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Original Article

Impacts of Age and Gender on Brain Edema in a Mouse Water Intoxication Model

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Brain edema causes abnormal fluid retention and can be fatal in severe cases. Although it develops in various diseases, most treatments for brain edema are classical. We analyzed the impacts of age and gender on the characteristics of a water intoxication model that induces pure brain edema in mice and examined the model's usefulness for research regarding new treatments for brain edema. C57BL/6J mice received an intraperitoneal administration of 10% body weight distilled water, and we calculated the brain water content by measuring the brain-tissue weight immediately after dissection and after drying. We analyzed 8-OHdG and caspase-3 values to investigate the brain damage. We also applied this model in aquaporin 4 knockout ($AQP4^-$) mice and compared these mice with wild-type mice. The changes in water content differed by age and gender, and the 8-OHdG and caspase-3 values differed by age. Suppression of brain edema by $AQP4^-$ was also confirmed. These results clarified the differences in the onset of brain edema by age and gender, highlighting the importance of considering the age and gender of model animals. Similar studies using genetically modified mice are also possible. Our findings indicate that this water intoxication model is effective for explorations of new brain edema treatments.

Key words: brain edema, water intoxication model, age, gender, AQP4

Brain edema is a disease in which the volume of the brain increases due to an abnormal accumulation of fluid in the brain parenchyma. It develops in multiple brain diseases such as head trauma, cerebral ischemia, and cerebral hemorrhage and in metabolic disorders such as liver failure. In severe cases, brain edema can result in death due to increased intracranial pressure and brain herniation. Although brain edema recovers with treatment of the primary disease, specific treatments for brain edema itself are sorely needed [1]. Current treatments for brain edema including osmotherapy, hyperventilation, and partial craniotomy have limitations, and it is thus important to establish an effective treatment method for brain edema itself.

Many pathogenic mechanisms of brain edema and its related molecules have been investigated with the use of brain edema model animals [1,2]. The water intoxication model, a commonly used model of brain edema, is known to reproduce hyponatremia caused by renal disease and excessive fluid intake, and it is a model that can induce cytotoxic brain edema without trauma [1]. The brain edema pathogenetic mechanism of the water intoxication model, which is created by an intraperito-

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neal administration of distilled water (10% of body weight), does not involve damage to the blood-brain barrier (BBB), and the edema is known to be due to the excessive influx of water into the cells due to a decreased extracellular Na^+ concentration [1]. Many studies have used water intoxication models, but most of those studies created models based on the animals' weight, without examining gender or age. There are differences in brain-injury mechanisms between human males and females, and it is known that after ~30 years of age, most human physiological processes begin to decline, and BBB function also changes [3].

We conducted the present study to investigate whether gender and age affect the formation and resolution of brain edema after water intoxication in a mouse model. Brain damage was investigated by an assessment of DNA damage and apoptosis at each age in the model. We also evaluated the impact of the knockout of aquaporin 4 (AQP4) on brain edema formation after water intoxication. AQP4 knockout ($AQP4^-$) is known to affect brain edema formation in other models, but the impact is model-dependent [4].

Materials and Methods

Animals. We purchases 5- to 52-week (wk)-old male and female C57BL/6J mice from CLEA Japan (Tokyo). All experimental procedures were conducted in accord with Japan's National Institutes of Health regulations and were approved by the Animal Research Committee of Kawasaki Medical School in compliance with the ARRIVE guidelines.

Water intoxication model mice. As a water intoxication model, sterile distilled water (DW) was injected intraperitoneally (i.p.; 10% of body weight) under isoflurane anesthesia to induce acute brain edema in mice at various ages [5]. At 0 h (no i.p. injection; control) and 1, 2, 3, and 6 h (each group n = 3) after the DW injection, the mice were sacrificed by cervical dislocation, and the brains were immediately dissected. The brains were placed on pre-weighed aluminum foil, and the pre-dried weight was measured within 2 min after sacrifice. The brains were then dried in a high-temperature dryer at 120°C for 24 h, and the brain water content (%) was calculated as the weight difference between before and after drying [6]. The cardiac blood serum osmolality was measured by SRL Laboratory (Tokyo).

