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Effects of Exposure to 1-Bromopropane on Astrocytes and Oligodendrocytes in Rat Brain

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Abstract: Effects of Exposure to 1-Bromopropane on Astrocytes and Oligodendrocytes in Rat Brain: Sahabudeen Sheik MOHIDEEN, *et al*. Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Nagoya University—Objectives: Human cases of 1-bromopropane (1-BP) toxicity showed ataxic gait and cognitive dysfunction, whereas rat studies showed pyknotic shrinkage in cerebellar Purkinje cells and electrophysiological changes in the hippocampus. The present study investigated the effects of 1-BP on astrocytes and oligodendrocytes in the rat cerebellum and hippocampus to find sensitive markers of central nervous system toxicity. **Methods**: Forty-eight F344 rats were divided into four equal groups and exposed to 1-BP at 0, 400, 800 and 1,000 ppm for 8 h/day; 7 days/week, for 4 weeks. Nine and three rats per group were used for biochemical and histopathological studies, respectively. **Results**: Kluver-Barrera staining showed pyknotic shrinkage in the cytoplasm of Purkinje cells and nuclei of granular cells in the cerebellum at 1,000 ppm. Immunohistochemical analysis showed increased length of glial fibrillary acidic protein (GFAP)-positive processes of astrocytes in the cerebellum, hippocampus and dentate gyrus at 800 and 1,000 ppm. The myelin basic protein (MBP) level was lower at 1,000 ppm. The numbers of astrocytes and granular cells per tissue volume increased at 400 ppm or higher. **Conclusion**: The present study showed that elongation of processes of astrocytes accompanies degeneration of granular cells and Purkinje cells in the cerebellum of the rats exposed to 1-BP. The decrease in MBP and number of oligodendrocytes suggest adverse effects on myelination. The increase in astrocyte population per tissue volume in the cerebellum might be a sensitive marker of 1-BP neurotoxicity, but the underlying mechanism for this change remains elusive.
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Key words: 1-bromopropane, Astrocyte, Myelin basic protein, Neurotoxicity, Oligodendrocyte

1-Bromopropane (1-BP; n-propylbromide, CAS Registry no. 106-94-5) is an organic solvent used as an alternative to ozone depleting solvents such as 1,1,1-trichloroethane and chlorofluorocarbons, which were banned in 1996 in developed countries. 1-BP is widely used for metal cleaning, foam gluing and dry cleaning[1]. However, it is neurotoxic[2–7] and exhibits reproductive toxicity[2, 8] in animals. Epidemiological studies on workers involved in the production of 1-BP showed sensory and motor deficits such as diminution of vibration sense, decrease in motor nerve conduction velocity, prolongation of distal latency in lower limbs, and lower cognitive function in neurobehavioral tests[9, 10]. Humans cases exposed to large doses of 1-BP showed abnormal T2-weighted signals on gadolinium-enhanced brain MRI[11], cognitive dysfunction and hyperreflexia in the lower limbs[12], diminished sensation[11–14] as well as symptoms of the central nervous system (CNS) such as headache[12–13]. While there is abundant evidence for 1-BP neurotoxicity in human, there is little support for these findings on morphological examination of 1-BP-exposed laboratory animals, apart from degeneration of preterminal axons in the medulla oblongata after exposure at 800 ppm for 12 weeks[3] and pyknotic shrinkage of Purkinje cells in the cerebellum after exposure at 1,000 ppm for 5–7 weeks[7] and 1,500 ppm for 5 weeks[5].

The present study was designed to examine the effects of 1-BP on astrocytes and oligodendrocytes in the cerebellum and hippocampus of rats with the aim of finding more sensitive markers of CNS toxicity

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than neuronal degeneration reported in previous studies[5, 7]. Since abnormalities in the white matter have been described in afflicted humans[11], the study investigated the morphological changes in myelin and the expression level of myelin and oligodendrocyte-related proteins. The study focused on the cerebellum and the hippocampus because the former was the region that exhibited morphological changes in neuronal cell bodies[5, 7] and the latter showed electrophysiological changes[15] and the clearest change in mRNA expression levels of neurotransmitter receptors[16].

Materials and Methods

1) Chemicals

1-BP (99.81% purity in the chart area of a capillary gas chromatograph with flame ionization detector [FID]) was kindly supplied by Tosoh Co., Japan. The chemical structure of 1-BP was confirmed with proton nuclear magnetic resonance (NMR).

2) Animals

Forty-eight male F344 rats (specific pathogen-free, 9-week-old, body weight 160–180 g) were purchased from Clea Laboratory Animal Center, Japan. They were housed and acclimatized to the new environment for one week and then divided at random into four groups of twelve each. They were housed in a room set on 12:12 light: dark cycle (lights on at 0900 hours and off at 2100 hours), stable relative humidity (67–60%), and constant temperature (23–25°C). Food and water were provided *ad libitum*.

3) Exposure to 1-BP

The four groups of rats were exposed to 0, 400, 800 and 1,000 ppm of 1-BP in inhalation chambers for 8 hours a day, 7 days/week, for 4 weeks. Daily exposure commenced at 1100 and was terminated at 1900 hours. The inhalation exposure system has been described in detail previously[3]. Briefly, a volume of the solvent was evaporated at room temperature and mixed with a larger volume of clean air to achieve the desired concentration. The vapor concentration was measured every 10 seconds by gas chromatography and digitally controlled within ± 5% of the target concentration. A rectification board with numerous custom-made holes was used for uniform distribution of the 1-BP-rich vapor. The concentration at 9 points was within 3% of the value of the central point in the chamber. The body weight of the rats was measured before the start, weekly and at the end of exposure. Two rats of the 1,000 ppm biochemistry group died because of severe debilitation on days 25 and 28 of the experiment, respectively. Japanese laws concerning protection and control of animals, the standards related to the care and management of experimental animals, and the Guide for Animal Experimentation of the Nagoya University School of Medicine were followed strictly throughout the experiment.

4) Fixation and staining for histopathological examination

Three rats from each group were deeply anesthetized with sodium pentobarbital and then perfused transcardially with 4% paraformaldehyde solution and maintained in ice for 1 hour for better fixation. The whole brain was dissected out *en bloc* and divided into three parts by cutting coronally at the frontal margin of the optic chiasma and just before the frontal margin of the cerebellum. These brain parts were postfixed overnight and the fixative was replaced with a series of 10, 20 and 30% sucrose solution (Sigma Aldrich, Corporation, St. Louis, MO, USA). Finally, the tissues were embedded in optimal cutting temperature [OCT] compound (Tissue-Tek®, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and stored at −80°C.

The frozen tissue processed after fixation was cut into 10 or 20 μm sections and mounted on glass slides (Matsunami MAS Superfrost Slides, Matsunami Glass Ind., Osaka, Japan). The sections were stored at −30°C until staining. The frozen sections from three rats were processed for Kluver-Barrera staining and Carazzi hematoxylin-eosin staining to find demyelination and pyknosis, as well as immunohistochemistry. For immunohistochemistry, sections were incubated overnight with diluted antibodies at 4°C. The antibodies used were myelin basic protein 1 : 200 (MBP-ab40390, Abcam, Cambridge, UK), APC/CC1 1 : 300 (APC/CC1-ab16794, Abcam) and glial fibrillary acid protein 1 : 400 (GFAP-G3893, Sigma-Aldrich Corporation, St. Louis, MO, USA). Briefly, the sections were kept at room temperature for 5 minutes before immunostaining, and then washed briefly in PBS (pH 7.4). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol. The antigen in the tissue was extracted using LAB solution by the method supplied by the manufacturer (Polysciences, Inc., Warrington, PA, USA). The tissues were stained using an RTU Vectastain Universal Quick Kit (Vector Laboratories, Burlingame, CA, USA) following the protocol provided and the reaction was visualized using AEC peroxidase substrate (ImmPACT AEC peroxidase substrate, SK-4205, Vector Laboratories, Burlingame, CA, USA). Finally, the slides were mounted with aqueous mounting medium (VectaMount Mounting Medium, H-5501, Vector Laboratories, Burlingame, CA, USA) following the protocol provided by the supplier. Photomicrographs of the tissues were taken using an FSX100 computer assisted microscope (Olympus Corporation, Tokyo, Japan). The cross-reactivity of

APC/CC1 antibody with astrocytes was found to be absent in double-immunofluorescence staining using GFAP and APC/CC1 antibodies.

5) Measurement of the area of GFAP-positively stained astrocytes and length of their processes

Areas of GFAP-positively stained astrocyte cell bodies, the length of their processes, and the shortest diameter of granular cell nuclei were measured. Furthermore, the numbers of astrocytes, oligodendrocytes and granular cells were counted on photomicrographs using the cellSens Dimension software (Olympus Corporation, Tokyo, Japan). Only astrocytes with clear nuclei and nucleoli and their entire processes visible within the section were measured and counted. The areas of astrocytes, including processes, were measured by drawing a borderline along the positively-stained astrocytes using the freehand circle tool for all of 6–15 different astrocytes that met the criteria of having nucleoli in the stratum radiatum layer of the CA1, CA2 and CA3 regions of the hippocampus and the molecular layer of the dentate gyrus in two slides per rat of three rats per group, as well as all of 6–15 astrocytes that met the criteria of having nucleoli in the inferior cerebellar peduncle at Bregma −10.32 following Paxinos and Watson[17]. The length of the process was measured by drawing a line over the process using the freehand line tool. Three to six processes per astrocyte were confirmed to be within one section and the average length of the process was calculated and used as the representative value for each astrocyte. The measurement of process length was conducted in the same astrocytes the area of which was measured as above. The averages of the area and process length of astrocytes per rat were used as representative values of each rat for statistical analysis of the four groups.

6) Astrocyte and oligodendrocyte counts

The numbers of astrocytes and oligodendrocytes were counted in the middle cerebellar peduncle at Bregma −10.32 following Paxinos and Watson[17] in GFAP- and APC/CC1-immunostained sections. Briefly, positively stained cells were counted in 9 photomicrographs under × 400 magnification (219 × 165 μm) in two sections per animal in three animals per group. The number of cells per volume of tissue was calculated. The average number of cells per rat was compared statistically between the groups.

