Cerebral excitability in pup rats prenatally exposed to 1-bromopropane is suppressed by bromide accumulated in the brain

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Abstract: Previously, we reported that prenatal exposure to 1-bromopropane (1-BP) causes the accumulation of bromide (Br) in the brain of rat pups. Here, we aimed to investigate the effects of Br accumulation in rat pups prenatally exposed to 1-BP vapor. Dam rats were exposed to 1-BP (400 or 700 ppm; 1-BP group) by inhalation, or to NaBr (20 mM; Br group) in drinking water during gestation days 1–20. We also analyzed pentylenetetrazole (PTZ, 60 mg/kg, ip)-induced behavioral changes in pups prenatally exposed to 1-BP or Br on postnatal day (PND) 14. PTZ-induced epileptic convulsions were inhibited in both 1-BP (700 ppm) and Br groups. The inhibition of neuronal excitability induced by Br was evaluated electrophysiologically using the hippocampal slices obtained from PND14–16 pups. PTZ (2 mM) failed to induce epileptiform discharge in the presence of 1.2 mM Br in the slices obtained from the control group. However, it induced epileptiform discharge following the removal of Br, by perfusing artificial cerebrospinal fluid into the slices obtained from the Br group. Our results indicate that Br accumulates in the brain of neonatal rat pups prenatally exposed to 1-BP vapor suppressed neuronal excitability.

Key words: Prenatal exposure, 1-bromopropane, Bromide, Neurotoxicity, Pentylenetetrazole, Hippocampal slice

Introduction

1-Bromopropane (1-BP) is a solvent substitute with various applications, including as a cleaning agent, and as an aerosol propellant and adhesive. In fact, in 2016 alone, 4,000 tons of 1-BP was produced and imported in Japan, with primary uses as an intermediate for drugs and agrochemicals, and as a vapor washing solvent1. Although the American Conference of Governmental Industrial Hygienists recommended an 8 h time-weighted average (TWA) threshold limit value of 0.1 ppm 1-BP, in 20142, the Japan Society for Occupational Health had previously recommended an occupational exposure limit (OEL) of 0.5 ppm in 20123. However, more recently, 1-BP exposure concentrations were investigated by the Japan Ministry of Health, Labor and Welfare in Japanese facilities4, showing that the actual exposure values of TWA were higher than the OEL in most cases; thus,
the risk of occupational accidents and exposures remain high. Maintaining the TWA value is recommended as a precautionary measure against potential neurotoxicity, hepatotoxicity, reproductive toxicity, and developmental toxicity[1]. However, data on the developmental neurotoxicity (DNT) of 1-BP are limited despite concerns and increasing interest regarding DNT induced by industrial chemicals[2].

To address this issue, we previously reported that prenatal 1-BP exposure may induce delayed DNT as the excitability observed in the hippocampal slices of rat pups exposed to prenatal 1-BP vapor was enhanced during the lactation period, whereas disinhibition was observed in adult rats after reaching sexual maturation[3]. Moreover, another study reported that Br− readily crosses the placenta in dams exposed to sodium bromide in their diet[4]. In addition, we found high concentrations of accumulated Br− in both breastfeeding dams exposed to 1-BP vapor during pregnancy[5]. Thus, Br− may represent a marker of both effect and exposure during the early postnatal period, including prenatal 1-BP exposure.

Furthermore, in humans, the blood and urine concentrations of Br− in some employees exposed to 1-BP via inhalation were reported to be significantly higher than those in the control group and compared to the recommended exposure concentrations[6, 7]. In most of these employees, 1-BP exerted neurotoxic effects. Similarly, in adult rats exposed to 1-BP vapor, the brain Br− concentration was significantly higher than that in controls[8].

The net brain excitability is a balance between excitation and inhibition. γ-Aminobutyric acid (GABA) is one of the most prominent synaptic neurotransmitters; its receptor activation has an inhibitory effect on neuronal circuits in the adult brain. Br− has been shown to interact with type A GABA receptors, allowing Cl− to enter through the pores of GABA receptors[9–11], thus demonstrating its antiepileptic therapeutic potential[12–15]. We previously reported that kainate (KA)-induced wet dog shake behavior was inhibited in suckling pups[16]. In adult male rats[17], the final concentration of Br−, to that of 1-BP, was achieved[18] demonstrating its antiepileptic properties[19–21]. Hence, in the current study, we used PTZ to examine brain excitability.

Subjects and Methods

Animals

Fifty-nine (42 females and 17 males) Wistar rats purchased from Kyudo Co. (Tosu, Japan) at 9–11 weeks of age were housed in plastic cages with paper-made chips (AL-PHA-dr; Shepherd Specialty Papers, Milford, USA) under a 12/12 h light/dark cycle (light period: 07:00-19:00 h). The room temperature was 23 °C ± 1 °C and relative humidity was 40%–70%. The rats had free access to food and water. Both breeding conditions and pregnancies in rats were achieved as previously described[22]. In the morning after mating day, the presence of sperm in the vaginal smear or vaginal plug was verified to confirm gestation day (GD) 0. The rats and pups were anesthetized with isoflurane vapor before being sacrificed. All experiments were approved by the Ethics Committee for Animal Care and Experimentation in accordance with the University of Occupational and Environmental Health, Japan (AE03-065).

Prenatal exposure to 1-BP

1-BP (CAS No. 106-94-5; Guaranteed Reagent, higher than 98% purity) was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). The exposure dosages were 0 (control), 400, and 700 ppm. The 400 ppm dosage was the lowest observed-adverse-effect level (LOAEL) for neurotoxicity of 1-BP in adult male rats[23]. In the 1-BP group, dams were exposed to 1-BP vapor for 6 h/day over 20 days from GD 1 to 20 in an exposure chamber, following a previously described method[24]. The day of delivery was defined as PDN 0 (GD21). 1-BP vapor exposure dosage was monitored using a gas chromatograph (GC535B FSL; GL Sciences Inc., Japan) equipped with a flame ionization detector. Exposure schedule and evaluation of Br− effects were described[25]. The lowest limit in the quantitative determination using a gas chromatograph (GC535B FSL; GL Sciences Inc., Japan) equipped with a flame ionization detector was 3.87 µg/g tissue. The remaining litters at PDN14 were injected with PTZ to examine brain excitability.

Prenatal exposure to Br− and analysis of brain Br− concentration

Sodium bromide (NaBr; CAS No. 7647-15-6; Guaranteed Reagent, >99.5% purity) was purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Br− was administered to rats via drinking water for 20 days from 11:00 h on GD 1 to 11:00 h on GD21. Pregnant rats were randomly divided into the following two groups: Control (purified water; n=6) and Br− exposed (Br group; n=9). We estimated Br− uptake rate via drinking water so that a similar concentration of Br− was achieved in the brains of pups prenatally exposed to 1-BP.

Subjects

Fetus number, litter size, and body weight (PDN 1, 7, and 14) of the pups were also recorded. At PDN14, brain weight was measured in two and four litters from the control and Br− groups, respectively; the brain samples were then cryopreserved at −80 °C to analyze Br− concentration.

The brain concentration of Br− was determined using a gas chromatograph (GC535B FSL; GL Sciences Inc., Japan) equipped with a flame ionization detector. The remaining litters at PDN14 were injected with PTZ to examine brain excitability.

Fig. 1 Exposure schedule and evaluation of Br− effects
the experiment, we recorded the evoke potentials (open arrow in Fig. 2).
All chemicals used in this study were of reagent grade and purchased from commercial sources.

Statistical analysis
Mann–Whitney U test and Chi-squared test of independence using 2 × 2 contingency table were used to evaluate the differences in litter size and the occurrence ratio of PTZ-induced generalized tonic contraction, respectively, between the control and Br groups. Mann–Whitney U test followed by Steel–Dwass test was used to compare the occurrence ratio of a series of PTZ-induced epileptic behaviors among the control, 400 ppm, and 700 ppm groups.

Results
We previously reported no difference in litter size between the prenatal 1-BP group and control group. Similarly, no differences were observed in the litter size between the Br and control groups (14 ± 3 fetuses, 15 ± 3 pups in the Br group; 14 (12 and 15) fetuses, 16 ± 2 pups in the control group, p > 0.5; Mann–Whitney U test).

Body weight is an effective index to evaluate developmental toxicity. As shown in the supplemental Fig. S1, prenatal 1-BP or Br had no effect on body weight at PND1, the day after birth; however, a significant inhibition of increased body weight was observed at PND 7 and 14, in both male and female pups.

PTZ-induced behavioral changes
The occurrence ratios for each behavioral episode measured in the 1-BP groups are summarized in Table 1. In the 1-BP control group, after body shaking and rapid backward walking, the first apparent epileptic sign was myoclonic jerks predominantly involving hind limb extension. This type of seizure occurred once to several times, followed by wild running for 1–2 s in the observation box. Subsequent-

Table 1. Pentylenetetrazole (PTZ)-induced behavioral changes and epileptic seizures in PND14 pups prenatally exposed to 1-bromopropane (1-BP) vapor

| Behavior                  | 0 ppm | 400 ppm | 700 ppm |
|---------------------------|-------|---------|---------|
| Occurrence rate           |       |         |         |
| Pup number                |       |         |         |
| %                         |       |         |         |
| Pop number                |       |         |         |
| %                         |       |         |         |

PTZ-induced discharges in the hippocampal slices
The hippocampal slices at PNDs 14–16 were prepared as previously described. The concentration of PTZ used in vitro was 2 mM, according to previous studies. Spontaneous and periodic discharge induced by PTZ, as well as field potentials evoked via electrical stimulation of Schaffer collateral/commisural fibers, were recorded from the CA1 region. To select the intact slices, an evoked PS amplitude > 3 mV, the average amplitude in the control group at PND14, was used. Our experimental design is shown in Fig. 2, where the ability of Br to inhibit brain hyperexcitability induced by PTZ is illustrated. First, we confirmed that PTZ (2 mM) induced epileptic discharge (Fig. 2A). Second, we examined the effects of PTZ in the presence of Br. To achieve this, we perfused the slices with 2.4 mM Br to a final concentration of 1.2 mM in the slices (Fig. 3A). Br concentration in the artificial cerebrospinal fluid (ACSF) was determined based on our results. Finally, after washing Br from the slices obtained from the Br group (Fig. 2C), we examined whether PTZ could induce epileptic discharge in these slices. We analyzed their latency, occurrence rate, amplitude, and discharge duration for comparison. This experimental design is capable of determining whether Br is associated with the observed changes in excitability in pup brains prenatally exposed to 1-BP. To ensure that the slice was intact throughout the experiment, we recorded the evoke potentials (open arrow in Fig. 2).

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ly, generalized tonic contraction in pups was characterized by the loss of postural control and strong contractions in the extensor muscles of the fore and hind limbs with cyanosis, followed by generalized SE.

The occurrence ratios for body shaking, rapid backward walking, and myoclonic jerks remained the same among the three groups. The 700 ppm group showed a significant decrease in wild running, generalized tonic contraction, and SE compared with the control group (p<0.01, Mann–Whitney U test followed by Steel–Dwass test).

We also analyzed the occurrence ratio of PTZ-induced generalized tonic contraction in the Br group and found it to decrease from 55% to 3% (p<0.01, Chi-squared test of independence using 2 × 2 contingency table) following PTZ injection in the Br group (in the control group).

PTZ-induced generalized tonic contraction was similarly suppressed in both 1-BP and Br groups.

**PTZ-induced epileptiform discharges in the hippocampal slices**

The brain Br concentration in the Br group at PND14 was approximately 1.9 mM (152.4 ± 28.1 µg/g tissue (n=23 pups from four litters)). The brain Br concentrations in pups prenatally exposed to Br were similar to those previously reported. Based on these findings, we selected the Br concentration in ACSF to perfuse the hippocampal slices.

First, we ascertained that PTZ (2 mM) induced characteristic epileptiform discharges in 60% of the tested slices (n=10) obtained from nine control pups. PTZ-induced discharges showed an isolated periodic pattern, followed by long-lasting tonic-like, epileptiform discharges. The isolated periodic epileptiform discharges (Fig. 4A) occurred for a short duration (0.23 ± 0.03 s), with an amplitude of 1.0 ± 0.3 mV and an occurrence rate of 13 ± 3/min with a duration of 60–240 s. Thereafter, long-lasting tonic-like epileptiform discharge (a single curly bracket) proceeded for 25–45 s with an amplitude of 1.7 ± 0.8 mV. The evoked potential was also epileptiform with multiple spikes (thick arrows) in all ten slices. Second, the perfusion of 2.4 mM (1.2 mM Br) to 60–240 s. Thereafter, long-lasting tonic-like epileptiform discharge was 9–22 s with an amplitude of 1.4 ± 0.6 mV. The evoked potential was epileptic in all ten slices (Fig. 4C).

**Discussion**

Our findings have significant implications for work and safety regulations implemented by governments, as well as regulations that govern food and drink industries. Furthermore, our study provides insights into the potential mechanisms of neuronal excitability, inhibition, and GABA function. In the field of medicine, our findings are important for the treatment of epilepsy and related neurological disorders that involve overexcitement of the CNS. Our previous studies demonstrated the possibility that pathological conditions after birth could be caused by chemicals to which the mother might have been exposed during pregnancy.

In the current study, we evaluated changes in neuronal excitability of the brain caused by Br in DNT induced by prenatal 1-BP exposure. A high concentration of Br was observed in the brain of pups prenatally exposed to 1-BP; thus, in the Br group, we first achieved the same brain Br concentration as that observed in the 1-BP group. Subsequently, we compared the developmental effects of prenatal Br exposure on neuronal excitability with those of 1-BP exposure in pups, and found that both exhibited similar effects in suppressing PTZ-induced tonic contraction in vivo and hyperexcitability induced by PTZ, using the brain slices prepared in vitro.

Exposure of pregnant rats to 1-BP resulted in fetuses brain Br concentrations up to ~10 mM at GD20 and ~7.5 mM in pups at PND3, which then gradually declined to approximately 1.3 mM at PNDs 13–15, according to the estimation using the one-compartment model. Moreover, the average concentration of Br in the brain of pups at PND14 was 1.9 mM, which is similar to the 1.3 mM Br reported to potentially enhance GABAergic inhibitory activity, which may explain the inhibition of increased body weight during lactation owing to weak suckling caused by the sedative effect of Br.

Considering that PTZ-induced epileptic episodes are typical (e.g., generalized tonic contraction following several myoclonic convulsions) in immature animals, the PTZ model is commonly used to induce brain hyperexcitability during lactation. In this study, PTZ-induced generalized tonic contractions were suppressed in PND14 rat pups. However, the brain region(s) involved in PTZ-induced tonic contraction in pups was characterized by the loss of postural control and strong contractions in the extensor muscles of the fore and hind limbs with cyanosis, followed by generalized SE.

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PTZ-induced generalized tonic contraction was similarly suppressed in both 1-BP and Br groups.
Table 2. Br concentration in each group and hippocampal slices

| Sample                        | Br concentration (nM) |
|-------------------------------|-----------------------|
| PND14 brain of the 1-BP       | 1.3                   |
| exposure group                |                       |
| PND14 brain of the NaBr exposure group | 1.9               |
| Hippocampal slices            | 1.2                   |

Finally, in vitro, we confirmed that Br, comparable with the accumulated concentration in the brains of pups prenatally exposed to Br, suppressed PTZ-induced epileptiform activities in the CA1 area in the hippocampal slices (Fig. 4B and Table 2). Considering that we only observed epileptiform activity in 60% of the tested slices (Fig. 4A), hippocampal CA1 may be less responsive to PTZ-induced hyperexcitability, as was also reported in a previous study. Additionally, the occurrence rate of generalized tonic contraction have yet to be identified. To the best of our knowledge, only one mapping study has been reported on rat pups at PND10 and PND21, with no evidence from c-Fos mapping or magnetic resonance imaging studies using PND14 rat pups. However, the underlying mechanism(s) induced by Br during brain development warrant further investigation.

Developmental toxicity has been reported in rat dams exposed to NaBr through drinking water. Although their precise exposure conditions differed from ours, the inhibition of increased body weight in pups was the same as our current study findings. In addition, the decrease in brain weight, and changes in brain structure, correspond to the effects observed on postnatal development of pup brains.

We have also been investigating the usefulness of electro-physiological DNT and reported cases of prenatal valproic acid and 1-BP exposure. In the present study, we aimed to evaluate the developmental effects of Br accumulated in the brains of pups prenatally exposed to 1-BP and used the hippocampal slices to directly examine the effects of Br as significant field potentials could be evoked. Although further research on other chemicals is necessary, the results of electro-physiological DNT studies can provide a basis for future studies on DNT.

Certain limitations were noted in the current study. First, it is unclear whether the hippocampus has the highest level of responsiveness to injected PTZ. Hence, once it is determined which sections contain the highest responsiveness, slices should be obtained for further analysis. Second, the underlying mechanisms at cellular concentrations were not elucidated in the current study and, thus, requires further investigation.

As noted previously, the Japan Society for Occupational Health recommends an OEL of 0.5 ppm, whereas in the present study, considering three factors (i.e., difference in species, LOAEL of 700 ppm, and developmental neurotoxicity), the exposure limit was calculated as 0.7 ppm using a default uncertainty factor. Therefore, based on our experimental data, the OEL appears to be appropriate. However, further studies on developmental neurotoxicity, based on different approaches, are required for the establishment of a standardized human risk assessment system.

To allow for accurate biological monitoring, the relationship between chemical concentrations in the blood/urine and the brain should be examined in animal models, and subsequently adapted for humans, as targeted brain samples are not available in humans. Accordingly, a mathematical simulation study examining the relationship between the concentration of 1-BP in the blood and brain of rats, is underway. Once such techniques are adequately developed, we will be able to expand the findings to different kinds of chemicals that affect brain function.

In conclusion, Br, accumulated in the brain of pups prenatally exposed to 1-BP, affected neuronal excitability during lactation. Our findings may serve as a warning to women working in industries using Br. Meanwhile, xenobiotic Br is also found in medications, certain soft drinks, as well as dietary and herbal supplements containing bromide salt, which can cause bromism when consumed regularly. Although some of these products contain warning labels, the use of these labels has recently decreased. Nonetheless, several forms of bromide are readily available. Therefore, quantitative rating of adverse effects induced by complex exposure to chemicals is necessary, particularly in children and pregnant women.

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