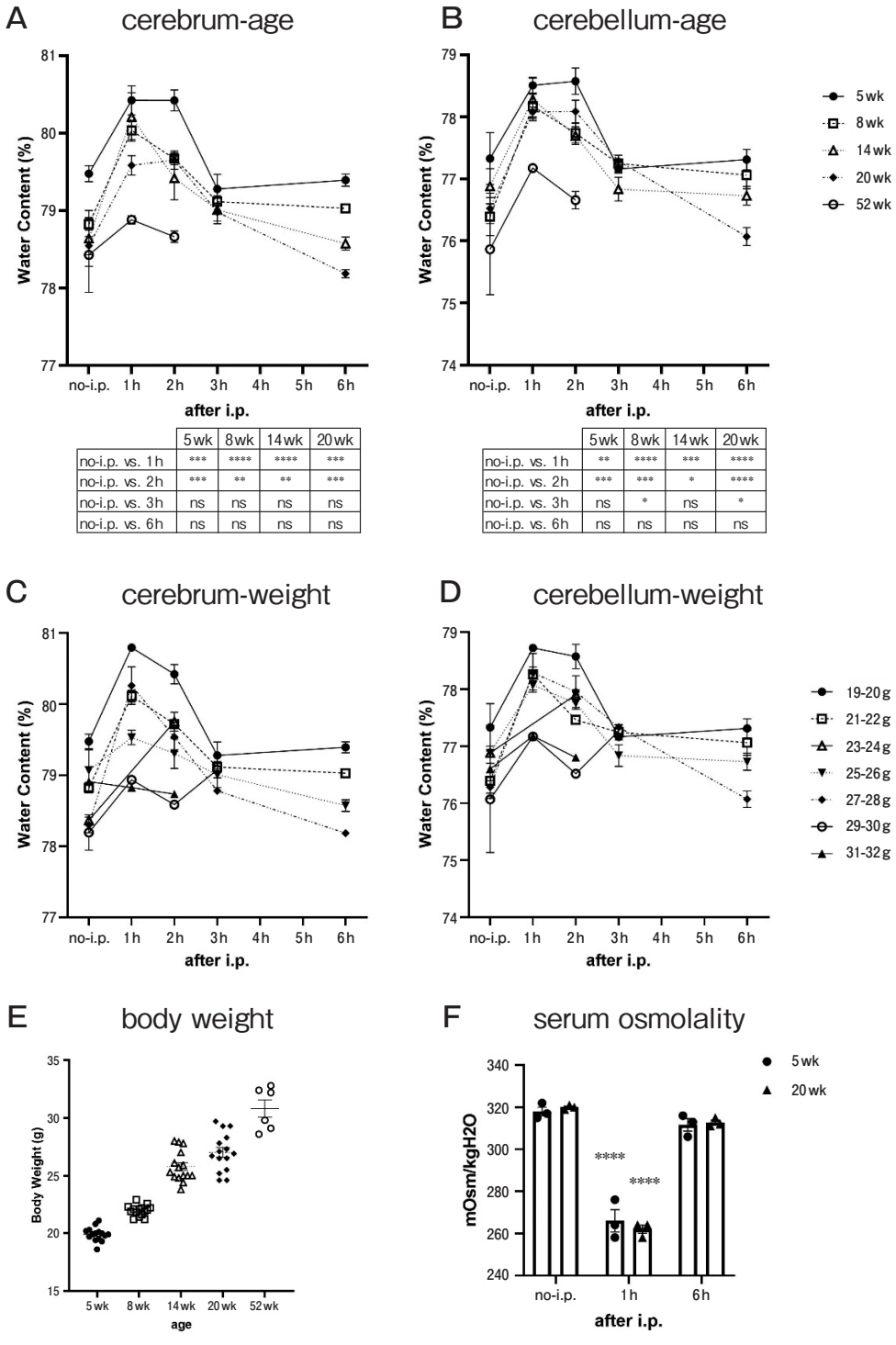
Measurement of DNA damage. For the investigation of DNA damage, we measured the 8-OHdG activity in the cerebrums of the no i.p. and 2 h groups (n = 3 each) with the use of the Highly Sensitive ELISA kit for 8-OHdG (KOG-HS10/E, Japan Institute for the Control of Aging, Shizuoka, Japan). For the investigation of apoptosis, we measured the protein levels of caspase 3 and cleaved caspase 3 (1 : 500, no. 19677-1-AP, Proteintech, Chicago, IL, USA) by conducting a western blot analysis (each group n = 6) [7]. Protein expression detected by chemiluminescence was quantified by the Image J software program and normalized to the expression of GAPDH (1 : 10000, #2118, Cell Signaling Technology, Beverly, MA, USA) by reprobing (stripping buffer, No. 21059, Thermo Fisher Scientific, Waltham, MA, USA) [8].

Aquaporin 4 (AQP4). The aquaporin 4 knockout mouse strain ($AQP4^-$ mouse, RBRC10053) was provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan [9]. As did the wild-type C57BL/6J mice, the $AQP4^-$ mice received an i.p. injection of sterile DW equivalent to 10% of body weight, and the cerebrum and cerebellum water content was measured 1 h later (8 week, males, no i.p. -$AQP4^-$, 1 h-$AQP4^-$, n = 3 each group). The expression level of AQP4 protein in the cerebrum was measured by the same method as caspase 3 (5-20 week, males, n = 4-6 each group) using AQP4 primary antibody (1 : 1000, no. 16473-1-AP, Proteintech).

Statistical analysis. All data are expressed as the mean ± SEM. A statistical analysis with multiple comparisons was performed by a two-way analysis of variance (ANOVA) followed by the Dunnett's (Fig. 1A, B and 3) or Tukey's (Fig. 1F, 2, 4, 5 and 6) post hoc test. Statistical significance was set as $p < 0.05$. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc, Boston, MA, USA).

Results

Impacts of age on water content. Regarding changes in the behavior of the mice, there was almost no movement for 1 h in any of the mice after the induction of water intoxication. By 3 h post-induction, movement was restored to the same level as that observed before the water administration (data not shown). Our analysis of the brain water content in male C57BL/6J mice of different ages revealed that the brains'



	5wk	8wk	14wk	20wk
no-i.p. vs. 1h	***	****	****	***
no-i.p. vs. 2h	***	**	**	***
no-i.p. vs. 3h	ns	ns	ns	ns
no-i.p. vs. 6h	ns	ns	ns	ns

	5wk	8wk	14wk	20wk
no-i.p. vs. 1h	**	****	***	****
no-i.p. vs. 2h	***	***	*	****
no-i.p. vs. 3h	ns	*	ns	*
no-i.p. vs. 6h	ns	ns	ns	ns

Fig. 1 Water content (%) of the cerebrum and cerebellum of male C57BL/6 mice. A, B: Changes in the brain water content after a single intraperitoneal (i.p.) injection of distilled water (DW) (n = 3; 52 week, n = 2). At all ages, the water content increased significantly by 2 h and returned to near-normal values after 3 h. The 5-week-old mice and 20-week-old mice showed no decrease in water content after 2 h post-injections. C, D: Replots by weight of the mice shown in panels A, B. F: The cardiac blood serum osmolarity at each time point: no age difference was observed. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

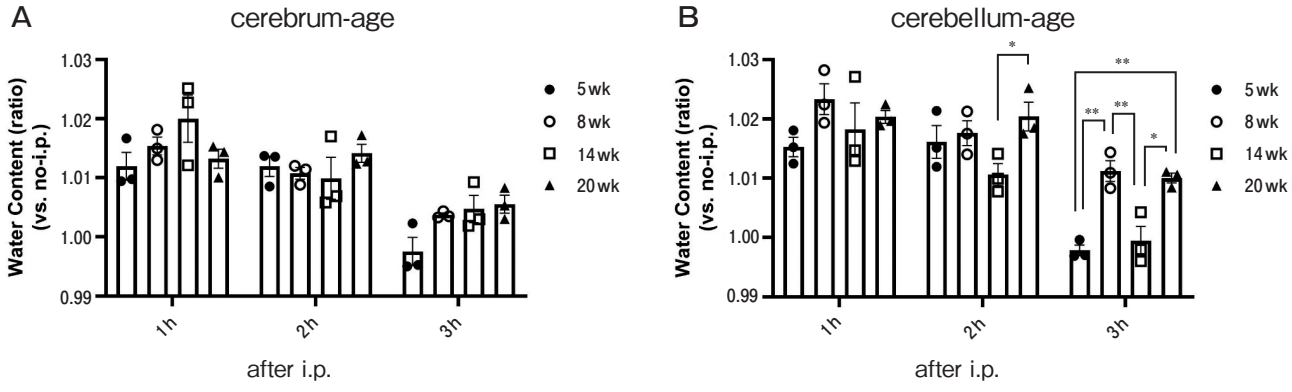
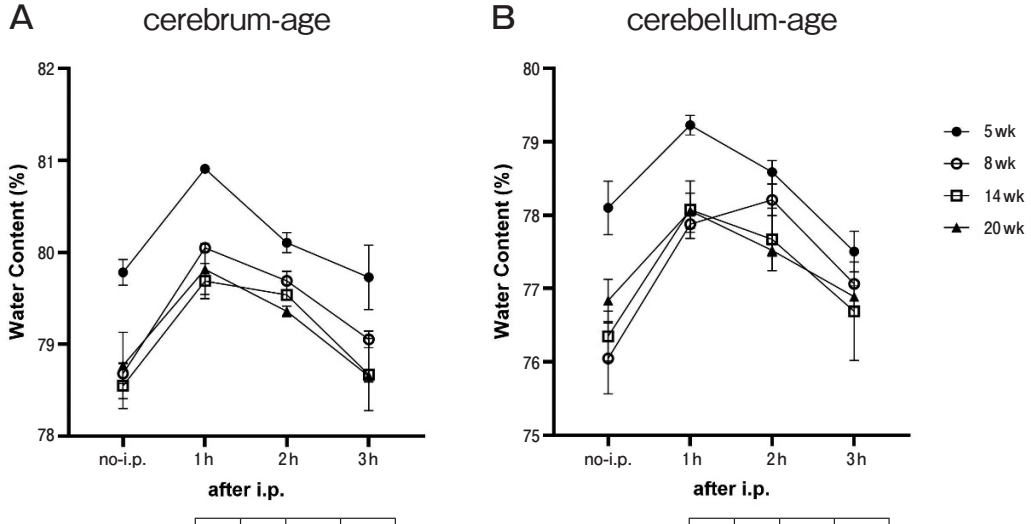


Fig. 2 Water contents (as a ratio) of the cerebrum and cerebellum of the male mice. The values shown in panels A and B of Fig. 1 were calculated as a ratio to 1 (as the control no-i.p. group). The rate of increase in the water content at 1 h after the DW injection was similar among most of the groups. In the cerebrum, however, only the brain water content in the 5 week mice returned to its original content after 3 h post-injection (A). The water content in the cerebellum of 5 week and 14 week mice returned to normal after 3 h post-injection, whereas that of the 8 week and 20 week did not decrease significantly (B). $*p<0.05$, $**p<0.01$.



	5wk	8wk	14wk	20wk
no-i.p. vs. 1h	***	****	****	***
no-i.p. vs. 2h	ns	***	***	ns
no-i.p. vs. 3h	ns	ns	ns	ns

	5wk	8wk	14wk	20wk
no-i.p. vs. 1h	**	****	****	**
no-i.p. vs. 2h	ns	****	**	ns
no-i.p. vs. 3h	ns	*	ns	ns

Fig. 3 The water content (%) of the cerebrum and cerebellum of the female C57BL/6 mice. The changes in brain water content after an i.p. injection of DW are shown (n=3). Two h after the injection, both the cerebral and cerebellar water contents had returned to normal levels in the females at the ages of 5 weeks and 20 weeks, but not in the males. The cerebellum of the 8-week-old females showed a further increase in water content after 2 h post-injection. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

water contents after the administration of DW showed a time frame similar to the level of locomotory activity (Fig. 1A, B). In both the cerebrum (Fig. 1A) and cerebellum (Fig. 1B), the brain water content was increased at 1 h after administration and returned to or was near to the original levels by 3 h.

In the 5-week-old male C57BL/6J mice, the water content remained at the same level at 1 h and 2 h, and it decreased after 2 h at 8 and 14 weeks. At 20 weeks, there was no decrease in the content after 2 h, as at 5 weeks. In the no-i.p. group, the water content of both the cerebrum and cerebellum was almost the same at all

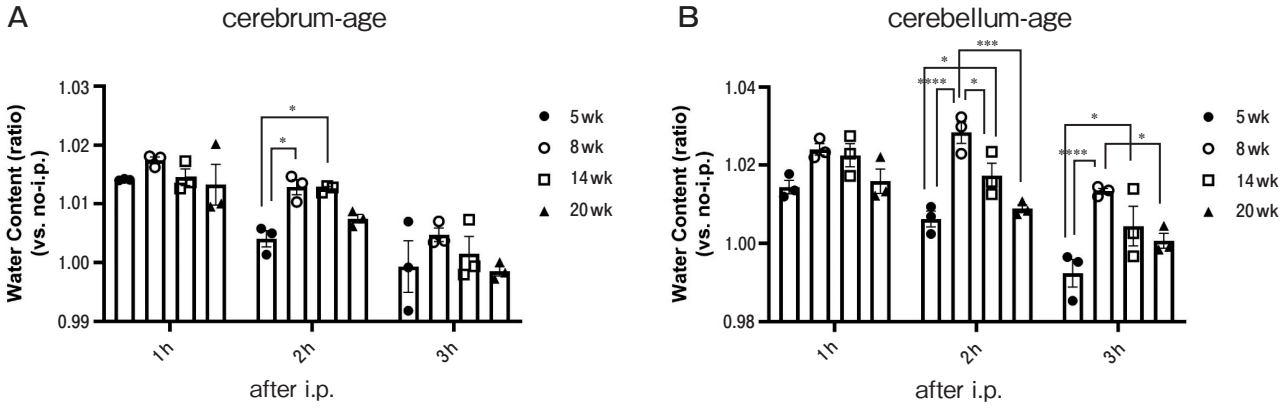


Fig. 4 The water content (as a ratio) of the cerebrum and cerebellum of the female C57BL/6 mice. The values shown in panels A and B of Fig. 3 were calculated as a ratio to 1 for the control no-i.p. group. The rate of increase in the water content at 1 h after an injection of DW was similar among most of the groups. At 2 h post-injection, the water content of the cerebrum recovered faster in the 5 week and 20 week groups of females compared to the male mice. In the cerebellum, the water content decreased significantly at all ages compared to 8 weeks, and this trend continued after 3 h post-injection. Both brains showed fluctuations that differed from those of the males. $*p<0.05$, $***p<0.001$, $****p<0.0001$.

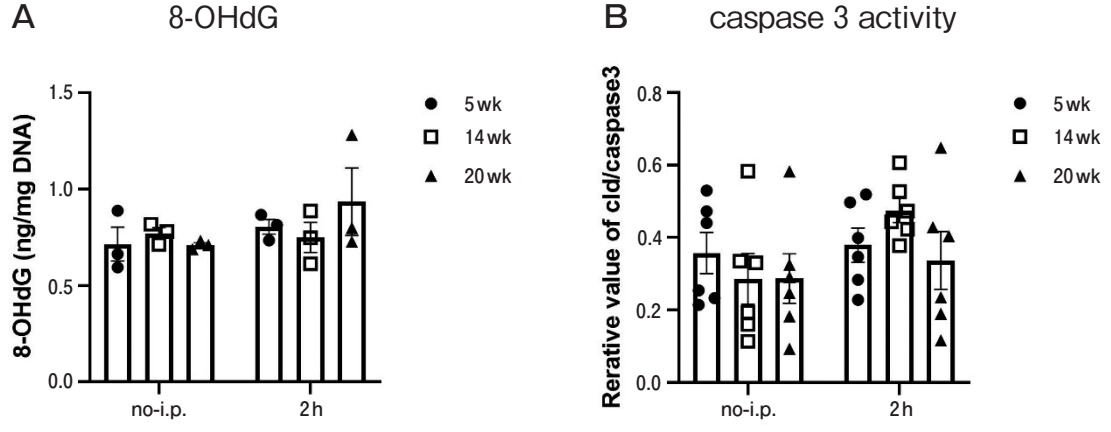


Fig. 5 The production of 8-OHdG and the activity of caspase 3 (cld/caspase 3) in the cerebrum of male C57BL/6J mice were measured (n=3, n=6, respectively) at 2 h after an i.p. injection of DW.

ages, but at 6 h after the DW administration, the water content decreased with the increase in the age of the mice (Fig. 1A, B). The serum osmolality level was significantly decreased at 1 h after the DW administration and returned to the original level 6 h later. The no-i.p., 1 h, and 6 h groups showed no age-related differences in serum osmolality (Fig. 1F). The data presented in panels a and b of Fig. 1 are grouped by weight in Figure 1C, D. The inclination of the graph of the water content increase at 1 h after the i.p. DW administration was similar by age (Fig. 1A, B) but varied by weight (Fig. 1C, D).

Figure 2 provides the calculated ratios of the results in Fig. 1A, B in relation to the no-i.p. group. At 1 h after the DW administration, the cerebrum showed an increasing trend in content compared to other ages at 14 weeks. At 2 h, the content was almost the same for all ages, and at 3 h, it returned to normal only at 5 weeks (one-way ANOVA, $p=0.0578$; 5 week vs. 20 week, Fig. 2A). In the cerebellum, on the other hand, the decrease in water content at 14 weeks was comparable to that at 5 weeks, which returned to normal after 3 h post-injection, and both the 8 week and 20 week groups' values remained significantly increased relative to the 5

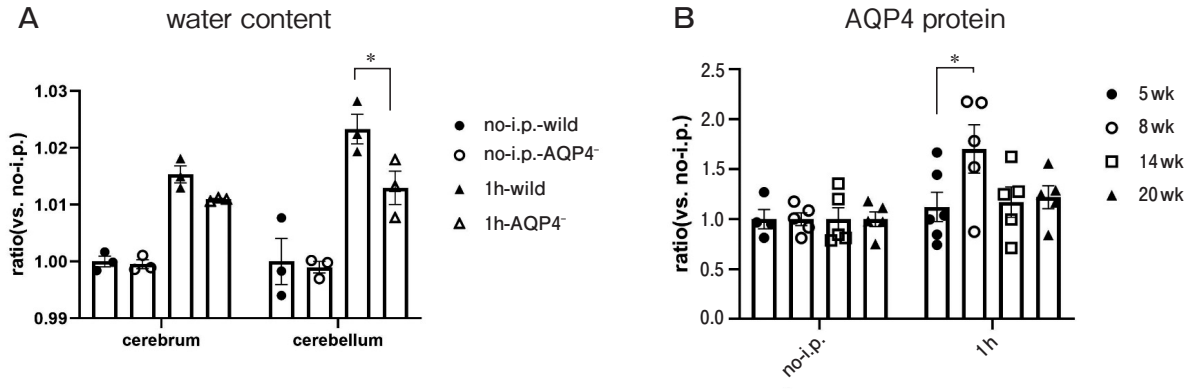


Fig. 6 The water contents in the brains of AQP4^{-} (knockout) mice. **A**, The water content in the cerebrum and cerebellum of 8 week wild-type and 8 week AQP4^{-} male mice at 1 h after the injection of DW was calculated as a ratio compared to the control no-i.p. group, and the degree of brain edema was compared (n = 3 each group). The cerebellums of the AQP4^{-} mice had significantly lower water content compared to those of the wild-type mice after water intoxication; **B**, In the cerebrum of the wild-type male mice (n = 4–6), the expression of AQP4 protein was increased by the DW administration, and it was significantly increased in the 8 week mice compared to the 5 week mice. $*p < 0.05$.

week and 14 week values (Fig. 2B).

Impacts of gender on water content. We examined the differences in brain edema by gender (Figs. 3, 4, n = 3 in all groups). In the female cerebrum, the water content began to decrease after 2 h at the ages of 5, 8, and 20 weeks. However, the 14 week mice retained a high volume of brain edema (Fig. 3A). In the murine female cerebellum, the water content increased further after 2 h only at 8 weeks (Fig. 3B). Figure 4 shows the calculated ratios of the results in Fig. 3 in relation to the no-i.p. group. At 1 h, the rate of the increase in the water content after the infusion of DW was similar in all groups in the cerebrum, and it tended to be higher in the 8 week and 14 week groups in the cerebellum. After 2 h, the cerebral water content recovered quickly at 5 and 20 weeks, and especially at 5 weeks, the water content decreased significantly compared to 8 and 14 weeks. There was a significant decrease in the cerebellum at all ages compared to 8 weeks, a trend that continued after 3 h.

Impacts of age on brain edema-induced DNA damage. To investigate whether the induction of brain edema was associated with cell damage and cell death, we examined the production of 8-OHdG and the activity of caspase 3 (cld/caspase 3) in the cerebrum of 5-, 14-, and 20-week-old male mice at 2 h after DW administration (Fig. 5). The cld/caspase 3 values were significantly increased in the 14 week group compared to the control no-i.p. group (*t*-test, $p = 0.0347$). The values of 8-OHdG did not differ among the different ages.

AQP4 in the water intoxication model. The brain water content of male AQP4^{-} mice was compared with that of wild-type male mice in the same water intoxication model. As shown in Fig. 6A, the increases in the brain — especially in the cerebellum water content of AQP4^{-} mice — at 1 h after the DW administration was smaller than that in the wild-type mice. At the same time point, the expression level of cerebral AQP4 protein in the wild-type male mice tended to be increased at all ages and was significantly increased at 8 weeks (Fig. 6B).

Discussion

The brain edema pathogenesis mechanism of the water intoxication model created by an intraperitoneal administration of DW at 10% of the body weight includes the speculation that BBB damage does not have an important role, and that this model causes an excessive inflow of water into the cells due to a decrease in extracellular Na^{+} concentration [1]. However, changes in the BBB due to physiological aging begins in humans in their 30s [2], and age-related physiological changes appear to be involved in the age-related differences in water influx and discharge function that we observed in this study. We first examined the water content of the brain after the administration of DW in male mice by

age. What was interesting about the experimental results was that the water content at 2 h after the DW injection differed depending on the age of the mice. This indicates that the peak timing of water inflow varies with age. In addition, there was no significant difference in serum osmolality at 6 h after the DW administration (Fig. 1F), and the decrease in water content differed depending on age (Fig. 1A, B). It is possible that not only changes in renal function [10, 11] but also changes in water homeostasis in the central nervous system, such as the drainage mechanism of the brain, are affected by aging.

We also observed that the grouping by body weight showed no regularity in water content changes (Fig. 1C, D). The variation in body weight increases with aging (Fig. 1E), resulting in a mix of mice of the same weight but various ages. We thus infer that changes in physical and physiological functions due to aging were not clearly expressed. When the brain water content is expressed as a ratio to the control no-i.p. group, the rate of increase in the water content and the time required to return to normal levels differed greatly depending on the age of the mice, and it also differed between the cerebrum and cerebellum (Fig. 2). The reasons for these results merit further study.