7) Measurement of diameter of granular cells and count of nuclei of granular cells

The shortest diameter of the nuclei of granular cells in the cerebellar vermis in the part anterior to the precumulative fissure at Bregma −10.20[17] was measured by drawing a line using the straight line tool. One-hundred granular nuclei, which were in focus, were measured per rat in photomicrographs in three rats per group after Carazzi hematoxylin-eosin staining. The number of nuclei of granular cells in the cerebellar vermis in the part anterior to the precumulative fissure was counted in 9 photomicrographs under × 600 magnification (109 × 80 μm) in two sections per rat in three rats per group. The number of nuclei per volume of tissue was calculated. The average number of nuclei per rat was compared statistically between the groups.

8) Processing brain samples for biochemical studies

After exposure to 1-BP for 4 weeks, 9 rats were sacrificed by decapitation and the hippocampus and cerebellum were harvested and immediately frozen in liquid nitrogen, then stored at −80ºC until analysis. The frozen samples were processed later to powder form without thawing, working over liquid N_2. Two heavy metal rods and all other utensils, such as spatula, were precooled in liquid N_2 before the process. The fine powder was collected into clean RNase and DNase-free plastic sample tubes that were also precooled in liquid N_2.

9) mRNA extraction

An appropriate amount of the frozen powdered sample was weighed under dry ice and processed for RNA extraction using an RNeasy Lipid Tissue Mini Kit (Qiagen Benelux B.V., Venio, Netherlands), following the standard protocol provided by the manufacturer. DNA contamination was eliminated using an RNase free DNase kit (Qiagen Benelux B.V., Venio, Netherlands). The concentration of the extracted RNA sample was determined using a NANO Drop (NanoDrop Technologies Inc., Wilmington, DE, USA), and stored at −80ºC. The RNA samples were employed for reverse transcription to obtain cDNA, using SuperScript II and Oligo(dT)12–18 (Invitrogen, Carlsbad, CA, USA), by the standard protocol, and then stored at −20°C.

10) Primers

Primers were designed using the OLIGO software (Molecular Biology Insights, Inc., Cascade, CO, USA). Primers were purchased from Sigma Genosys (Sigma-Aldrich Corporation, St. Louis, MO, USA), and confirmed for single product banding by agarose gel electrophoresis after PCR (PerkinElmer GeneAmp PCR System 9600, Foster City, CA, USA), using Taq DNA polymerase (Roche Applied Science, Indianapolis, IN, USA) at a concentration of 10 μM. The search parameters followed were Tm: 67–69º, salt concentration: 1,000 mM, nucleotide concentration:

Table 1. Primers used in RT-PCR

Gene	Length (bp)	Primer	Gene no.
MBP	62	TCG GCT TCT TAA TAT AAC TGC CCCGAGGAAACTCAATCTTC	NM_001025291
OLIG2	224	CGA AAGGTGTGGATGCTTAAC TCA ACCTTCCGA ATGTGA	NM_001100557
MOG	314	TCCGTGCAGAAGTCGAGA CCAAGAACAGGGACA ATA ACA	NM_022668
NG2	258	CAAGCGTGGCAACTTTATCTA AGGCACGCTGTTACGAAG	X56541
GFAP	115	CCGTTCTCTGGAAGACACTGAAAC TTGGAAGGATGGTTGTGGATTC	RNU03700
β-actin	127	CACTGTGTTGGCATAGAGGTCTTTA TGTGGCATCCATGAAACTACATTC	NM_031144

MBP, myelin basic protein; OLIG 2, oligodendrocyte lineage factor 2; MOG, myelin oligodendrocyte glycoprotein; NG2, chondroitin sulfate proteoglycan 4; GFAP, glial fibrillary acid protein.

100 mM, product length: 50−150 bp (<400 bp) and P.E. >400, 3' end delta G (−5.5 to −9.8) (optimal −8.0 to −9.2) and 3' dimmer delta G >−3.5 kcal, Hairpin loop delta G >0 kcal and Hairpin stem <3 bp, length of acceptable dimmers <3 and oligonucleotide length: 18−21 bp.

11) Real-time quantitative PCR

RT-PCR analysis was carried out using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) and TAKARA SYBR Premix Ex Taq (Perfect real Time) (Takara Bio Inc., Kyoto, Japan) under the following temperature conditions, (a) initial denaturation at 95°C for 15 seconds, (b) denaturation at 95°C for 5 seconds and (c) annealing and extension at 60°C for 30 seconds, with repetition of (b) and (c) for 40 cycles. A standard curve was constructed by serial dilutions of a cDNA sample. Measurement was repeated in duplicate and the mean of the two values were used as the representative value for each rat.

