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# Vasopressin escape and memory impairment in a model of chronic syndrome of inappropriate secretion of antidiuretic hormone in mice

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**Abstract.** Recently, chronic hyponatremia, even mild, has shown to be associated with poor quality of life and high mortality. The mechanism by which hyponatremia contributes to those symptoms, however, remains to be elucidated. Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) is a primary cause of hyponatremia. Appropriate animal models are crucial for investigating the pathophysiology of SIADH. A rat model of SIADH has been generally used and mouse models have been rarely used. In this study, we developed a mouse model of chronic SIADH in which stable and sustained hyponatremia occurred after 3-week continuous infusion of the vasopressin V2 receptor agonist 1-desamino-8-D-arginine vasopressin (dDAVP) and liquid diet feeding to produce chronic water loading. Weight gain in chronic SIADH mice at week 2 and 3 after starting dDAVP injection was similar to that of control mice, suggesting that the animals adapted to chronic hyponatremia and grew up normally. AQP2 expression in the kidney, which reflects the renal action of vasopressin, was decreased in dDAVP-infused water-loaded mice as compared with control mice that received the same dDAVP infusion but were fed pelleted chow. These results suggest that "vasopressin escape" occurred, which is an important process for limiting potentially fatal severe hyponatremia. Behavioral analyses using the contextual and cued fear conditioning test and T-maze test demonstrated cognitive impairment, especially working memory impairment, in chronic SIADH mice, which was partially restored after correcting hyponatremia. Our results suggest that vasopressin escape occurred in chronic SIADH mice and that chronic hyponatremia contributed to their memory impairment.

*Key words*: SIADH mice, Chronic hyponatremia, Vasopressin escape, Aquaporin 2, Memory impairment

**HYPONATREMIA** is among the most common electrolyte abnormalities encountered in clinical practice [1]. Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) is the most frequent cause of hyponatremia in hospitalized patients [2, 3]. Recently, many lines of evidence have shown that chronic hyponatremia, even when mild, is associated with poor quality of life (QOL) and high mortality. It has been reported that chronic hyponatremia is associated with falls, fracture, osteoporosis, and neurocognitive impairment [4-8]. In clinical trials, correction of chronic hyponatremia led to improvement of self-assessed mental health status (vitality, social functioning, calmness, and sadness) [9]. However, little is known about the underlying mechanism and whether hyponatremia itself can induce those symptoms.

Animal models that mimic human diseases are very important for understanding disease pathogenesis and pathophysiology [10]. Verbalis and colleagues developed a rat model of SIADH that is widely used: moderate to severe hyponatremia is induced by continuous injection of the V2 receptor (V2R) agonist 1-desamino-8-D-arginine vasopressin (dDAVP) and feeding of a liquid diet to produce chronic water loading [11-13]. However, because genetic engineering techniques are more prevalent in the mice than rats, a mouse model of chronic SIADH would be useful to investigate the function of specific genes in the pathophysiology of SIADH. In addition, many experimental settings are designed only for mice, especially for behavioral analysis in the field of neuroscience. However, research employing a mouse model of SIADH has rarely been reported. Bouchat and

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associates observed osmotic demyelination in a model of SIADH in mice [14, 15]. Hyponatremia was induced in the study using dDAVP infusion and liquid diet feeding, and was rapidly corrected by hypertonic saline 4 days later [14, 15]. We were unable to identify any reports using a mouse model of chronic SIADH sustained for several weeks. Therefore, we intended to generate a chronic SIADH model in mice.

It is well known that vasopressin escape is an important phenomenon in humans and rats which limits severe hyponatremia to prevent further water retention and hyponatremia that could otherwise be fatal [16-20]. It has been reported that escape from the action of vasopressin-induced antidiuresis (vasopressin escape) occurs a few days after water loading in a rat model of SIADH, and that the onset of vasopressin-escape is associated with a decrease in the expression of vasopressin-regulated water channel aquaporin-2 (AQP2) in the renal collecting duct, a hallmark of vasopressin escape, despite high circulating vasopressin levels [18, 21, 22]. However, there have been no reports of vasopressin escape accompanied by a decrease in AQP2 expression in the kidney in a chronic mouse model of SIADH.

Acute hyponatremic brain swelling is responsible for causing neurologic symptoms [23, 24]. Conversely, brain swelling is absent in chronic hyponatremia [25] and the basis for the neurologic and cognitive symptoms in this context is less clear. Using a model of chronic SIADH in rats, we have reported that chronic hyponatremia induces gait disturbances, memory impairment, and reduction of long-term potentiation in the hippocampus [26]. We also reported that elevation of the extracellular glutamate concentration in the hippocampus of chronically hyponatremic rats may be associated with memory impairment [26].

The aim of this study was to develop a mouse model of chronic SIADH in which chronic hyponatremia is stably sustained for several weeks. Additionally, using the chronic SIADH mouse model, we investigated AQP2 expression in the kidney, a hallmark of vasopressin escape, and memory impairment by behavioral analysis.

## Materials and Methods

### *Animal experiments*

All of the procedures were performed in accordance with the institutional guidelines for animal care at Fujita Health University, Japan, which, in turn, conform to the National Institutes of Health animal care guidelines and were approved by the Institutional Animal Care and Use Committee of Fujita Health University (Approval number AP19051).

Male 8-week-old C57BL/6J mice (Chubu Science Materials, Nagoya, Japan) were housed in a standard animal facility kept at constant temperature (23 ± 2°C) with a 12/12 hour light/dark cycle. Mice had *ad libitum* access to standard chow (MF®; Oriental Yeast Co., Ltd., Tokyo, Japan; 23.1 g protein, 5.1 g fat, 58.1 g carbohydrate, trace minerals and vitamins per 100 g) and tap water until the induction of hyponatremia according to previously described methods with some modifications [13, 26] (Fig. 1). Briefly, osmotic minipumps (Alzet® model 1004; Durect Corporation, Cupertino, CA) containing dDAVP (10 mg/mL; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) or saline were implanted subcutaneously into mice under isoflurane anesthesia. Injection rates of dDAVP were as follows, 0.03 ng/h in Group 4, 0.3 ng/h in Group 5, and 0.5 ng/h in Group 3 and 6. Mice in Group 1 and 2 were injected with saline (Fig. 1). After 1 day of dDAVP administration, mice in Group 2, 4, 5 and 6 were water loaded by substituting their daily feed with a liquid formula (Isocal Plus®; