TOXICOLOGICAL SCIENCES **136(2)**, 478–486 2013
doi:10.1093/toxsci/kft200
Advance Access publication September 5, 2013

Developmental Subchronic Exposure to Diphenylarsinic Acid Induced Increased Exploratory Behavior, Impaired Learning Behavior, and Decreased Cerebellar Glutathione Concentration in Rats

Takayuki Negishi,*,†,[1,2] Yuki Matsunaga,†,[2] Yayoi Kobayashi,‡ Seishiro Hirano,‡ and Tomoko Tashiro†

*Department of Physiology, Faculty of Pharmacy, Meijo University, Nagoya-shi, Aichi 468–8503, Japan; †Department of Chemistry and Biological Science, Aoyama Gakuin University, Sagamihara-shi, Kanagawa 252–5258, Japan; and ‡Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba-City, Ibaraki 305–8506, Japan

[1]To whom correspondence should be addressed at Department of Physiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya-shi, Aichi 468–8503, Japan. E-mail: taka-u@yayoi.club.ne.jp.
[2]These authors contributed equally to this work.

Received August 4, 2013; accepted August 27, 2013

In Japan, people using water from the well contaminated with high-level arsenic developed neurological, mostly cerebellar, symptoms, where diphenylarsinic acid (DPAA) was a major compound. Here, we investigated the adverse effects of developmental exposure to 20 mg/l DPAA in drinking water (early period [0–6 weeks of age] and/or late period [7–12]) on behavior and cerebellar development in male rats. In the open field test at 6 weeks of age, early exposure to DPAA significantly increased exploratory behaviors. At 12 weeks of age, late exposure to DPAA similarly increased exploratory behavior independent of the early exposure although a 6-week recovery from DPAA could reverse that change. In the passive avoidance test at 6 weeks of age, early exposure to DPAA significantly decreased the avoidance performance. Even at 12 weeks of age, early exposure to DPAA significantly decreased the test performance, which was independent of the late exposure to DPAA. These results suggest that the DPAA-induced increase in exploratory behavior is transient, whereas the DPAA-induced impairment of passive avoidance is long lasting. At 6 weeks of age, early exposure to DPAA significantly reduced the concentration of cerebellar total glutathione. At 12 weeks of age, late, but not early, exposure to DPAA also significantly reduced the concentration of cerebellar glutathione, which might be a primary cause of oxidative stress. Early exposure to DPAA induced late-onset suppressed expression of NMDAR1 and PSD95 protein at 12 weeks of age, indicating impaired glutamatergic system in the cerebellum of rats developmentally exposed to DPAA.

Key Words: **diphenylarsinic acid; rat; cerebellum; glutathione.**

Diphenylcyanoarsine and diphenylchloroarsine, arsenic chemicals harmful to humans, were synthesized as chemical weapons during World War II in Japan, United States, and Europe (Pearson and Magee, 2002; Szinicz, 2005); they cause severe vomiting and sneezing. In the year 2003, a number of people living in Kamisu, Ibaraki, Japan suffered from some neurological symptoms suggestive of cerebellar dysfunction, such as vertigo and coordination problems as well as myoclonus, somnipathy, visual disorder, and recent memory and cognitive disturbance (Ishii *et al.*, 2004). Further analysis revealed that well water that the patients used had been contaminated with arsenic chemicals leaked from a concrete block buried near the well. Health-care authorities thereby determined that this incident was arsenic poisoning. Among arsenic compounds detected in the well water, diphenylarsinic acid (DPAA), a degradation or ingredient product of the above-mentioned emetic weapons, was a major compound. With regard to the neurological symptoms suspected to be caused by organoarsenic exposure, a recent study reported abnormal eye movements (upbeat nystagmus) in the victims of the accident as a residual, persistent, and chronic central nervous system (CNS) damage due to organoarsenic poisoning (Nakamagoe *et al.*, 2013).

Since then, several toxicological studies have elucidated some adverse effects of DPAA on mammals *in vitro* and *in vivo*. DPAA was cytotoxic with a LC_{50} value of 121μM in primary mouse hepatocytes (Sumi *et al.*, 2007) and 700μM in HepG2 cells (Kita *et al.*, 2009), and it induced oxidative stress in rat cerebellar Purkinje cells (Kato *et al.*, 2007). Other studies reported that DPAA downregulates glutaminase C in HepG2 cells (Kita *et al.*, 2007), promoting its degradation by the mitochondrial Lon protease (Kita *et al.*, 2012).

As mentioned above, patients who ingested water containing DPAA exhibited neurological symptoms that were distinct from typical clinical symptoms of chronic poisoning by other arsenic chemicals (abdominal pain, nausea, hyperpigmentation, etc.)(Guha Mazumder, 2003), which implies the adverse neurological effect(s) specific to DPAA. The effects of DPAA on the CNS are poorly understood although there are a few reports

regarding the effects of DPAA on the CNS of rat (Kato *et al.*, 2007) and mouse (Umezu *et al.*, 2012). We previously reported that primary cultured cerebellar cells were highly sensitive to the cytotoxic effects of DPAA (LC_{50} was 30μM) and that 10μM DPAA induced oxidative stress and aberrant synthesis of neuroactive and vasoactive peptides in cultured astrocytes (Negishi *et al.*, 2012). Besides, a high dose of DPAA (100 mg/l DPAA in drinking water for 14–21 days) induced behavioral abnormalities, such as gait disturbance and deficient acclimatization against to a novel condition that was detected in the open field test in young rats (9 weeks of age), and it also caused oxidative stress and altered synthesis of the neuroactive and vasoactive peptides in the cerebellum *in vivo* (Negishi *et al.*, 2012).

Utilization of experimental animals to prove that exposure to DPAA causes the neurological symptoms reported in the Kamisu accident would establish a useful model for searching for preventive and/or therapeutic drugs against the neurological symptoms induced by DPAA. These studies are important for not only the victims of the Kamisu accident but also people affected by similar accidents that might occur elsewhere. In this study, we investigated the effect of subchronic developmental exposure to a relatively low dose of DPAA (20 mg/l DPAA in drinking water) on behavior and cerebellar development in rats.

MATERIALS AND METHODS

Chemicals

DPAA (> 99% pure, Fig. 1A; Tri Chemicals, Yamanashi, Japan) was provided by the Japan Science Foundation (Tokyo, Japan).