12) Western blotting of Ng2 protein

The frozen powdered tissue samples were homogenized using T-PER® Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) together with Protease Inhibitor Cocktail P8340 (Sigma-Aldrich Corporation, St. Louis, MO, USA) according to the procedure recommended by the supplier. The resulting supernatant was collected and the protein concentration was measured using a BCA protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Then, 20 μg of protein was subjected to 6−10% polyacrylamide gel electrophoresis and transferred to PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). After blocking with 3% nonfat milk in Tween TBS buffer, the membranes were incubated overnight at 4°C with the respective primary antibodies for β-actin A1978 (Sigma-Aldrich Corporation, St. Louis, MO, USA) at a dilution ratio of 1 : 10,000 or anti-NG2 chondroitin sulfate proteoglycan AB5320 (Millipore, Billerica, MD, USA) at a dilution ratio of 1 : 750. After washing the membranes with TBS-Tween buffer, they were again incubated with the respective secondary antibodies, anti-mouse/rabbit IgG, Amersham ECL horseradish peroxidase-linked species-specific whole antibody (GE Healthcare, Buckinghamshire, UK), at a dilution ratio of 1 : 10,000. The specific immune complex was detected using SuperSignal® West Pico Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Each band was quantified by densitometry using the UN-SCAN-IT Gel & Graph Digitizing software version 6.1 (Silk Scientific Inc., Orem, UT, USA).

13) Enzyme-linked immunosorbent Assay (ELISA) for MBP and GFAP

Protein levels of MBP and GFAP in the cerebellar homogenates were quantified using anti-rat MBP ELISA-E90539Ra and anti-rat GFAP ELISA-E90068Ra (Uscn Life Science, Wuhan, P.R. China) following the protocol supplied by the manufacturer. Briefly, 100 μl of the standards (10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 ng/ml for MBP and 1,000, 500, 250, 125, 62.5, 31.2 and 15.6 pg/ml for GFAP) and samples were added to the anti-MBP/GFAP pre-coated plate and incubated for 2 hours at 37°C. After incubation, the solutions in the plate were discarded and

100 μl of detection reagent A was added to each well and further incubated for 1 hour at 37°C. The solutions in the plate were discarded and the plates were washed three times using 350 μl of wash buffer provided in the kit. Then 100 μl of detection reagent B was added and incubated for 30 minutes at 37°C. The solutions were discarded and the plate was washed five times using 350 μl of wash buffer. Subsequently, 90 μl of substrate solution was added and the plate was incubated for 15 minutes at 37°C in the dark. Finally, 50 μl of stop solution was added and absorbance was measured at 450 nm immediately. The sample values were calculated using a standard curve.

14) Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison with the control was conducted to assess differences between the exposed groups and the control. For the value of the GFAP-positive area, process length of astrocytes and quantitative real-time PCR analysis, the log transformation was performed before the ANOVA. Regression analyses were conducted to check the dose-dependent effect using exposure concentration as the independent variable and each of the examined parameters as the dependent variable. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed using the JMP® version 7 statistical analysis software (SAS Institute, Cary, NC, USA).

Results

1) Kluver-Barrera staining

Kluver-Barrera staining showed pyknosis and shrinkage of Purkinje cells and nuclei of granular cells of the cerebellum in the 1,000 ppm exposed group (Fig. 1D). No such change was found in the hippocampal neurons. Furthermore, no clear demyelination was found in the cerebellum or hippocampus (Fig. 1B).

2) Carazzi hematoxylin-eosin staining

Carazzi hematoxylin-eosin staining showed pyknotic shrinkage of nuclei of the granular cells of the cerebellum in rats of the 1,000 ppm exposure group (Fig. 1F). A shrunk and rough or inhomogeneous eosinophilic area, which represented either neuropil or glial cytoplasm, was found between granular cells in the 1,000 ppm exposure groups (Fig. 1F). The numbers of granular cells per volume in the cerebellum were significantly higher in the 800 and 1,000 ppm groups than the control group (Table 3). The shortest diameter of the nuclei of the granular cells in the cerebellum was significantly smaller in rats exposed to 1,000 ppm than that in the control rats (Table 3).

3) Immunostaining

The area of GFAP-positive astrocytes was significantly larger in the 800 and 1,000 ppm groups in the inferior cerebellar peduncle and the CA1 and CA3 regions (Table 2), in the 1,000 ppm group in the CA2 region and in all the three exposure groups in the DG region (Table 2). The lengths of processes of GFAP-positive astrocytes were significantly longer in the 800 and 1,000 ppm groups in the inferior cerebellar peduncle and CA1 region and in the 800 ppm group in the CA2 region and in all the three exposure groups in the CA3 and DG regions (Table 2). The number of astrocytes in the middle cerebellar peduncle was significantly higher in all the three exposure groups, compared to the control (Table 3).

In rats of the 800 and 1,000 ppm exposure groups, MBP immunoreactivity tended to be weaker in the granular layer of the cerebellar vermis in the part anterior to the primary fissure and hippocampus (Fig. 2), compared with the respective control groups. A similar pattern was noted in the medulla, granular layer of the vermis and cerebellar hemisphere. In the hippocampus, MBP immunoreactivity of the exposed groups tended to be lower than that of the control, especially in areas with relatively higher background expression of MBP than other areas, relative to the control, including the stratum lacunosum-moleculare of the CA1, the stratum lucidum of the CA3 (Fig. 2) and the polymorphic cell layer of the DG. No clear demyelination was identified based on MBP immunostaining.

