Neurotoxic Damage of Granule Cells in the Dentate Gyrus and the Cerebellum and Cognitive Deficit Following Neonatal Administration of Phenytoin in Mice

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Abstract. The use of antiepileptic drugs during human gestation probably increases the risk of causing CNS disorders in later life. In brain, granule cells in the dentate gyrus (DG) and cerebellum are still developing in the last trimester of human gestation and a similar development is taking place during the mouse perinatal period. We treated newborn C57BL/6 mice orally with 35 mg/kg phenytoin (PHT) daily during postnatal days (PD) 5 to 14. Histopathological investigation revealed that the layer of mature granule cells in the DG that was immunoreactive to anti-calbindin D28k was thinner in PHT-treated mice. Purkinje cells in the treated group also had poor, immature arborls with an irregular arrangement. A number of TUNEL-positive cells were observed in the DG and cerebellum during the treatment. PHT-treated mice were impaired in the acquisition of a hidden platform task in the water maze and committed significantly more errors during the learning process in the radial arm maze. These findings demonstrate that neonatal administration of PHT interferes with the development of granule cells in the hippocampus and the cerebellum and causes spatial learning deficits in later life. Cautious clinical use of this drug for pregnant patients is warranted, especially in the last trimester.

Key Words: Cerebellum; Dentate gyrus; Granule cells; Neonates; Phenytoin; Spatial learning.

INTRODUCTION

Maternal treatment with antiepileptic drugs can lead to cognitive dysfunction in later life of offspring in addition to the increasing risk of malformations. Granule neurons in the hippocampus and the cerebellum are noted for their exceptionally late development (1–3). These neurons in both brain areas develop in the last trimester of human gestation (4), corresponding to the neonatal period in mice and rats, namely from embryonic day 10 to postnatal day (PD) 20 (5–8). This period is vulnerable to various insults that may result in permanent brain damage. The critical role of the hippocampal formation in learning and memory is widely accepted. The classical studies of the patient (9) and a large number of lesion studies (10–13) support the involvement of this brain structure in certain kinds of mnemonic function. Conversely, the function of the cerebellum had been thought to be limited to motor regulation, although recent findings have indicated that the cerebellum is involved in some aspects of cognitive processing (14, 15). In this study, we describe developmental disorders of granule cells in the hippocampus and the cerebellum accompanied by a spatial learning deficit after treatment with phenytoin (PHT) during this neonatal period.

PHT is a primary anti-epileptic drug used for all types of epilepsy except absence seizures (16) and is one of the most commonly prescribed antiepileptic drugs at present. There is also a growing body of evidence that PHT causes brain damage when administered early in development in laboratory animals. Gestational exposure of PHT in rats can reduce whole brain weight (17), delay maturation of reflexes (18), and alter postnatal behaviors such as increased spontaneous locomotion (19), as well as learning impairments (20, 21). Although much research has been conducted to evaluate the effects of prenatal PHT treatment in rodents, little work has been done to examine the effects of neonatal PHT treatment (22). The purpose of this study is to clarify the effects of PHT administered during mouse neonatal period (which corresponds to the last trimester of human gestation) on spatial learning and the morphology of related brain structures, the hippocampus, and the cerebellum.

MATERIAL AND METHODS

Animals

C57BL/6 mice (Japan CLEA Co., Ltd., Tokyo, Japan) were bred in our laboratory (temperature: 22°C ± 2°C; humidity: 50% ± 10%). The mice were mated overnight and gestational day 0 was determined when a vaginal plug was present in the morning. We used only mice that delivered spontaneously on gestational day 19. The day of birth was designated as PD 0. At birth, the size of each litter was 4 to 7 pups per dam. Pups in each litter were randomly divided into treatment and control groups, at a ratio of about 1:1. In total, 35 litters were used for morphological, immunohistological, pharmacokinetic, and behavioral studies.
Drug Treatment

A fine powder of PHT with a mean particle size of 17 μm was specially prepared from commercial PHT (Dainippon Pharmaceutical Co., Ltd., Tokyo Japan) by passing it through a fine mesh. PHT was suspended in sesame oil (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) using an ultrasonic homogenizer. PHT was administered orally through a polyethylene tube (0.28 mm i.d.) at a dose of 35 mg/kg (volume; 10 ml/kg body weight) once a day during PD 5 to 14. This dose was considered effective based on our previous study (23). In the control group, sesame oil alone was administered once a day during the same period. The pups were weighed every day from birth to PD 14, and weighed weekly thereafter.

Histopathological Investigation

For morphological studies, mice were killed with overdoses of diethyl ether. The brains were removed and weighed.

Immunohistochemistry: Brains were immersed in Bouin’s fixative at 4°C for 2 hours (h). The brains were dehydrated, embedded in paraffin, and sectioned serially at a thickness of 8 μm in parasagittal or horizontal planes. The sections were immunostained with an anti-inositol 1, 4, 5-trisphosphate receptor type 1 (IP3R1) antibody (24–26) for cerebellum or an anti-calbindin D28k antibody (Swant, Bellinzona, Switzerland) (27) for hippocampus. In order, brain sections were immersed in solutions of the following: (a) 0.3% hydrogen peroxide/methanol for 30 min to block endogenous peroxidases; (b) 2% normal blocking serum/phosphate-buffered saline for 1 h; (c) monoclonal antibody against IP3R1 (18A10) (24, 26) or calbindin D28k for 2 h; (d) biotinylated anti-rat IgG (IP3R1) or anti-rabbit IgG (calbindin D28k) for 1 h; and (e) avidin-biotin-peroxidase complex (ABC) solution for 1 h. The immunoreacted sections were stained by incubating them with diaminobenzidine solution (Amersham International, Buckinghamshire, England). The sections were then counterstained with hematoxylin. Biotinylated anti-IgG and ABC solutions were from the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). To detect apoptosis a method based on specific labeling of DNA strand breaks (28) by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method (29) was used. Briefly, samples were processed using the terminal deoxynucleotidyl transferase kit (Apop Tag™, ONCOR Inc., Gaithersburg, MD). The deparaffinized sections were treated with 0.3% H2O2 in order to inactivate endogenous peroxidases and with TdT in a humidified chamber at 37°C for 1 h. The sections were then incubated with the anti-digoxigenin-peroxidase in a humidified chamber for 30 min at room temperature and developed with a diaminobenzidine solution. These sections were counterstained with methyl green for 10 min at room temperature. Labeled cells were considered apoptotic if they exhibited condensed or fragmented nuclei (30, 31). Some sections were stained with hematoxylin and eosin (H&E).

Behavioral Studies

All of the behavioral studies were performed by a well-trained experimenter blind to treatment condition. Fourteen 2-month-old male mice per group were used.

Spontaneous Motor Activity: Locomotor activity was measured by placing individual animals in a clear Plexiglas box (30 × 20 × 13 cm) that was then positioned in a frame mounted with infrared beams (SCANET SV-10, Toyoy Industry Co., Japan). Beam interruptions were summed in 10-min bins over a period of 60 min.

Rotating Rod Test: Motor coordination was assessed with a rotating rod apparatus (KN-75, Natsume Seisakujo Co., Ltd., Tokyo, Japan), which consisted of a plastic rod (3-cm diameter, 8-cm long) with a gritted surface flanked by 2 large discs (40-cm diameter) (32, 33). A mouse was placed on the rod and the rod was rotated at a speed of 0 (stationary), 5, 10, and 20 rpm. Latency until a fall occurred was recorded for 4 trials at each speed.

Footprint Test: To obtain footprints, black ink was applied to the hindpaws of each mouse and the mouse was allowed to walk forward in a narrow alley (9 × 10 (high) × 25 cm) on white paper.

Forced Swimming Test: A mouse was gently placed into a cylinder (14.5-cm diameter, 20-cm high), which was filled with water (23°C) to the depth of 13 cm. Swimming time was measured automatically for 10 min by using a movement detection system (SCANET SV-10AQ, Toyoy Industry Co.) (34).

Water Maze Test: Spatial learning was assessed by 3 variants of the Morris water maze task (35, 36) adapted for mice. The maze was a 150-cm plastic pool filled to a depth of 31 cm with 23°–25°C water. Cue platform task: In this task, a circular platform (12-cm diameter) was made visible by attaching a black board (9 × 19 cm) on the platform and the mouse was required to locate the visible platform. This task consisted of 4 trials per day for 3 consecutive days. The location of the platform varied for each of the 4 daily trials. Each mouse was always started at the east position and allowed 60 s to locate the platform. If the mouse found the platform within 60 s it was allowed to stay there for 30 s. Mice that failed to find the platform in 60 s were placed onto the platform by hand and remained on it for 30 s. Hidden platform task: A circular transparent acrylic platform (12-cm diameter) was submerged 1 cm below the surface of the water in the southeast quadrant throughout the hidden platform task. Each mouse was subjected to 4 trials per day over 7 days. There were 4 starting points located at the center of each quadrant and the mouse was put at a different starting point for each of the 4 daily trials. The time taken to reach the platform (escape latency) was recorded. Other procedures were the same as for the cue platform task. Probe test: A single trial of probe test was carried out after the hidden platform task had been completed. In this trial, the platform was removed and the movement of each mouse in the pool was monitored using a computer-based video tracking system (BTA-2, Muromachi Kikai Co., Ltd., Tokyo, Japan). Each mouse was placed in the northwest position of the pool and allowed to swim for 60 s. Swimming path length, the number of times the platform site was crossed, and the time spent in the training (southeast) quadrant were calculated.

Radial Arm Maze Test: Each mouse was fostered individually and their body weights were maintained within 80% to 85% of their initial values by mild food restriction. Water was available ad libitum. Mice were fed food pellets (20 mg, Bioserv, San...
TABLE
Effects of Phenytoin (PHT) on Brain Development of 56-day-old Mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PHT</th>
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<tr>
<td>No. of mice examined</td>
<td>13</td>
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<tr>
<td>Body weight (g)</td>
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<td>22.2 ± 0.6</td>
<td>93</td>
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<tr>
<td>Total brain weight (mg)</td>
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<td>371.2 ± 5.7**</td>
<td>82</td>
</tr>
<tr>
<td>Cerebral weight (mg)</td>
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<td>280.3 ± 4.0**</td>
<td>83</td>
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<tr>
<td>Cerebellar weight (mg)</td>
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<td>39.4 ± 1.3**</td>
<td>71</td>
</tr>
<tr>
<td>Brainstem weight (mg)</td>
<td>56.7 ± 0.8</td>
<td>49.2 ± 1.8**</td>
<td>87</td>
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** p < 0.01.

Diego, CA) in their home cage days prior to testing. The apparatus used was an elevated 8-arm radial maze (36–38) adapted for mice. Briefly, each arm was 30-cm long and 6-cm wide and the center arena was 15 cm in diameter. A food pellet, used as a reinforcer, was placed in a well at the tip of each maze arm and a mouse was placed into the central arena of the maze to start the trial. The animal was left in the maze until all 8 pellets were obtained or 5 min elapsed. Each mouse received 2 trials per session each day for 7 days. A correct choice was defined as entering a previously unvisited arm on that trial, while re-entering an arm after the food in that arm had already been removed was recorded as an error. Running time per choice was obtained from total running time for a trial divided by the number of choices. The choice angle between the chosen arms (45°, 90°, 135°, or 180°) was evaluated for every first 8 choices throughout testing (a total of 112 angles per animal).

** Determination of Plasma PHT Level**

Phenytoin concentrations in the plasma and brain were determined by HPLC (30). In our previous study, plasma PHT concentrations reached a steady state 3 h after dosing on the third day of PHT treatment (30). Consequently, PHT levels in the plasma and brain were determined at this point in the present experiment.

**Statistical Analyses**

Behavioral scores were subjected to ANOVA with repeated measures for the time factor. Parametric comparisons between PHT-treated mice and control mice were carried out using two-tailed unpaired t-tests. Otherwise, the nonparametric Mann-Whitney U-test was used. A value of p < 0.05 was considered statistically significant.

Fig. 1. Immunostaining with calbindin D28k antibody in the DG on PD 14. The width of calbindin-positive mature granule layer in the DG increased more in the control group (A, B) than in the PHT-treated group (C, D). Scale bars: A–D = 50 μm.
**Fig. 2.** Immunostaining with calbindin D28k antibody in the DG on PD 21. Immunoreactive mature cells grew to occupy almost the entire granule cell layer in the DG in the control group (A, B). In the PHT-treated group (C, D), however, calbindin-negative immature granule cells were still observed in the innermost portion of DG (C, D). Scale bars: A–D = 50 μm.

**RESULTS**

**Body and Brain Weights**

Some of the pups treated with 35 mg/kg PHT showed an acute behavioral deterioration, including signs of anorexia, hyperactivity, and motor coordination problem. Total mortality in the PHT group was 38%. At the end of PHT treatment the acute symptoms induced by PHT disappeared and pups body weight gradually returned to normal. At 56 days of age, the body weight of the PHT-treated mice was not significantly different than that of control mice (Table). Total brain weight was lower in the PHT group than in the control group, and the reduction of brain weight was more remarkable in comparison to body weight (Table). The most marked weight reduction was observed in the cerebellum (71% of control), followed by the cerebrum (83%). The brainstem weight reduction was less (87% of control) compared with the other 2 brain regions.

**Granule Cells in the Dentate Gyrus (DG) and Dendritic Development of Purkinje cells in the Cerebellum**

The width of the calbindin-immunoreactive granule layer in the dentate gyrus (DG) was larger in the control group (Fig. 1A, B) than that of the PHT-treated group (Fig. 1C, D) on PD 14. The calbindin-positive mature granule cells grew to occupy almost the entire granule cell layer in the DG of control mice by PD 21 (Fig. 2A, B). In the PHT-treated group, however, calbindin-negative, immature granule cells were still observed in the innermost part of the DG on PD 21 (Fig. 2C, D).

Cell bodies and dendrites of Purkinje cells were specifically immunostained with IP3R1 in the control cerebellum on PD 14 or 21 (Fig. 3A, B). In the PHT-treated group, dendrites of Purkinje cells were poorly developed and showed a dispersed and indistinct staining pattern on PD 14 or 21 (Fig. 3C, D).

In sagittal sections of PD 7 mouse cerebella stained with H&E, pyknotic cells in the external granular layer
in the vermis area appeared in the PHT-treated group (Fig. 4). These pyknotic changes were not observed in the internal granular layer of the PHT-treated mouse (Fig. 4B).

Apoptotic Cell Death in the DG and the Cerebellum

Assessment of apoptotic cell death over the whole brain was done using TUNEL staining that detects DNA strand breaks. A marked number of apoptotic cells were observed particularly in the hippocampus (Fig. 5) and the cerebellum (Fig. 6) of PHT-treated mice. These TUNEL-positive cells were clearly seen at PD 7, but not at PD 14 in PHT-treated mice. There were some TUNEL-positive cells in the olfactory bulb and the periventricular areas, but they were also seen invariably in control mice.

In the hippocampus, TUNEL-positive granule cells were found in the polymorphic layer of the DG (Fig. 5C, D). This is in sharp contrast to the fewer number of cells that were TUNEL-positive in the granule layer of DG and other hippocampal areas. It suggests that the differentiation and the maturation of granule cells were affected. We also observed many apoptotic granule cells in the cerebellum in PHT-treated mice compared to control animals (Fig. 6B). This is particularly evident in the external granule layer of the vermis on PD 7.

Behavioral Symptoms

PHT-treated mice did not manifest any easily observed abnormal behavior at the time of behavioral testing (2 months of age). Spontaneous locomotor activity in a new environment was indexed by infrared beam breaks. Overall, PHT-treated mice tended to be less active, but this effect was not significant compared to controls (F[1,26] = 2.79, p = 0.107) (Fig. 7A).

The rotating rod test was used because this test has been found to be sensitive for detecting motor dysfunction in mice neonatally treated with PHT in a previous study (30). Retention time of PHT-treated mice at every speed (0–20 rpm) of rotating rod was comparable to that of control mice (Fig. 7B). In addition, walking patterns in both groups of mice were not different as determined by the footprint test. There was no significant difference between the 2 groups in stride length (63.8 ± 1.5 mm vs 66.0 ± 1.7 mm for control and PHT-treated mice, respectively; p = 0.354) or step width (23.5 ± 0.6 mm vs 24.5 ± 0.7 mm for control and PHT-treated mice, respectively; p = 0.281). These results indicate that PHT-treated mice displayed no apparent motor dysfunction.

Swimming time in the forced swim test can be interpreted as an index of willingness or motivation (34). Mice in both groups swam gradually less as a function of time (F[9,26] = 53.92, p < 0.0001), but there were no differences between the 2 groups (F[1,26] = 0.004, p = 0.95).

Spatial Learning Tasks

We investigated spatial learning by the Morris water maze task (Fig. 8). This test has often been used to study the ability to acquire spatial information (12, 39). Mice were trained first on a visible platform task to assess swimming ability, as well as learning function, because PHT-treated mice were expected to have motor problems due to cerebellar damage, as found in our previous study (30). Mice in both groups improved in their ability to locate a platform across sessions (F[2,26] = 81.23, p < 0.0001). Escape latency of the PHT-treated mice was comparable to control mice (F[1,26] = 0.36, p = 0.55). Thus, PHT-treated mice could swim and learn this task as well as control mice.

After mice in both groups were able to reach the visible platform within 5 s, their spatial learning ability was examined using a hidden platform task. Mice can learn the location of a hidden platform using several distal visual cues surrounding the water pool. PHT-treated mice took more time to locate the hidden platform than control mice (F[1,26] = 14.38, p = 0.0008) (Fig. 8A). The ability of control and PHT-treated mice to find the hidden platform improved significantly with repeated testing (F[6,26] = 6.56, p < 0.0001), and there was no significant interaction between group and session (F[6,26] = 0.70, p = 0.648), meaning that both control and PHT-treated mice learned the hidden platform task of water maze, although spatial learning capacity of PHT-treated mice was inferior to that of control mice.

The probe test demonstrated more clearly the deficit of PHT-treated mice in spatial learning. Tracking analysis of swimming revealed that control mice spent a significantly greater percentage of the trial swimming in the quadrant where the platform had been placed in the previous task (F[3,39] = 44.35, p < 0.0001), while PHT-treated mice did not focus on the training quadrant (Fig. 8B). Time spent in the training quadrant by PHT-treated mice was significantly shorter than that of control mice (U[14,14] = 38.5, p = 0.0063) (Fig. 8B). Platform crossing of PHT-treated mice was not significantly lower than that of control mice (t = 1.50, p = 0.147). These results indicate an impairment of spatial learning ability in PHT-treated mice. Furthermore, swimming distance of PHT-treated mice was not significantly shorter than that of control mice during the probe test (t = 0.77, p = 0.446), suggesting that PHT-treated mice did not have any deficits in motor function or motivation.

To confirm the apparent impairment of spatial learning in PHT-treated mice, a radial arm maze test was performed (Fig. 9). This test is also used to evaluate spatial learning, but in this case, mice are required to remember and avoid previously visited arms to get rewards. The number of errors made by PHT-treated mice was significantly greater compared to control mice (F[1,22] =
was 31.9 after the last dose of PHT. Brain concentration of PHT higher than the plasma level.

Running time per choice in PHT-treated mice was significantly shorter than in the control mice (F[1,22] = 24.58, p < 0.0001) (Fig. 9B). In addition, control mice preferred to choose 45 and 90 degree angles of arms, while the PHT-treated mice chose significantly wider angles of arms than the control mice (45°: U[12,12] = 16, p = 0.0012; 90°: U[12,12] = 69.5, p = 0.89; 135°: U[12,12] = 18, p = 0.0018; 180°: U[12,12] = 5.5, p = 0.0001) (Fig. 9C).

Concentration of PHT

Plasma PHT level was 20.0 ± 2.8 μg/ml (n = 4) 3 h after the last dose of PHT. Brain concentration of PHT was 31.9 ± 10.3 μg/g (n = 4), which was 1.6 times higher than the plasma level.

**DISCUSSION**

Several studies suggested that the children of epileptic women who took antiepileptic drugs during pregnancy may have mild to moderate mental retardation (40–42). These studies, however, could not completely attribute the mental alteration to the epileptic drugs, because maternal seizures themselves might increase the risk of specific cognitive dysfunction (43, 44). In experimental animals a great deal of effort has been made to confirm the effects of prenatal exposure to PHT, but little attention has been given to its effects during the neonatal period, especially on cognitive function. To address these issues, we demonstrated in this study that PHT treatment during mouse neonatal period, which is developmentally equivalent to the last trimester of human gestation, caused developmental disturbance and apoptotic death of granule cells in the hippocampal DG and the cerebellum and caused spatial learning deficits. These findings suggest that PHT treatment during this period can have harmful effects on cognitive function and development of the related brain structures.

Increased immature granule cells were observed in the DG in PHT-treated mice, indicating that neonatal PHT administration affected the development of granule cells in the DG. The granule cells in the DG are derived from the neuroepithelium in the embryonic hippocampus. The proliferative cells migrate toward the DG, forming a secondary germinal matrix halfway in between. The secondary matrix produces cells forming the superficial border of the DG layer and cells of the tertiary germinal matrix in the hilus of the DG. After birth, the tertiary matrix produces granule cells that accumulate beneath the older superficial neurons (5–7). The mature granule cells are immunopositive to anti-calbindin antibody (27, 45). The layer of calbindin-positive mature granule cells was thin in PHT-treated mice and there were more calbindin-negative immature cells in PHT-treated mice than in control mice. Furthermore, we found that some immature granule cells in the polymorphic layer of DG were TUNEL-positive, suggesting that PHT caused apoptotic death of these cells. Therefore, PHT appears to interfere with the differentiation of granule cells, and even led some of them to apoptotic death in the DG.

The acquisition of spatial learning, as assessed by 2 different kinds of tasks, was impaired as a consequence of neonatal exposure to PHT in mice. In the water maze, the ability of PHT-treated mice to learn the location of the hidden platform was markedly inferior to that of control mice, while in the visible platform task, the performance of PHT-treated mice was normal. This kind of dissociation has often been observed in hippocampus-lesioned animals (12, 39), suggesting that the PHT-induced learning deficit may be a result of a dysfunctional hippocampus. The other spatial task we employed is relatively independent of motor ability. The radial arm maze assesses spatial learning as well, but does not require timed responses like the water maze. PHT-treated mice made more errors than control mice and preferred to choose wide angles in this test. Animals with hippocampal dysfunction behave in a similar manner to the PHT-treated mice in the present study, i.e. increase in error choices and a bias to choose arms with rather wider angles (46–48). Results obtained from these 2 tasks suggest that a hippocampal dysfunction underlies the deficit in spatial learning caused by prior PHT exposure.

It is important to note the potential contribution of the cerebellum to the spatial learning deficit in the PHT-treated mice. The cerebellar weight of PHT-treated mice was reduced and we observed morphologically immature dendritic development of Purkinje cells following PHT administration. In addition, TUNEL-positive cells were observed in the external granular layer of the cerebellum as seen in a previous study (30). Mice treated with PHT in the present study showed no motor skill dysfunction in any tested situations. Therefore, it is unlikely that the learning deficit in this study is attributable simply to motor dysfunction. Nevertheless, the possibility remains that the cerebellar damage might exacerbate the impairment in spatial learning ability. The role of the cerebellum in learning has been discussed by others, and many spontaneous mutant mice with cerebellar morphological disorders exhibit learning deficits (49, 50).

We have previously studied a cerebellar disorder in ICR mice that were treated with PHT at PD 2 to 5 (23, 30, 51). We observed apoptotic cells in the external granular layer and apparent dysfunction of motor ability in these mice. In the present study, C57BL/6 mice were used because of their higher learning ability in the water.
Fig. 3. Developmental expression pattern of IP3R1 in control and PHT-treated mice in the vermis of the cerebellum on PD 14 or PD 21. In the control group (A, B), cell bodies and dendrites, including arbors of Purkinje cells, are specifically immunostained with IP3R1. In the PHT-treated group (C, D), dendritic arbors appear as an immature pattern. Scale bars: A–D = 50 μm.

Fig. 4. Pathological changes of the vermis in PD 7. Sections were stained with H&E. Substantial changes were not seen in the control group (A), whereas some pyknotic cells (arrows) were observed in the PHT-treated group (B). EL: external granular layer. IL: internal granular layer. Scale bar: 50 μm.
Fig. 5. Apoptotic cell death in the DG on PD 7. No TUNEL-labeled cells were seen in the control group (A, B), whereas many TUNEL-positive apoptotic cells (arrows) were observed in the innermost portion of DG in PHT-treated group (C, D). Scale bars: A–D = 25 μm.

Fig. 6. Apoptotic cell death in the cerebellum on PD 7. TUNEL-labeled granule cells are not seen in the control group (A), whereas many TUNEL-positive apoptotic cells (arrows) emerged in the external granular layer of the vermis in PHT-treated group (B). Scale bars: A, B = 50 μm.
Fig. 7. Behavioral analysis of PHT-treated mice. A: Spontaneous motor activity in a novel environment. B: Retention time for remaining on the rotating rod. Each point represents a mean ± SEM of 14 animals. The performance of PHT-treated mice (open circles) were comparable to control mice (solid squares) in both tests.

Fig. 8. Impairment of PHT-treated mice in the acquisition of the hidden platform task in the water maze. A: Mean escape latencies in the hidden platform version of the water maze task averaged over 4 trials per session. B: Mean time spent in the 4 quadrants during the probe trial. NE, SE, SW, and NW indicate northeast, southeast, southwest, and northwest position of quadrants, respectively. Each point and column represents a mean ± SEM of 14 animals per group. PHT-treated mice (open circles) took significantly more time to locate the hidden platform than control mice (solid squares) (**p < 0.001, ANOVA). In the probe trial, PHT-treated mice spent significantly less time in the training quadrant (SE) compared to controls (**p < 0.01, Mann-Whitney U-test).

A battery of behavioral tests was conducted to determine if any other factors beside spatial learning ability may have contributed to deficits observed in the learning tasks. First, immobility in the forced swimming situation was tested because changes in motivation (i.e. to escape the water) affect learning in the water maze. There was no change in escape behavior in PHT-treated mice in this study. Second, performance of PHT-treated mice in the visible platform task was normal, indicating that PHT-treated mice possessed adequate visual function to solve a water maze. Taken together, these observations further support the idea that PHT-treated mice had a specific impairment in the acquisition of spatial learning.

The plasma concentration of PHT (20 ± 2.8 μg/ml) in treated neonates seems to be within the therapeutic range to control seizures in humans, i.e. 10 to 20 μg/ml (16).
Fig. 9. Impairment of PHT-treated mice in the acquisition of the 8-arm radial maze task. Mean total errors in a trial (A) and running time per choice (B) averaged over 2 trials per session. C: Percentage of choice angles by calculating 112 angles over the testing. Each point or column represents a mean (± SEM) of 12 animals per group. PHT-treated mice (open circles) made significantly more errors compared to controls (solid squares) with respect to total errors (**p < 0.01, ANOVA). Running time of PHT-treated mice was shorter than control mice (**p < 0.01, ANOVA). Note that the distribution of choice angles differed between PHT-treated (open columns) and control mice (shaded columns) (**p < 0.01, Mann-Whitney U-test).

We also confirmed that PHT easily penetrated the brain of mouse neonates. Short-lived intermediates of PHT metabolites are thought to be responsible for the toxic effects of PHT on brain development (53, 54). The high concentration of PHT in the brain may elicit microsomal induction, increase these intermediates (55), and emphasize the neurotoxicity of PHT. Prenatal exposure to PHT has been reported to be correlated with lower IQ scores and problems in cognitive functions (42, 56, 57). Although the possibility of a different susceptibility to PHT among species must be considered, our findings emphasize the necessity of taking precautions against the possible neurotoxic effects of PHT on brain development if administered to epileptic pregnant women.

In conclusion, developmental disorders of granule cells in the DG and the cerebellum accompanied by a spatial learning deficit was observed in mice treated orally with PHT during early neonatal days. We consider that the spatial learning deficit in PHT-treated mice is primarily due to a developmental disorder in the hippocampus and the cerebellum. Our findings suggest that the developing nervous system is vulnerable to the harmful effects of PHT and the careful clinical use of this drug for pregnant patients is warranted even in the last trimester of gestation.

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