Animals

Pregnant Wistar rats (n = 10) were purchased from Japan SLC (Shizuoka, Japan) and maintained under controlled temperature (24 ± 1°C) and humidity (55 ± 5%) with a 12-h light/12-h dark cycle. All animal experiments were approved by the Animal Experimentation Committee of Aoyama Gakuin University and performed under veterinary supervision. DPAA was first dissolved in distilled water at 100 mg/l. The DPAA solution was brought to pH 7.0 using sodium hydroxide, and immediately before use, the solution was diluted 1:4 with tap water. When rats delivered (postnatal day 0; PND0), all male pups were once gathered. Six male pups were randomly picked up and then assigned to a maternal rat. Five maternal rats received DPAA at 20 mg/l in drinking water starting on PND0; the pups were exposed to DPAA via milk. Five control maternal rats received tap water containing 20% distilled water (Fig. 1B). Drinking water was renewed every 3 days. The amount of water and food consumed was monitored throughout the experiment. At 21 days after birth, male pups were separated from the mothers. The offspring weaned from the DPAA-exposed mothers were continuously fed with the DPAA-containing water until PND42 (6 weeks of age). The control pups (from control mothers) received drinking water without DPAA (tap water and distilled water 4:1, by volume). As shown in Fig. 1B, eight control rats from four litters and the same number of DPAA-exposed rats were randomly selected (two rats per litter) and subjected to the behavioral tests and histological and biochemical analyses described below. From PND42, a half of the male rats remaining in the control group continuously received the control drinking water (C-C; n = 8 from four litters [two per litter]), whereas the other half received DPAA-containing water (C-D; n = 8 from four litters [two per litter]) until 12 weeks of age. Similarly, from PND42, a half of the DPAA-exposed rats received the control drinking water (D-C; n = 8 from four litters [two per litter]), whereas the other half

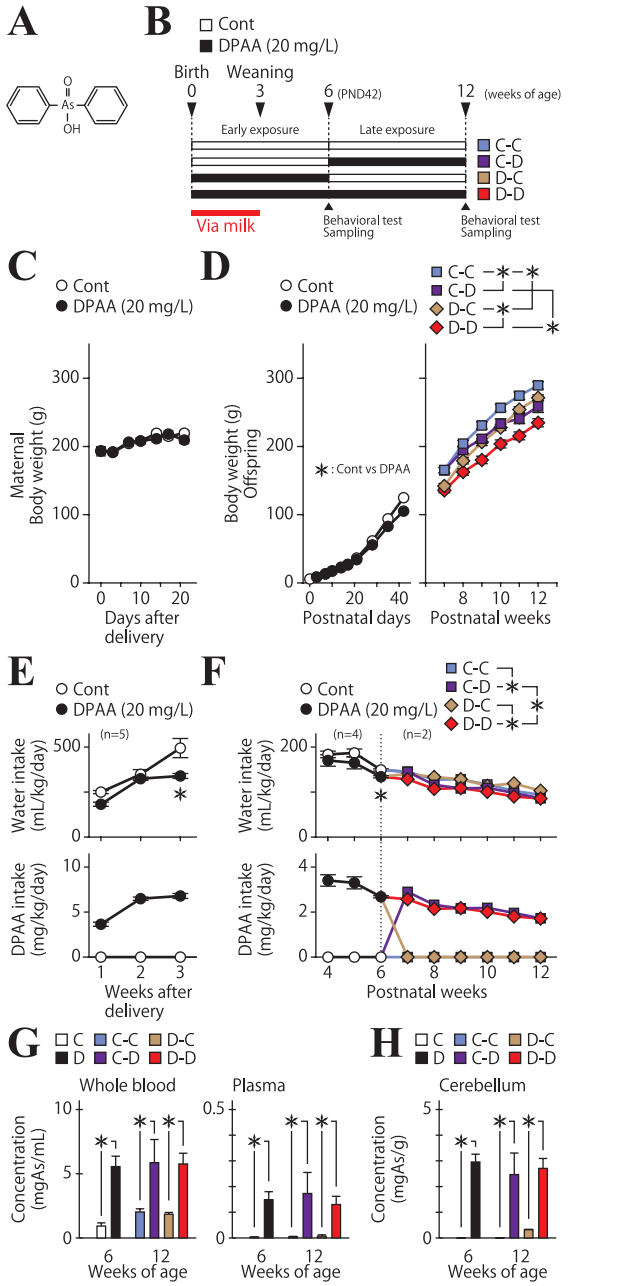

FIG. 1. A, Chemical structure of DPAA. B, Experimental schedule of exposure to DPAA in this study. C, Body weight change in mothers exposed to DPAA during the suckling period (mean ± SEM; n = 5). D, Body weight gain in offspring exposed to DPAA during the early period (left panel; mean ± SEM) and the late period (right panel; mean ± SEM) (n = 5 before weaning [a mean body weight of littermates was used], n = 24 (six per litter) from 3 to 6 weeks of age, and n = 8 (two per litter) from 6 to 12 weeks of age). E, Water intake (ml/kg/day) of mothers and an estimated dose of DPAA (mg/kg/day, mean ± SEM; n = 5 from five cages). F, Water intake (ml/kg/day) of offspring and their estimated dose of DPAA (mg/kg/day, mean ± SEM; n = 4 or 2 from four or two cages, respectively). G, Arsenic levels in whole blood (left) and plasma (right) collected from offspring at 6 and 12 weeks of age (mean ± SD; n = 4 from four litters). H, Arsenic levels in the cerebellum of offspring at 6 and 12 weeks of age (mean ± SD; n = 4 from four litters). Note that the dose of DPAA in animals drinking DPAA-free water is shown as zero in (E) and (F). *p < .05. Abbreviation: DPAA, diphenylarsinic acid.

consumed DPAA-containing water (D-D; $n = 8$ from four litters [two per litter]) until 12 weeks of age. At this age, all four groups (C-C, C-D, D-C, and D-D) were subjected to the experiments.

Behavioral Tests

Open field test. The open field test was performed at 6 and 12 weeks of age (time of day 18:00–20:00) as described in our previous study (Negishi *et al.*, 2012). Spontaneous locomotor activity of the animals in the test apparatus was recorded using a CCD camera for 5 min per trial, and trajectory was analyzed using the SMART video tracking system (Panlab, S. L., Barcelona, Spain). Three tests were performed on each animal, and each test was performed every other day.

Step-through passive avoidance test. A step-through passive avoidance test was performed at 6 or 12 weeks of age, and the apparatus consisted of light and dark compartments. When each animal experienced this apparatus for the first time, an electric foot shock (0.25 mA, 3 s) was applied to the animal through the grid floor, immediately after the rat crossed from the light to the dark from the light compartment completely (both hind legs landed on the grid floor in the dark). The latency of entrance into the dark compartment was recorded (max. 30 min) both during the first experience of the apparatus (acquisition trial) and in a retention trial conducted 24 h later.

Biochemistry

Sample collection. Male rats were sacrificed by collecting the whole blood into an anticoagulant (EDTA)–treated syringe under deep anesthesia. Plasma was prepared by centrifugation at 3000 × g for 15 min. Whole blood and plasma were stored at −80°C until use. After perfusion with iced calcium- and magnesium-free PBS (PBS(−)), the cerebellum was isolated. The right side of the cerebellum (for immunohistochemistry) was embedded in the O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and frozen rapidly on dry ice. The left side of the cerebellum (for biochemical assays) was flash frozen in liquid nitrogen. The frozen cerebella were stored at −80°C until use.