Our examination of the differences by gender revealed several interesting findings. As shown in Fig. 4, the rate of water increase in the female mice was highest at 8 weeks for both the cerebrum and cerebellum at all time points. The transition of water content in the females at 2 and 3 h after the DW injection is a phenomenon not seen in the males (Figs. 2, 4). These results suggest that even among mice at the same age, the water dynamics of the central nervous system differ by gender. Differences in damage between males and females have been reported in a rat water intoxication model [12], a traumatic brain injury model [13], and human brain injuries [14]. Since differences in therapeutic effects are often observed between men and women in clinical practice, it is necessary to further explore gender differences in research as well.

We compared the production of 8-OHdG and the activity of caspase 3 in the cerebrum of male mice to determine whether there are differences in brain tissue damage dependent on age (Fig. 5). As a marker of DNA oxidative stress, 8-OHdG is an indicator of DNA damage. We compared the activity of caspase 3, an indicator of apoptosis, by calculating the ratio of caspase 3 to the active form, *i.e.*, cleaved caspase 3 (cld/caspase 3). At 2 h after the induction of edema, the values of 8-OHdG did not differ significantly among the mice of different ages, and the 14 week group had increased caspase 3 activity. In this model, only brain edema is induced without traumatic injury; the difference in damage is thus largely due to age. One possible reason for the difference is the existence of senescent cells [15]. It was also reported that the deletion of REST (repressor element 1-silencing transcription factor) in the aging brain causes age-related neurodegeneration [16]. These reports suggested that the degree of brain damage caused by increased water content due to brain edema may vary at each age.

As shown in Fig. 5, the apoptotic cascade in the model used in our present study was indeed enhanced at 2 h after the induction edema, but as shown in Fig. 1, the brain edema itself had almost resolved 3 h later, suggesting that the enhancement is not sustained to a level that is sufficient to actually lead to cell death. The model thus induces brain edema to the extent that DNA is affected, but unlike other models, it is not affected by other factors such as tissue damage and is suitable for exploring treatments that purely control water dynamics.

To explore whether the present water intoxication model might also be useful in studies of altered water handling in genetically modified mice, we examined aquaporin 4 (AQP4), which is known to be associated with brain edema (Fig. 6). AQP4 is involved in the pathophysiology of brain edema associated with various types of brain injury and disease, and in a brain edema model of water intoxication or hyponatremia, AQP4 knockout mice clearly had less severe brain edema [17]. AQP4 has also been observed to promote astrocyte swelling ("cytotoxic swelling") and the reabsorption of extracellular edema fluid ("vasogenic edema") during the formation of brain edema [4]. We compared changes in brain water content in AQP4 knockout mice with those in wild-type mice after water intoxication, and as shown in Fig. 6A, the $AQP4^{-}$ mice had lower brain water content at 1 h after administration of DW compared to the wild-type mice, which is consistent with a role of AQP4 in parenchymal cell swelling. In the cerebrums of the wild-type male mice aged 5 to 20 weeks, the expression of AQP4 protein tended to increase upon the induction of brain edema, and the degree of this increase varied depending on the age of

the mice (Fig. 6B). AQP4 is a member of a large family of water channel proteins that allow a bidirectional transport of water across the phospholipid bilayer of the plasma membrane [18], and our results suggest that the excessive influx of water into the central nervous system may promote AQP4 synthesis in order to enhance efflux mechanisms. The model is suitable for investigating the involvement of various target substances without the influence of other pathological conditions and is useful for various basic studies on brain edema.

In conclusion, physiological conditions in vivo continue to change in various ways due to aging and gender differences. Based on our present findings, we propose that (*i*) animal models of water intoxication for brain edema research must consider the age and gender of the animals, and (*ii*) the model used in the present study is useful for examining these physiological differences.

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