The oligodendrocyte marker APC/CC1 decreased dose-dependently in the medulla and granular layer of the cerebellar vermis in the part anterior to the precumulative fissure (Fig. 2). A similar trend was observed in the medulla, granular layer of the vermis and cerebellar hemisphere. The number of positively-stained oligodendrocytes was significantly lower in the middle cerebellar peduncle in the 1,000 ppm group (Table 3). No change in immunostaining for APC/CC1 was detected in the hippocampus.

4) Results of quantitative real-time PCR

In the cerebellum, the mRNA expression levels of MBP and MOG genes were significantly lower in the ≥400 ppm groups while those of OLIG2 and NG2 were significantly lower in the 800 and 1,000 ppm groups, compared with the control. Regression analysis using the 1-BP concentration as the independent variable and the mRNA level of MBP, NOG, NG2 or OLIG2 as the dependent variable showed significant relationships between the concentration and every examined gene, suggesting the dose-dependency of the expression. The level of GFAP mRNA expression was significantly higher in the cerebellum of rats

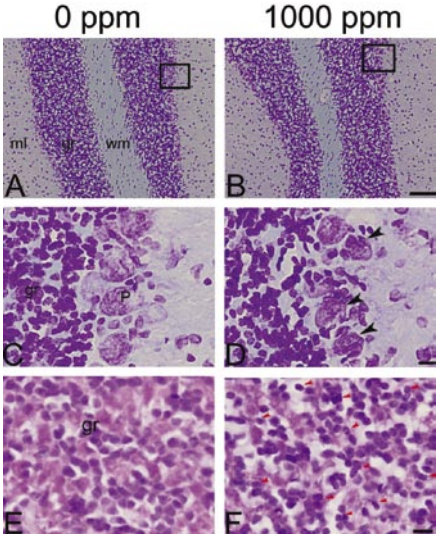

Fig. 1. Photomicrographs of the cerebellar vermis in the part anterior to the precumulative fissure: (A, C, E) control, (B, D, F) 1,000 ppm group. (A, B, C, D) Kluver-Barrera staining, (E, F) hematoxylin-eosin staining. Black squares at the borders of the granular layer and molecular layer in A and B correspond to C and D, respectively. (E, F) Granular layer. Scale bar=100 μm in B, 10 μm in D and F. Magnification: A, C and E are similar to B, D and F, respectively. (B) No clear demyelination was found in the white matter. (D) Black arrows depict pyknotic shrinkage in Purkinje cells. (F) Red arrows depict pyknotic shrinkage in the nuclei of granular cells. Note shrinkage and inhomogeneity of the eosinophilic area between granular cells. gr: granular layer, ml: molecular layer, wm: white matter, and P: Purkinje cells.

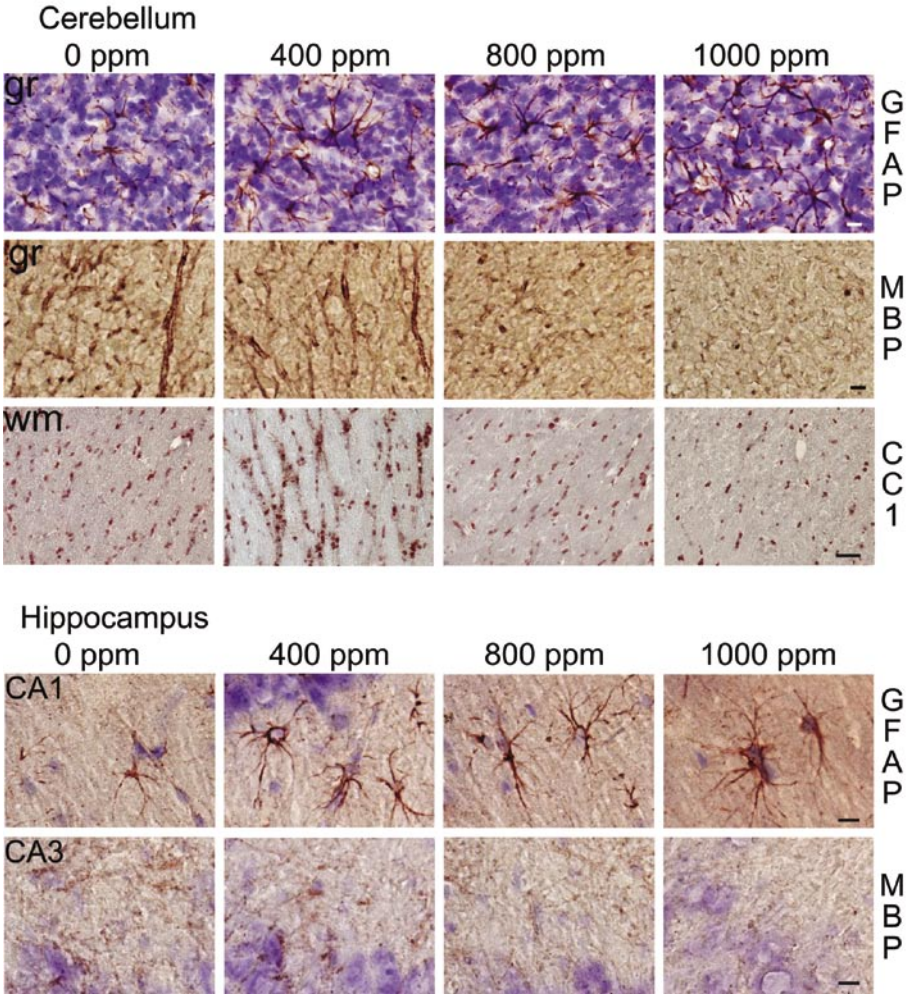

Fig. 2. Photomicrographs of the cerebellar vermis showing immunostaining for glial fibrillary acid protein (GFAP) and myelin basic protein (MBP) in the part anterior to the primary fissure. and APC/CC1 in the part anterior to the internal precumulative fissure. Scale bars: 10 μm for GFAP and MBP immunostaining, 50 μm for APC/CC1 immunostaining. gr: Granular layer; wm: white matter. Photomicrographs of the hippocampus showing immunostaining for glial fibrillary acid protein (GFAP) in the stratum radiatum layer of the CA1 region and myelin basic protein (MBP) in the stratum lucidum layer of the CA3 region. Scale bars=10 μm.

Table 2. Quantitative analysis of GFAP-positive area and process length of astrocytes after 4-week exposure to 1-bromopropane (1-BP)

	Exposure to 1-BP at			
	0 ppm	400 ppm	800 ppm	1,000 ppm
Area (μm^2)				
Cerebellum	125.7 ± 4.2	154.9 ± 19.7	248.6 ± 38.4*	228.1 ± 10.2*
CA1	166.0 ± 28.9	206.5 ± 9.6	232.0 ± 36.0*	320.4 ± 39.4*
CA2	191.0 ± 5.5	178.9 ± 26.7	181.8 ± 21.9	296.2 ± 40.4*
CA3	109.1 ± 22.1	223.0 ± 17.2	192.6 ± 31.1*	236.6 ± 78.4*
DG	114.0 ± 26.0	231.1 ± 40.9*	254.0 ± 19.0*	294.7 ± 88.4*
Length (μm)				
Cerebellum	18.8 ± 0.6	22.9 ± 3.0	29.7 ± 4.7*	24.1 ± 1.1*
CA1	20.5 ± 3.6	23.5 ± 1.1	30.3 ± 4.7*	31.1 ± 3.8*
CA2	20.9 ± 0.6	22.9 ± 3.5	27.3 ± 3.3	31.0 ± 3.6*
CA3	19.7 ± 4.2	25.2 ± 1.9*	28.9 ± 4.7*	31.2 ± 4.6*
DG	18.8 ± 4.2	29.5 ± 5.2*	33.5 ± 2.6*	35.9 ± 6.1*

Data are mean ± SD of 3 rats in each group. The area and length of processes were measured for all of 6–15 different astrocytes that met the criteria of having nucleoli in the stratum radiatum layer of the CA1, CA2 and CA3 regions of the hippocampus and the molecular layer of the dentate gyrus in two slides per rat of three rats per group, as well as all of 6–15 astrocytes that meet the criteria of having nucleoli in the inferior cerebellar peduncle.
*$p<0.05$, compared with the control (by ANOVA followed by Dunnett's multiple comparison test). Log transformation was performed for the value before ANOVA. Regression analysis showed significant trends for exposure concentration in all the listed parameters. GFAP, glial fibrillary acid protein; DG: dentate gyrus.

Table 3. Effect of 4-week exposure to 1-BP on the number of cells, the shortest diameter of nuclei of the granular cells and protein levels in the cerebellum

	Exposure to 1-BP at			
	0 ppm	400 ppm	800 ppm	1,000 ppm
No. of cells in cerebellum				
Astrocytes	6.7 ± 1.0	9.0 ± 0.6*	10.6 ± 1.6*	12.3 ± 1.0*
Oligodendrocytes	29.8 ± 4.0	23.6 ± 4.8	22.2 ± 1.7	18.3 ± 1.7*
Granular cells	138.0 ± 12.9	156.4 ± 8.2	186.6 ± 9.4*	216.6 ± 6.3*
Granular cell nucleus diameter (μm)	3.7 ± 0.1	3.7 ± 0.4	3.6 ± 0.2	3.0 ± 0.2*
ELISA value in cerebellum				
GFAP (ng/g wet tissue)	313 ± 16	350 ± 16	333 ± 16	441 ± 18*
MBP (mg/g wet tissue)	1.39 ± 0.15	1.34 ± 0.28	1.29 ± 0.29	1.10 ± 0.10*

Data are mean ± SD of 3 rats in each group for the number of astrocytes and oligodendrocytes ($10^2/mm^3$) counted in the middle cerebellar peduncle. Data are mean ± SD of 3 rats in each group for the number of granular cells ($10^6/mm^3$) counted in the granular layer of the cerebellar vermis in the part anterior to the precumulative fissure. Data are mean ± SD of 3 rats per group for the shortest diameter of the nuclei of granular cells (μm). The mean of the shortest diameters of 100 granular cells was used as the representative value for each rat. Data for ELISA are mean ± SD of 9 rats in the 0, 400 and 800 ppm groups and 7 rats in the 1,000 ppm group. *$p<0.05$, compared with the control (by ANOVA followed by Dunnett's multiple comparison test). Regression analysis showed significant trends for exposure concentration in all the listed parameters. GFAP, glial fibrillary acid protein; MBP, myelin basic protein.

Table 4. Effect of 4-week exposure to 1-BP on mRNA levels in the cerebellum

	Exposure to 1-BP at			
	0 ppm	400 ppm	800 ppm	1,000 ppm
MBP	2.4 ± 2.5	0.7 ± 0.3*	0.6 ± 0.1*	0.6 ± 0.1*
OLIG2	3.9 ± 2.4	2.4 ± 1.4	0.9 ± 0.8*	0.5 ± 0.2*
MOG	2.5 ± 1.8	1 ± 0.4*	0.9 ± 0.3*	0.7 ± 0.1*
NG2	15 ± 1.6	4.5 ± 3.2	1.4 ± 1.3*	0.9 ± 0.2*
GFAP	0.19 ± 0.11	0.14 ± 0.07	0.17 ± 0.06	0.31 ± 0.07*

Data are mean ± SD of the expression level relative to β-actin of 9 rats in the 0, 400 and 800 ppm groups and 7 rats in the 1,000 ppm group. *p<0.05, compared with the control (by ANOVA followed by Dunnett's multiple comparison test). Log transformation was performed before ANOVA.
Regression analysis showed significant trends for the exposure concentration in all the listed parameters, except GFAP (p=0.083). MBP, myelin basic protein; OLIG 2, oligodendrocyte lineage factor 2; MOG, myelin oligodendrocyte glycoprotein; NG2, chondroitin sulfate proteoglycan 4; GFAP, glial fibrillary acid protein.

exposed to 1,000 ppm (Table 4), but dose-dependency could not be confirmed by regression analysis (p=0.083). The mRNA levels of all the examined genes in the hippocampus were not significantly different between the exposed groups and the control (data not shown).

5) Western blot of NG2 protein

Quantitative analysis of western blots showed that exposure to 1-BP tended to reduce NG2 protein expression, irrespective of the dose, but the difference from the control was not significant (data not shown).

6) ELISA for MBP and GFAP

Exposure to 1-BP at 1,000 ppm significantly reduced MBP expression levels and significantly increased the GFAP level in cerebellar homogenates (Table 3).

Discussion

The present study showed that exposure to 1-BP increased the GFAP-positive area of astrocytes and the length of their processes in the cerebellum and hippocampus, suggesting that 1-BP induces astrocyte activation. The results also showed a decrease in MBP and down-regulation of mRNA expression of oligodendrocyte-related genes and NG2 in the cerebellum, together with a decrease in the number of oligodendrocytes, although clear morphological changes in oligodendrocytes or myelin sheath were not identified. The study also demonstrated pyknotic shrinkage of granular cells, degeneration of Purkinje cells in the cerebellum, and shrinkage of nuclei of the granular cells as confirmed by quantitative analysis.

Astrocytes carry out many critical functions in the normal brain, including formation of the blood-brain barrier[18] and expression of transporters for neurotransmitters. Pathologically, it is well known that astrocytes fill in the space upon CNS injury and form a glial scar[19]. Recent studies showed that both astrocytes and microglia respond to intracellular generation of reactive oxygen species and glutathione depletion, which are produced by exposure to environmental chemicals, such as methylmercury[20]. These astrocytic responses include uncoupling of Nrf2. Since exposure to 1-BP is reported to deplete glutathione[6] and produces oxidative stress[21], it is reasonable to assume that the changes seen in astrocytes in the present study represent a response to exposure to 1-BP. However, the relationship between such response and morphological changes in astrocytes observed in the present study remains elusive.

Astrocytes are also known to induce remyelination of the spinal cord after ethidium bromide-induced demyelination in rats[22]. The present study hypothesized that if exposure to 1-BP resulted in the induction of demyelination, then this should activate the myelination process. Rather, the results showed a decrease in the expression of myelin- and oligodendrocyte- related genes of MBP, MOG and OLIG2, which does not suggest activated myelination. Although a previous study showed developmental toxicity with increased expression of GFAP and decreased expression of myelin-related genes in SD rats treated with chlorpyrifos from PND1 through PND6[23], the relationship between activation of astrocytes and down-regulation of myelin-related proteins remains elusive.

The results of KB staining and MBP immunostaining did not show clear demyelination in the examined regions of the cerebellum and hippocampus. However, care should be taken when interpreting the results of the effect of 1-BP on demyelination, and further systematic investigation on possible demy-

elination using complete serial sections is needed. The present study showed that the numbers of oligodendrocytes per tissue volume, which play a role in myelination, were lower in the cerebellum of the 1,000 ppm group, but did not demonstrate how this change translates in functional changes in the CNS of rats exposed to 1-BP. Although the mRNA expression of oligodendrocyte- and myelin-related genes or NG2 was observed at levels lower than 1,000 ppm, further studies are needed to interpret these phenomena as well as the decrease in MBP protein level in relation to the CNS toxicity of 1-BP.

The present study not only confirmed the degeneration of Purkinje cells reported previously[5, 7] but also demonstrated degeneration of nuclei of granular cells in the cerebellum of the 1,000 ppm group. The shrinkage of nuclei of cerebellar granular cells in the 1,000 ppm group was confirmed, while the increase in the population of granular cells in the 800 and 1,000 ppm exposure groups suggests shrinkage of eosinophilic substance between the granular cells. The number of granular cells per volume increased at concentrations lower than the level that induced shrinkage of nuclei of granular cells in the cerebellum, but such increases could be secondary to shrinkage of the aforementioned eosinophilic substance. Thus, one can hypothesize that shrinkage of the eosinophilic material is due to exposure to 1-BP, but confirmation of this by electron microscopy is needed before one can conclude that the eosinophilic substance in the exposed group is neuropil or glial cytoplasm.

With regard to the sensitivity of the examined parameters in the present study, exposure to 1-BP induced elongation of astrocyte processes at 800 ppm, which is lower than the level of 1,000 ppm that induced shrinkage of granular cells. The morphological changes in astrocytes also seem more sensitive than protein assay of MBP and GFAP, which changed significantly only at 1,000 ppm. The discrepancy in GFAP expression between morphometric study and ELISA may be due to the experimental setup in which samples used for ELISA were homogenates of the whole cerebellum. It is also noted that the number of astrocytes per tissue volume increased at the lowest exposure level of 400 ppm, but possible effect of shrinkage between astrocytes should be also considered as discussed above for the number of granular cells, thus the significance of the increased population of astrocytes per tissue volume remains elusive in relation to neurotoxicity of 1-BP.

Since neuronal degeneration was found in the cerebellum, microglial response to neuronal degeneration should be also of interest. The present study did not investigate microglia, but a recent study using Wistar male rats in our laboratories demonstrated morphological changes in microglia after exposure to 1-BP[21].

The relevance of the present result on cerebellar change should be explained in comparison with human cases of 1-BP neurotoxicity. One of the latest case reports showed predominance of damage to large myelinated fibers in sensory nerves, which was compatible with the symptoms of sensory-ataxic neuropathy[14], while possible cerebellar dysfunction as the cause of ataxia could not be excluded completely. Another case also described cerebellar ataxia following exposure to 1-BP[24]. It is likely that the site of 1-BP-induced damage in the nervous system depends on both the level and duration of exposure to 1-BP.

The maximum exposure level of 1,000 ppm in the present study was selected because it was reported previously to induce morphological changes in the cerebellum[7]. For example, no morphological changes were found in the CNS of rats exposed to 1-BP at 400 ppm for 8 h/day, 7 days/week for 12 weeks, while degeneration of preterminal axons of spinal ganglion neurons in the gracile nucleus was found in the medulla oblongata of rats exposed to 1-BP at 800 ppm for 8 h/day, 7 days/week for 12 weeks[3]. Pyknotic shrinkage of Purkinje cells was detected in the cerebellum of rats exposed to 1-BP at 1,000 ppm for 8 h/day, 7 days/week for 5 weeks[7], but not at exposure levels less than 1,000 ppm[3]. On the other hand, exposure to 1-BP caused severe debilitation after 5–7 weeks[7]. Accordingly, we considered that 1,000 ppm is the maximum level that can be applied for 8 h/day, 7 days/week for 4 weeks. Human cases in Utah who showed neurologic deficits were exposed to 1-BP at ambient concentration of 130 ppm (range 91–176 ppm) with a time-weighted average of 108 ppm (range 92–127 ppm)[12]. The time weighted average of 1-BP level in another case in North Carolina was 133 ± 67 (mean ± SD) ppm (range 60–261 ppm) after improvement of ventilation[13]. Other victims from Utah, USA, developed cognitive dysfunction after exposure to 1-BP, which was estimated to be 130 ppm (range 91–176 ppm) with a time-weighted average of 108 ppm (range 92–127 ppm)[12]. The latest report on human case of 1-BP intoxication reported an estimated exposure level of 553 ppm (mean of time-weighted averages, range 353–663 ppm)[14] with an ambient concentration of 1-BP in front of the washing tank of around 1,500 ppm (data not shown). Thus, the concentrations of 1-BP used in the present study of 400–1,000 ppm are not "extremely higher" than those found in poorly controlled workplaces associated with the reported human cases of neurotoxicity.

In conclusion, the present study showed morphological changes in astrocytes, including elongation of processes, accompanied by degeneration of granular cells and Purkinje cells in the cerebellum of the rats

exposed to 1-BP. The decrease in MBP and the number of oligodendrocytes with decrease in mRNA expression of myelin/oligodendrocyte-related genes and NG2 suggest that 1-BP adversely affects myelination, although clear demyelination was not found. While the increase in the number of astrocytes per tissue volume in the cerebellum might be a sensitive marker, the underlying mechanism of this change remains elusive.

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