Measurement of the arsenic level in the whole blood, plasma, and cerebellum. Whole blood, plasma, and weighed cerebellum were wet-ashed with nitric acid and hydrogen peroxide 3:1 (vol:vol). The samples were diluted with water, and total arsenic concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS) according to previous studies (Kobayashi and Hirano, 2013; Kobayashi *et al.*, 2008). Arsenic levels in liquid samples (whole blood and plasma) and tissue samples (cerebellum) were calculated as mg/ml and mg/g, respectively.

Immunohistochemistry. The following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP) chicken polyclonal antibody (Abcam, Cambridge, UK); anti-NeuN mouse monoclonal antibody (clone A60, Sigma-Aldrich); anti-GAD67 mouse monoclonal antibody (clone K-87, Abcam); anti-heme oxygenase 1 (HO-1) rabbit polyclonal antibody (Abcam); anti-8-hydroxydeoxyguanosine mouse monoclonal antibody (clone N45.1, JaICA, Shizuoka, Japan); and anti-4-hydroxynonenal mouse monoclonal antibody (clone HNEJ-2, JaICA).

Cryosections were cut at 20μm and fixed in methanol at −20°C for GFAP, hemeoxygenase-1 (HO-1), and NeuN. The cryosections were then permeated by 0.1% Triton X-100 in PBS(−) and incubated in the blocking solution consisting of 4% bovine serum albumin (BSA, Sigma-Aldrich) and 4% normal goat serum (Dako, Glostrup, Denmark) in PBS(−) for 30 min. Subsequently, the sections were incubated with diluted primary antibodies, which were visualized using an appropriate secondary antibody (Alexa 488- or Alexa 546-conjugated goat anti-mouse or rabbit IgG polyclonal antibody). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich).

Western blotting. The following primary antibodies were used: anti-HO-1 antibody (Abcam); anti-GFAP mouse monoclonal antibody (clone GA5, Cell Signaling Technology Japan, Tokyo, Japan); anti-GAD67 mouse monoclonal antibody (Abcam); anti-NMDAR1 rabbit polyclonal antibody (Cell Signaling Technology); anti-PSD95 rabbit monoclonal antibody (Cell Signaling Technology); anti-glutathione synthetase (GSS) rabbit polyclonal antibody (Abcam); anti-glutamate-cysteine ligase catalytic subunit (GCLC) mouse monoclonal antibody (Abcam); anti-glutamate-cysteine ligase regulatory subunit (GCLM) rabbit monoclonal antibody (clone EPR6667, Abcam); anti-synaptophysin rabbit monoclonal antibody (Abcam); anti-synapsin I rabbit polyclonal antibody (Life Technologies Corporation, CA); anti-NMDAR2A rabbit polyclonal antibody (Cell Signaling Technology); anti-NMDAR2B rabbit polyclonal antibody (Cell Signaling Technology); anti-VGLUT1 rabbit polyclonal antibody (Synaptic Systems, Goettingen, Germany); anti-VGLUT2 rabbit polyclonal antibody (Synaptic Systems); anti-GLAST (EAAT1) rabbit polyclonal antibody (Abcam); anti-GLT-1 (EAAT2) rabbit polyclonal antibody (Cell Signaling Technology); anti-GABA(A)Ra-1 rabbit polyclonal antibody (Thermo Fisher Scientific, Massachusetts); anti-Gephyrin mouse monoclonal antibody (clone 3B11, Synaptic Systems); anti-VGAT rabbit polyclonal antibody (Merck Millipore, Massachusetts); anti-GAT-1 rabbit polyclonal antibody (Abcam); anti-calbindin-D-28k mouse monoclonal antibody (clone CB-955, Sigma-Aldrich); anti-β-actin mouse monoclonal antibody (clone AC-15, Sigma-Aldrich). Sample preparation and Western blotting were performed according to a previous report (Negishi *et al.*, 2012). In brief, protein extracted from the cerebellum was separated by SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane (Immobilon P, Millipore). The membrane was incubated in the blocking solution consisting of 5% BSA and 0.1% Tween-20 (Wako Pure Chemicals) in Tris-buffered saline (TBS) and then incubated with a primary antibody. Depending on the primary antibody, horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG or HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and Immobilon Western Chemiluminescence HRP Substrate (Millipore) were used for visualization of the immunoreactive molecules.

Glutathione assay. The concentration of cerebellar total glutathione was measured using the Total Glutathione Assay Kit (JaICA). Cerebellar tissue was homogenized in ice-cold TBS. The soluble fraction was separated after centrifugation, and protein concentration was normalized for all samples. Subsequently, the samples were subjected to the assay, which was performed in accordance with the product manual. The concentration of cerebellar total glutathione was calculated as nanomole per milligram protein.

Statistical Analysis

Numerical data were calculated as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) for a bar graph or line graph, respectively. In all the following statistical procedures, α was set at .05.

Body weight. Body weights (g) of mothers during the suckling period and their pups up to 6 weeks of age were analyzed by two-way repeated measures ANOVA, in which the between-subject and within-subject effects were the exposure to DPAA and age, respectively. Body weights of offspring at 7 weeks of age or older, when they were assigned to the four groups, were analyzed by three-way repeated measures ANOVA, in which the between-subject effects were early exposure to DPAA (from PND0 to 6 weeks of age) and late exposure to DPAA (from 6 to 12 weeks of age; L-DPAA), whereas the within-subject effect was age. Estimated daily water intake was analyzed in a similar manner.

Behavioral tests. Distances traveled (m) and numbers of entries in the center area in the open field test were analyzed by 2-way or 3-way repeated measures ANOVA at 6 or 12 weeks of age, respectively. The between-subject effect was the exposure to DPAA as described above, and the within-subject effect was the trials. Latency (min) in the passive avoidance test was also analyzed by 2-way or 3-way repeated measures ANOVA, in which the between-subject and within-subject effects were the same as those in the open field test.

Arsenic level, protein expression level, and total glutathione concentration. All biochemical experiments were duplicated and a mean value of them was applied to the statistical analyses as a value of each sample. The arsenic level, protein expression level, and total glutathione concentration

in the DPAA-exposed and control rats at 6 weeks of age were analyzed by Student's *t* test, and those in C-C, C-D, D-C, and D-D groups at 12 weeks of age were assessed by two-way ANOVA in which the two main effects were early and late exposure to DPAA.

