contrast, the distribution changes of reelin- and PCNA-positive cells at PND 21 in our AA-exposed cases here disappeared at PND 77, as with the recovery in the distribution of target progenitor cells, suggestive of a weak and reversible influence of AA on neurogenesis itself. Because reelin plays a role for repositioning of fully differentiated neurons to stabilize mature neuron circuitry (Frotscher 2010), no excess population of reelin-expressing cells at later stages observed here could be the reflection of no effect on the maintenance of neuron circuitry of migrated granule cells. Considering no effect on the mRNA expression levels in the hippocampus, in terms of reelin and related molecules, neuronal stage-defining marker molecules, cell proliferation marker molecule, and epigenetic event-related enzymes, observed cellular distribution changes on PND 21 by AA may be subtle and gradually progressive ones. However, no changes in the distribution of reelin- and PCNA-positive cells in dams suggest that offspring neurogenesis is much more sensitive to AA than the adult animals through maternal exposure.

In the present study, we found a sustained increase in cellular density of NeuN-positive mature neurons in the hilus after cessation of maternal exposure to AA from 20 ppm as well as ip-injected cases, while the reelin-positive population returned to the control level. In the

rodent dentate hilus, there are approximately equal numbers of GABAergic interneurons and other neurons (Houser 2007). We have found an increase in glutamic acid decarboxylase (GAD) 67-immunoreactive cells on PND 21 after maternal AA-exposure, confirming an increase in GABAergic interneurons (Ogawa et al. 2011). In the hypothyroidism model (Saegusa et al. 2010), we have found an increase in GAD67-positive population only at the adult stage, and the calbindin-D-28K-expressing population increased at the end of exposure on weaning. These results suggest that there are heterogeneous populations of interneurons showing different cellular potency depending on the type or magnitude of the insult on neurogenesis. We have recently found a transient increase in the density of reelin-producing interneurons associated with a sustained increase in mature neurons in the hilus by developmental exposure to brominated flame retardants (Saegusa et al., data submitted). However, we could not find fluctuations in the GAD67-positive populations either at the end of exposure or at the adult stage by these compounds. Because reelin is an inducible molecule and its expression level can be modified during the disease process (Kundakovic et al. 2009), it may be reasonable to consider that the excess population of reelin-producing interneurons during the AA-exposure halted their reelin production thereafter, reflecting a recovery from the effect on neurogenesis. Alternatively, the sustained increase in interneurons may be the signature of impaired neurogenesis during the developmental stages.

With regard to distribution changes in the neuronal progenitor cells in the SGZ, we revealed here decreases in Dcx- and Dpysl3-positive cells with 100 ppm AA on PND 21, while the distribution of progenitor cells expressing Pax6, Tbr2 and NuroD1 were unchanged. Pax6 and Tbr2 are markers for earlier stage progenitor cells (Breunig et al. 2007; Hodge et al. 2008). In contrast, NeuroD1 and Dcx are not neurogenesis markers with high specificity and are expressed from type-2b progenitor cells to immature granule cells with peak expression in type-3 cells and immature granule cells, respectively (Breunig et al. 2007; Hodge et al. 2008; Knoth et al. 2010). Dpysl3 is an early postmitotic neuronal marker of immature granule cells (Knoth et al. 2010). Therefore, we consider the decreased Dpysl3 by 100 ppm AA reported here represents immature granule cells. These cells already have both dendritic growth cones and recurrent basal dendrites, suggesting an entry into the process of synaptogenesis (Ribak et al. 2004). It is well known that AA targets nerve terminals due to impairment of neurotransmission by affecting diverse nerve terminal processes (Lopachin et al. 2008), suggesting the possibility of direct injury of immature granule cells by affecting the newly generating nerve terminals. However, we did not observe increase in apoptosis. Importantly, we observed suppression of progenitor cell proliferation by AA in the present study. Because immature granule cells can no longer proliferate, AA may rather target earlier type-3 progenitor cells to suppress their proliferation causing a reduction in immature granule cells.

We have shown that high doses of AA by direct ip-injections during the lactation period are necessary to induce an increase in reelin-producing interneurons, in contrast to the much lower doses to stimulate reelin expression if administered maternally from the gestation period onwards (Ogawa et al. 2011). We reproduced that result in this study. In humans, the internal level of AA in neonates by transplacental exposure was estimated to be at least equal to that of the mother (Schettgen et al. 2004). Considering that only about a tenth of maternal AA is transferred to offspring through lactation (Takahashi et al. 2009), transplacental rather than lactational transfer of AA may be responsible for the disruption of neurogenesis. On the other hand, in this study, we found that the number of reelin-positive cells distributed in the hilus and the molecular layer of the dentate gyrus, the former being interneurons and the latter Cajal–Retzius cells (Nakajima et al. 1997), was unchanged by maternal exposure to AA in culled pups on PND 4. Considering the immaturity of the postnatal "adult neurogenesis" in the dentate gyrus, target cell toxicity in the SGZ or responses of reelin-producing neurons would not appear at this time point. Although the putative mechanism of AA-action, whether through its known genotoxic potential or unknown epigenetic modification, should further be addressed, these results suggest a late onset for inducing aberrant neurogenesis after transplacental AA-exposure.

Epidemiological studies of human industrial and accidental exposures suggest that the nervous system is a principal site for toxicity in humans. At the sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives, the committee concluded that based on national estimates, an intake of 0.001 mg/kg body weight/day of AA could be taken to represent the average for the general population and that an intake of 0.004 mg/kg body weight/day could be taken to represent high consumers (WHO/IPCS 2006). The committee also concluded that the non-observed effect level (NOEL) was determined to be 0.2 mg/kg body weight/day for the induction of distal axonopathy detected using electron microscopy in rats exposed to AA in drinking water for 90 days (Burek et al. 1980). The overall NOEL for reproductive and developmental effects and other non-neoplastic lesions was determined as 2 mg/kg body weight/day (Tyl et al. 2000). Based on the calculated margins of exposure, the committee concluded that adverse effects based on these endpoints are unlikely at the estimated average intakes, but that distal axonopathy changes cannot be excluded for some individuals with very high intake (WHO/IPCS 2006). In the

present study, the mild but sustained increase in mature neurons in the hilus from 20 ppm was considered to be irreversible, and thus, 4 ppm was determined to be the non-observed adverse effect level (NOAEL), translating into 0.36–0.89 mg/kg body weight/day. Considering the intake values for infants and children as 2–3 times higher than for adults when expressed on a body weight basis (WHO/IPCS 2006), the effect level in our present study may be equivalent to that detecting distal axonopathy by Burek et al. (1980). Nonetheless, administered AA is mutagenic and acts as a genotoxic carcinogen targeting multiple organs in rats (Rice 2005), and appropriate efforts to reduce AA concentrations in foodstuffs should continue as concluded by the Committee above mentioned (WHO/IPCS 2006).

In conclusion, while the neurotoxic effect of AA on neurogenesis and following neuronal migration in the dentate gyrus observed from 20 ppm was subtle and reversible, the sustained increase in mature neurons in the hilus at the later stages after AA-exposure from 20 ppm was considered to be irreversible. Because dysfunctional GABAergic interneurons are central contributors to a number of diseases such as schizophrenia and epilepsy (Dudek and Sutula 2007; Jones 2010), the biological significance of the excess population of inhibitory interneurons by AA on the neural circuits should further be assessed with regard to functional endpoints of the hippocampal dentate gyrus on learning, memory, and motivation.

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