RESULTS

General Observations

Wistar rats were exposed to DPAA (Fig. 1A) via drinking water (20 mg/L) as shown in Fig. 1B. In brief, for the first 3 weeks (before weaning), offspring received DPAA via breast milk from their mother, and then directly via DPAA-containing drinking water after weaning up to 6 weeks of age (Fig. 1B). At 6 weeks of age (PND42), the rats exposed to DPAA up to this time point were then assigned to either D-C or D-D group, which received DPAA-free water or DPAA-containing water, respectively, from 6 to 12 weeks of age. Similarly, the rats drinking DPAA-free water (control) up to 6 weeks of age were assigned to either C-C or C-D group, which received DPAA-free or DPAA-containing water, respectively, from that time point (Fig. 1B). Oral exposure of the maternal rats to DPAA during the suckling period had little effect on their body weights (Fig. 1C). However, early exposure to DPAA attenuated the increase in the body weight of offspring, especially from 3 to 6 weeks of age when they directly consumed DPAA-containing water (Fig. 1D). Late exposure to DPAA from 6 to 12 weeks of age attenuated the increase in the body weight of DPAA-exposed rats regardless of early exposure to DPAA (Fig. 1D). DPAA in drinking water caused slight avoidance of water intake both in dams (Fig. 1E) and offspring (Fig. 1F). The average oral dosage of DPAA estimated by the daily water intake was 5.64 ± 1.49 mg/kg/day in the DPAA-exposed dams (Fig. 1E). After weaning, the average dosage of DPAA in the DPAA-exposed offspring from 4 to 6 weeks of age was 3.13 ± 0.38 mg/kg/day and that in the DPAA-exposed rats (late exposure) from 6 to 12 weeks of age, C-D and D-D, was 2.23 ± 0.38 and 2.07 ± 0.28 mg/kg/day, respectively (Fig. 1F). Both at 6 and 12 weeks of age, early exposure to DPAA slightly inhibited the brain growth measured as weight, which was statistically significant (Supplementary Fig. 1). The DPAA-exposed rats showed a high arsenic level in whole blood and plasma (Fig. 1G). Cerebellar arsenic levels in the DPAA-exposed rats both at 6 (D) and 12 (C-D and D-D) weeks of age reached 3 µg/g wet tissue (Fig. 1H). No difference in cerebellar arsenic levels was observed between C-D and D-D.

DPAA-Induced Abnormal Behavior

Because people in the Kamisu accident presented with neurological symptoms (Ishii *et al.*, 2004), it seemed logical to examine behavior of DPAA-exposed rats. In this study, the open field test and the passive avoidance test were performed at 6 and 12 weeks of age. In the 5-min open field test

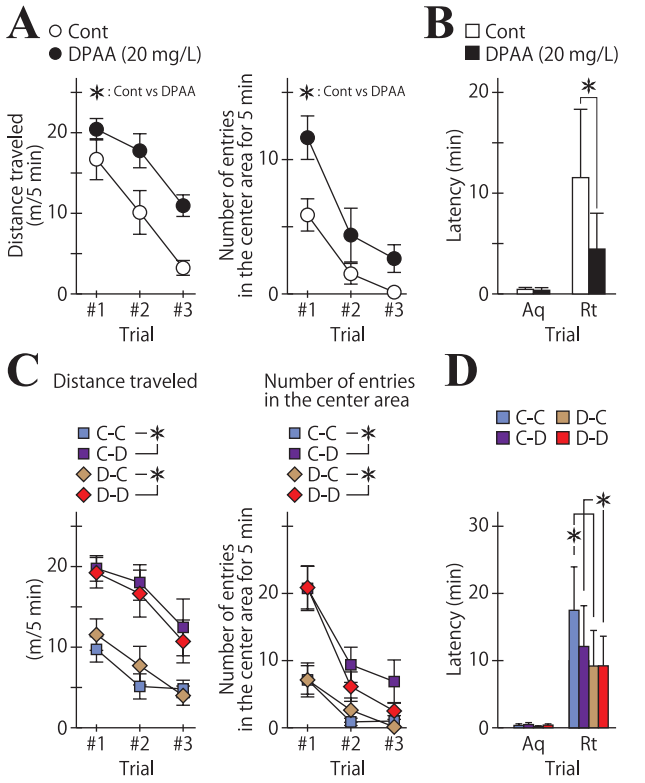

FIG. 2. A, The distance traveled (m, left panel) and the number of entries in the center area (right panel) in 5 min in the open field test performed at 6 weeks of age (mean ± SEM; $n = 8$ from four litters [two per litter]). B, The latency (min) until an animal entered the dark compartment from the light compartment in the Aq and the Rt in the passive avoidance test at 6 weeks of age (mean + SD; $n = 8$ from four litters [two per litter]). C, The distance (left) and the number of entries in the center area (right) in the open field test at 12 weeks of age (mean ± SEM; $n = 8$ from four litters [two per litter]). D, The latency (min) in the passive avoidance test at 6 weeks of age (mean + SD; $n = 8$ from four litters [two per litter]). *$p < .05$. Abbreviations: Aq, acquisition trial; DPAA, diphenylarsinic acid; Rt, retention trial.

at 6 weeks of age, exposure to DPAA increased the distance traveled (Fig. 2A, left panel) and the number of entries in the center area (Fig. 2A, right panel). Simultaneously, early exposure to DPAA shortened the latency in the retention trial of the passive avoidance test performed at 6 weeks of age (Fig. 2B). Late exposure to DPAA also significantly increased the distance traveled and the number of entries in the center area (Fig. 2C, left and right panel, respectively) at 12 weeks of age. On the other hand, early exposure had little or no effect at 12 weeks of age (see C-C vs D-C and C-D vs D-D). In the passive avoidance test at 12 weeks of age, 2-way repeated measures ANOVA showed that early, but not late, exposure to DPAA significantly reduced the latency in the retention trial at 12 weeks of age ($p < .05$, Fig. 2D). It appeared that, at 12 weeks of age, late exposure to DPAA also could have a weak effect on the latency in rats free from DPAA during the early period (see C-C vs C-D), whereas it had no effect in rats exposed to DPAA during early period (D-C vs D-D).

DPAA-Induced Oxidative Stress in the Cerebellum

Our previous study (Negishi *et al.*, 2012) suggested that HO-1, a protein getting rid of oxidative stress in various tissues, in the fibers of Bergmann glia, a subpopulation of astrocyte branching its fibers radially over the cerebellar molecular layer, of adult rats exposed to a high dose of DPAA (100 mg/l in drinking water) for 21 days. Accordingly, in this study, we examined HO-1 protein expression in the cerebellum of rats developmentally exposed to a relatively low dose of DPAA (20 mg/l) using immunohistochemistry and Western blotting analyses. Immunohistochemistry for HO-1 and GFAP revealed that early exposure to DPAA induced HO-1 immunoreactivity in the GFAP-positive Bergmann glial fibers at 6 weeks of age (Fig. 3A). Late exposure to DPAA also induced HO-1 immunoreactivity in Bergmann glia at 12 weeks of age (Fig. 3A). Western blotting of HO-1 and GFAP (Fig. 3B) also showed a significant increase in the expression of cerebellar HO-1 and GFAP proteins caused by early exposure to DPAA at 6 weeks of age and significant increases by late-period exposure to DPAA at 12 weeks of age. In addition, immunoreactivity of an oxidative DNA adduct, 8-hydroxydeoxyguanosine (8OHdG), was observed in the cerebellar capillary as a result of exposure to DPAA at 6 and 12 weeks of age (Fig. 3C). The DNA adduct did not disappear after a 6-week recovery (see C-C vs D-C). Furthermore, in order to estimate oxidative stress in the cerebellum of DPAA-exposed rats, the total tissue glutathione concentration was measured, which revealed that early exposure to DPAA reduced the total glutathione concentration at 6 weeks of age (Fig. 3D). At 12 weeks of age, early as well as late exposure to DPAA also significantly reduced the concentration of cerebellar total glutathione. Nevertheless, neither early nor late exposure to DPAA affected the cerebellar protein expression of glutathione-synthesizing enzymes (GSS, GCLC, and GCLM) at 6 and 12 weeks of age (Fig. 3E).

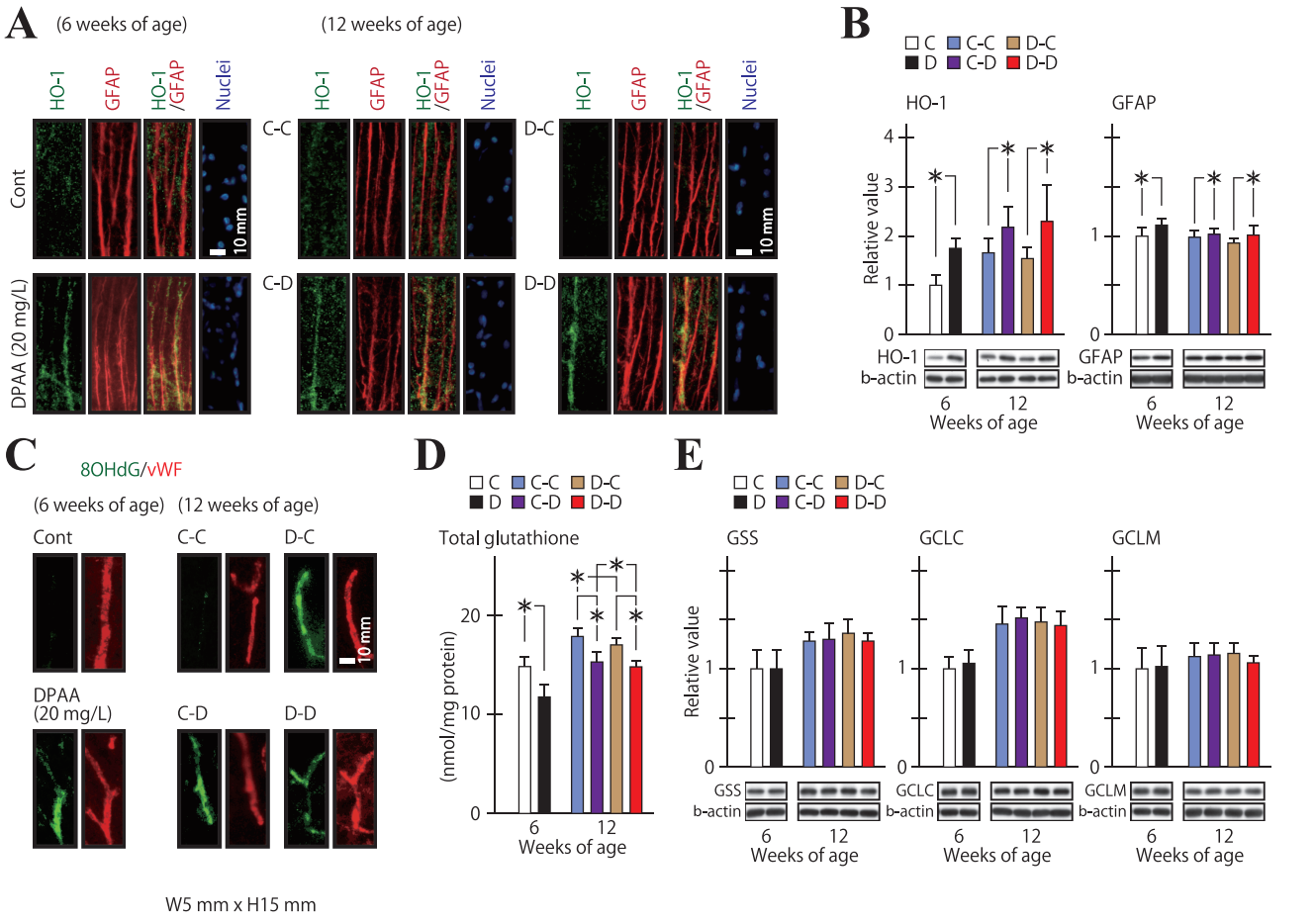

FIG. 3. A, Immunohistochemistry for HO-1 (green) and GFAP (red) expression in the fibers of Bergmann glial cells in the cerebellum of DPAA-exposed rats. Nuclei were visualized with DAPI. B, Semiquantification of HO-1 (left panel) and GFAP (right panel) protein expression by Western blotting in the cerebellum of DPAA-exposed rats. Data are shown as the ratio to the control rats at 6 weeks of age (mean ± SD; $n = 8$ from four litters [two per litter]). C, Immunohistochemistry for 4-hydroxynonenal (green) in the cerebellum of DPAA-exposed rats. Capillary endothelial cells were visualized by immunohistochemistry for vWF (red). D, Total glutathione concentration (nmol/mg protein) in the cerebellum of DPAA-exposed rats (mean ± SD; $n = 8$ from four litters [two per litter]). E, Protein expression of GSS, GCLC, and GCLM in the cerebellum (mean ± SD; $n = 8$ from four litters [two per litter]). $^*p < .05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DPAA, diphenylarsinic acid.

Expression of Neuronal and Astroglial Proteins in the Cerebellum as a Result of Exposure to DPAA

No apparent histological abnormality was observed in the cerebellum of the rats exposed to DPAA at both 6 and 12 weeks of age (Fig. 4A). Exposure to DPAA had little effect on the number of GAD67-positive Purkinje cells (Fig. 4B) and GAD67 protein expression (Fig. 4C) both at 6 and 12 weeks of age. However, when protein expression of several neuronal and astroglial proteins was examined, early exposure to DPAA suppressed NMDAR1 and PSD95 protein expression significantly at 12 weeks of age although it had little effect at 6 weeks of age (Fig. 4D). It appeared that early and late exposure to DPAA also suppressed NMDAR2A and NMDAR2B protein expression (Fig. 4D) although this effect was not statistically significant. Neither early nor late exposure to DPAA affected the expression levels of other proteins examined (synaptophysin, VGLUT1, VGLUT2, GLAST, GLT-1, GABA(A)Rα-1, gephyrin, VGAT, GAT-1, calbindin-D28k) at both 6 and 12 weeks of age although synapsin I expression was significantly reduced by early exposure to DPAA at 12 weeks of age (Supplementary Fig. 2).

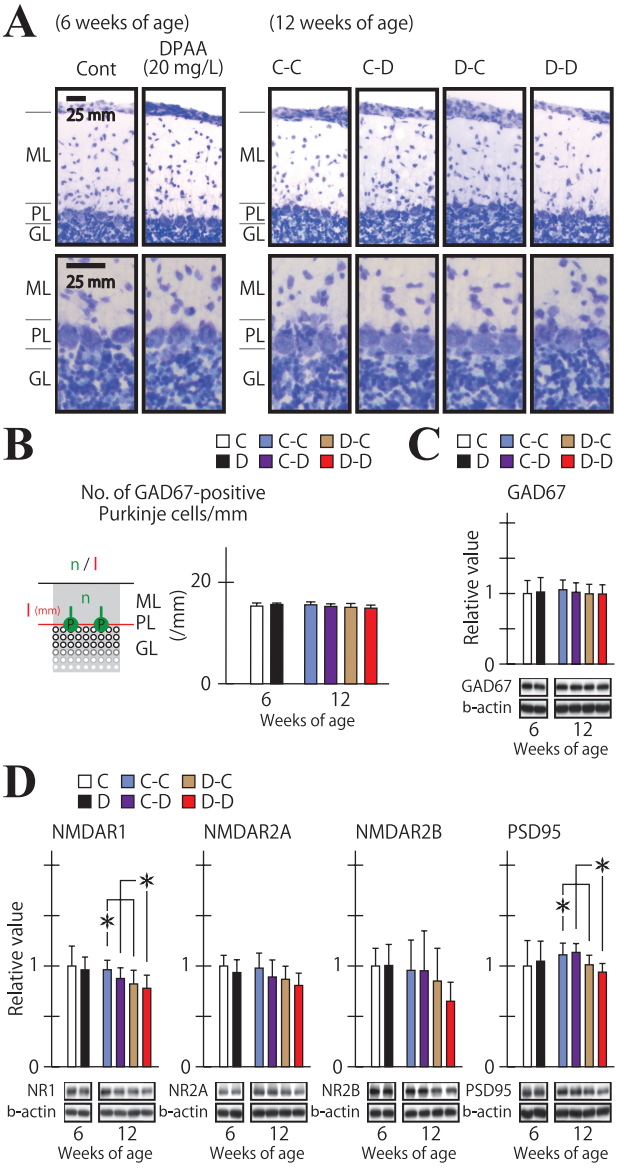

FIG. 4. A, Immunohistochemistry for GAD67 (upper) and NeuN (lower) localization in the cerebellum of DPAA-exposed rats. B, The number of Purkinje cells in the cerebellum of DPAA-exposed rats, which is shown as the number of cells per length of the layer (mm) (mean ± SD; $n = 6$ from four litters [one or two per litter]). C, GAD67 protein expression in the cerebellum (mean ± SD; $n = 8$ from four litters [two per litter]). D, NMDAR1 and PSD95 protein expression in the cerebellum (mean ± SD; $n = 8$ from four litters [two per litter]). *$p < .05$. Abbreviations: DPAA, diphenylarsinic acid.

DISCUSSION

In this study, we demonstrated that subchronic oral exposure of neonatal, juvenile, and young adult rats to DPAA caused behavioral abnormalities; it increased exploratory behavior in the open field test and decreased performance on the passive avoidance test. The behavioral aberrations were accompanied by biochemical abnormalities, activation of cerebellar astrocytes against DPAA-induced oxidative stress (increase in GFAP and HO-1 protein expression), and a reduction in the concentration of cerebellar total glutathione. In addition, exposure to DPAA during the early period induced the late-onset suppressed expression of one of the glutamate receptors, NMDAR1, and its anchoring protein, PSD95. It is important to note here that this study aimed to investigate and understand the adverse effects of DPAA in the arsenic poisoning incident in Kamisu, which was caused not by the buried chemical weapon but by a degradation or ingredient product of the arsenic weapon.

General Toxicity of DPAA

The highest arsenic concentration measured in the well water samples of Kamisu was 4.5 mg/l (Ishizaki *et al.*, 2005), and a well water sample contained approximately 8 mg/l (= μg/ml) DPAA (Kinoshita *et al.*, 2005). Furthermore, the predominant form of the arsenic compound in the well water was DPAA (Ishizaki *et al.*, 2005). These previous studies estimated the daily arsenic intake in adults (50 kg body weight) to be approximately 0.07–0.18 mg/kg/day, which corresponds to 0.24–0.63 mg/kg/day. In this study, the rats were exposed to DPAA via drinking water containing 20 mg/l DPAA (5.7 mg/l arsenic), and the daily DPAA intake by rats was approximately 2.5 mg/kg/day (0.71 mg/kg/day as arsenic). We previously reported the severe reduction of body weight, the drastic alteration in behaviors (gait disturbance), and the upregulation of antioxidative stress factors (HO-1) in the rats exposed to DPAA at a high dose (100 mg/l) subacutely (14–21 days) (Negishi *et al.*, 2012). Thus, to examine the adverse effect of more chronic exposure to DPAA at lower dose than those used in the previous study, we set a dose of DPAA and a period of exposure at 20 mg/l and 12 weeks, respectively. However, in consideration of a

significant suppression of body weight increase in juvenile rats exposed to 20 mg/l DPAA and a high estimated daily DPAA intake in DPAA-exposed rats (approximately 2.5 mg/kg/day), we have to say that this dose of DPAA is still high for considering the adverse effects of DPAA in humans in the accident. It is important to conduct a further study examining a dose-response relationship of the adverse effect of DPAA, in which, for instance, three doses (20 mg/l [high positive control], 10 mg/l [the dose close to the well water in the accident], and 5 mg/l) should be examined. On the other hand, in this study, we used eight subjects from four litters (two per litter) in each group for behavioral and neurological assessments, notwithstanding that developmental toxicity studies that focus on the adverse effects of exposure to the test chemicals during pregnancy (*in utero* for offspring) are encouraged to regard the number of litters used as the number of subjects. However, we performed substantially a postnatal exposure to DPAA and placed importance on the exposure to DPAA mainly after weaning. Moreover, it was relevant that, during suckling period, the level of DPAA in the body of pups exposed to DPAA via breast milk of DPAA-exposed mother was much lower than that of offspring that directly took DPAA via their drinking water after weaning. There was a possibility that early exposure to DPPA during suckling period via DPAA-containing breast milk had no or only a little effect on the development of offspring, which would be supported by the apparent DPAA-induced suppression of body weight increase only after weaning. We tried to make the condition of care environment for mother and pups equal among litters as much as possible as described in Materials and Methods section. Indeed, when we performed statistical analyses of all numerical data yielded from eight from four litters using ANOVA in which main effect was the foster mothers, we observed no significant effect of the foster mother in each experimental group examined. Thus, we decided to use data from eight subjects (two per litter) in the statistical analyses in this study. However, in any case, further studies especially focusing on the effect of "prenatal exposure" to DPAA on early brain development should be conducted using appropriate number of subjects from the same number of littermates, ie, one per litter, to provide more statistically reliable data. In view of these weak points, although this study demonstrated several clear evidences about the adverse effect of DPAA, it might still be placed as a pilot study for the risk assessment of exposure to DPAA in humans.

When the blood arsenic concentration was measured in the DPAA-exposed rats, 5–7 µg/ml arsenic was found in their whole blood, which was the same level as that in the DPAA-containing drinking water used in this study (5.7 mg/l arsenic). In contrast, only 0.2 µg/ml arsenic was detected in plasma. These results suggest that DPAA could enter the body easily via digestive organs and appears to be located on or in the circulating blood cells, which is in agreement with the findings of previous studies in rats (Kobayashi and Hirano, 2013) and monkeys (Kobayashi *et al.*, 2008). In the case of cerebellar tissue, 3 µg/g arsenic was found, which corresponded approximately to 3 mg/l arsenic. This amounts to 40µM DPAA, given that all arsenic detected was derived from unmodified DPAA. Comparison of arsenic concentrations in rats at 12 weeks of age with those at 6 weeks of age shows that 6-week exposure to 20 mg/l DPAA in drinking water was sufficient for blood and cerebellar arsenic concentrations to reach a plateau level. Simultaneously, a 6-week recovery (D-C) was sufficient to remove excessive arsenic compounds almost completely from the tissues. We previously reported that exposure to 10µM DPAA induced oxidative stress and increased the expression of neuroactive and vasoactive peptides in primary cultured cerebellar astrocytes although it was unclear whether these peptides promote or act against toxicity of DPAA (Negishi *et al.*, 2012).

Although chemical forms of arsenic including metabolites of DPAA in the cerebellum were not identified in this study, previous studies proposed a scenario where the pentavalent diphenylarsinic compound, DPAA, binds to cellular proteins and glutathione and becomes protein- or glutathione-bound trivalent organoarsenic compounds in the erythrocytes (Kobayashi and Hirano, 2013) and brain (Naranmandura *et al.*, 2009). The glutathione-conjugated DPAA is known to be highly toxic for the human hepatocarcinoma cell line HepG2 compared with unmodified DPAA (approximately 1000-fold difference). These complex trivalent arsenics should also be considered for fully understanding the adverse effects of DPAA.

DPAA-Induced Increase in Exploratory Behavior and Impairment of Learning Ability

We succeeded in inducing behavioral abnormalities in rats via exposure to DPAA, which included increased exploratory behavior in the open field test and impaired learning in the passive avoidance test. In the Kamisu accident, the afflicted people had some neurological problems including cerebellar symptoms (Ishii *et al.*, 2004). Although it was difficult to directly associate these behavioral abnormalities in rats with the neurological symptoms observed in people, we hypothesized that some common mechanisms must be underlying the DPAA-induced behavioral aberrations.

Rats exposed to DPAA during the early period showed enhanced exploratory behavior at 6 weeks of age in the open field test. At 12 weeks of age, rats exposed to DPAA during the late period (C-D and D-D) exhibited an increase in exploratory behavior in the open field test regardless of the DPAA status during the early period. In other words, D-C rats exposed to DPAA only during the early period showed exploratory behavior similar to that of C-C rats. These data suggest that this DPAA-induced hyperexploratory behavior might be a reversible adverse effect and that the presence of DPAA was crucial for inducing the hyperexploratory behavior in the open field test. A previous study in mice (Umezu *et al.*, 2012) also showed behavioral alterations as a result of exposure to DPAA, including increased ambulatory activity (30–100 ppm [mg/l] of DPAA via *ad libitum* intake of drinking water for 27 weeks),

although the doses were relatively high. Rats exposed to DPAA might display psychomotor excitement or restlessness in the open field apparatus. We can theorize that sleep disturbances observed in the patients in Kamisu may be a consequence of psychomotor agitation. We believe that measurement of locomotor activity in the home cage through night and day is worth undertaking in a future study. Although it is impossible here to propose the precise neurological or the neurochemical mechanism explaining DPAA-induced enhancement of exploratory behavior, nonetheless, locomotor activity in the open field test might be a useful endpoint for selecting and screening some preventive and/or therapeutic drugs against neurological symptoms in DPAA-exposed humans.

In addition to enhanced exploratory behavior, the rats exposed to DPAA during the neonatal and juvenile periods (0–6 weeks of age) showed lower passive avoidance performance in the retention trials than the control rats at 6 weeks of age. At 12 weeks of age, both the rats exposed to DPAA only during the early period (0–6 weeks of age: D-C) and those exposed in both the early and late periods (0–12 weeks of age: D-D) showed significantly lower passive avoidance performance compared with the control rats; the rats exposed only during the late period (C-D) showed slightly lower performance. These results imply that DPAA can affect performance on the passive avoidance test in rats and that the impairment caused by early exposure to DPAA cannot be restored to the control level even after a 6-week recovery (D-C vs C-C at 12 weeks of age), whereas the hyperexploratory behavior appears to be restored back to normal. In fact, as mentioned above, a few victims in the Kamisu accident developed short-term memory disturbances and cognitive impairment. On the other hand, the authors experimenting on mice (Umezu *et al.*, 2012) concluded that long-term exposure to DPAA (7.5–100 ppm) can not affect learning ability and/or memory. There might be species-specific differences between mice and rats in the behavioral sensitivity to DPAA. The passive avoidance performance in rats might serve as a relevant endpoint for assessing the risk of exposure to DPAA in humans.

DPAA-Induced Oxidative Stress in the Cerebellum

In this study, we confirmed the DPAA-induced increase in the expression of HO-1, an enzyme reducing oxidative stress, in the cerebellar astrocyte (Bergmann glia) as reported in our previous study using a higher dose of DPAA, 100 mg/l DPAA in drinking water for 21 days (Negishi *et al.*, 2012). The increased expression of HO-1 and accumulation of an oxidative adduct of DNA, 8OHdG, suggest that DPAA induces oxidative stress, such as increased production of reactive oxygen species in the cerebellum. Bergmann glia in D-C and C-C rats expressed the HO-1 protein at very similar levels, which suggests that a 6-week recovery in D-C rats may be sufficient to alleviate oxidative stress because this amount of time could also be sufficient to clear DPAA from the cerebellum.

At this point, we could discuss molecular evidence of oxidative stress in the cerebellum, ie, a DPAA-induced decrease in cerebellar total glutathione, because it is known that tissue glutathione plays a key role in the protection of living tissue from oxidative stress. This is the first report showing a decrease in glutathione concentration as an underlying cause of DPAA-induced oxidative stress. At present, precise molecular mechanisms underlying the DPAA-induced reduction in total glutathione remain unclear. Enzymatic activities of proteins related to glutathione synthesis might be reduced by DPAA without a change in the protein expression of enzymes involved in glutathione synthesis (GSS, GCLC, and GCLM). Alternatively, DPAA in the cerebellum might be bound to the reduced form of glutathione, and glutathione-conjugated DPAA was preferentially excluded from the cerebellar tissue. Although total glutathione concentration in the cerebellum was measured using the enzyme (glutathione reductase)–based assay in this study, measuring oxidized form of glutathione (GSSG) and reduced form of glutathione separately in the cerebellar tissue of DPAA-exposed rats using more sensitive methods such as liquid chromatograph mass spectrometer would provide more useful information to understand oxidative stress induced by exposure to DPAA.

DPAA-Induced Reduction in the Expression of Glutamatergic Synapse Proteins

No histological abnormality was observed in the cerebellum of DPAA-exposed rats in thisstudy, which suggests that the biological adverse effects of DPAA do not even include noticeable cell death. Nevertheless, in the cerebellum of rats at 12 weeks of age, early exposure to DPAA suppressed the expression of NMDAR1, an indispensable subunit of NMDA receptors, and PSD95, a postsynaptic protein anchoring NMDA receptors, which are known to be key players in glutamatergic neurotransmission in post synapses. These changes might contribute to the DPAA-induced neurological abnormalities. A previous *in vitro* study (Wang *et al.*, 2013) suggested that arsenic (arsenite) in astrocytes might impair synapse formation by perturbing the astrocyte function involving neuronal signal transduction. However, we could not propose the causal relationship between low expression of these postsynaptic proteins and behavioral abnormalities observed at both 6 and 12 weeks of age, such as hyperactivity and impaired memory. This is because early exposure to DPAA did not affect the expression of these proteins at 6 weeks of age. Nevertheless, considering the importance of NMDAR1 and PSD95 proteins in brain function including learning and memory, the suppressed expression of these postsynaptic proteins by early exposure to DPAA might also affect some cognitive functions that could not be examined in this study. Although brain regions other than cerebellum, such as the hippocampus and cerebrum, could be examined, we made it a priority to test the cerebellum because of the cerebellar symptoms induced by DPAA in humans. It is interesting to note that

the degree of DPAA-induced alteration in the behavior was more apparent than those in biochemical parameters such as NMDAR expression. We believed that behavior of rats would be a useful and suitable endpoint for the assessment of adverse effect of exposure to DPAA.

In conclusion, relatively low-dose exposure of rats to DPAA during development enhanced exploratory behavior, impaired learning ability similar to that in human victims of the Kamisu accident, and induced oxidative stress as suggested by the decreased glutathione level in the cerebellum.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

Ministry of the Environment, Government of Japan (research on the influence of diphenylarsinic acid and related compounds on human health); JSPS KAKENHI (Grant Number 20681005 [the Grant-in-Aid for Young Scientists (A), T.N.]; JSPS KAKENHI (Grant Number 23681010 [the Grant-in-Aid for Young Scientists (A), T.N.]). Note that the views expressed in this study do not necessarily reflect the positions or policies of the Ministry of the Environment, Government of Japan. The funding organizations have no control over this publication.

REFERENCES

Guha Mazumder, D. N. (2003). Chronic arsenic toxicity: Clinical features, epidemiology, and treatment: Experience in West Bengal. *J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng*. **38,** 141–163.

Ishii, K., Tamaoka, A., Otsuka, F., Iwasaki, N., Shin, K., Matsui, A., Endo, G., Kumagai, Y., Ishii, T., Shoji, S., *et al.* (2004). Diphenylarsinic acid poisoning from chemical weapons in Kamisu, Japan. *Ann. Neurol*. **56,** 741–745.

Ishizaki, M., Yanaoka, T., Nakamura, M., Hakuta, T., Ueno, S., Komura, M., Shibata, M., Kitamura, T., Honda, A., Doy, M., *et al.* (2005). Detection of bis(diphenylarsine)oxide, diphenylarsinic acid and phenylarsonic acid, compounds probably derived from chemical warfare agents, in drinking well water. *J. Health Sci*. **51,** 130–137.

Kato, K., Mizoi, M., An, Y., Nakano, M., Wanibuchi, H., Endo, G., Endo, Y., Hoshino, M., Okada, S., and Yamanaka, K. (2007). Oral administration of diphenylarsinic acid, a degradation product of chemical warfare agents, induces oxidative and nitrosative stress in cerebellar Purkinje cells. *Life Sci*. **81,** 1518–1525.

Kinoshita, K., Shida, Y., Sakuma, C., Ishizaki, M., Kiso, K., Shikino, O., Ito, H., Morita, M., Ochi, T., and Kaise, T. (2005). Determination of diphenylarsinic acid and phenylarsonic acid, the degradation products of organoarsenic chemical warfare agents, in well water by HPLC-ICP-MS. *Appl. Organomet. Chem*. **19,** 287–293.

Kita, K., Sato, M., Suzuki, T., and Ochi, T. (2009). Structure-effect relationship in the down-regulation of glutaminase in cultured human cells by phenylarsenic compounds. *Toxicology* **258,** 157–163.

Kita, K., Suzuki, T., and Ochi, T. (2007). Down-regulation of glutaminase C in human hepatocarcinoma cell by diphenylarsinic acid, a degradation product of chemical warfare agents. *Toxicol. Appl. Pharmacol*. **220,** 262–270.

Kita, K., Suzuki, T., and Ochi, T. (2012). Diphenylarsinic acid promotes degradation of glutaminase C by mitochondrial Lon protease. *J. Biol. Chem*. **287,** 18163–18172.

Kobayashi, Y., and Hirano, S. (2013). The role of glutathione in the metabolism of diphenylarsinic acid in rats. *Metallomics* **5,** 469–478.

Kobayashi, Y., Negishi, T., Mizumura, A., Watanabe, T., and Hirano, S. (2008). Distribution and excretion of arsenic in cynomolgus monkey following repeated administration of diphenylarsinic acid. *Arch. Toxicol*. **82,** 553–561.

Nakamagoe, K., Fujizuka, N., Koganezawa, T., Shimizu, K., Takiguchi, S., Horaguchi, T., Ishii, K., and Tamaoka, A. (2013). Residual central nervous system damage due to organoarsenic poisoning. *Neurotoxicol. Teratol*. **37,** 33–38.

Naranmandura, H., Suzuki, N., Takano, J., McKnight-Whitford, T., Ogra, Y., Suzuki, K. T., and Le, X. C. (2009). Systemic distribution and speciation of diphenylarsinic acid fed to rats. *Toxicol. Appl. Pharmacol*. **237,** 214–220.

Negishi, T., Takahashi, M., Matsunaga, Y., Hirano, S., and Tashiro, T. (2012). Diphenylarsinic acid increased the synthesis and release of neuroactive and vasoactive peptides in rat cerebellar astrocytes. *J. Neuropathol. Exp. Neurol*. **71,** 468–479.

Pearson, G. S., and Magee, R. S. (2002). Critical evaluation of proven chemical weapon destruction technologies—(IUPAC Technical Report). *Pure Appl. Chem*. **74,** 187–316.

Sumi, D., Manji, A., Shinkai, Y., Toyama, T., and Kumagai, Y. (2007). Activation of the Nrf2 pathway, but decreased gamma-glutamylcysteine synthetase heavy subunit chain levels and caspase-3-dependent apoptosis during exposure of primary mouse hepatocytes to diphenylarsinic acid. *Toxicol. Appl. Pharmacol*. **223,** 218–224.

Szinicz, L. (2005). History of chemical and biological warfare agents. *Toxicology* **214,** 167–181.

Umezu, T., Nakamiya, K., Kita, K., Ochi, T., Shibata, Y., and Morita, M. (2012). Diphenylarsinic acid produces behavioral effects in mice relevant to symptoms observed in citizens who ingested polluted well water. *Neurotoxicol. Teratol*. **34,** 143–151.

Wang, Y., Zhao, F., Liao, Y., Jin, Y., and Sun, G. (2013). Effects of arsenite in astrocytes on neuronal signaling transduction. *Toxicology* **303,** 43–53.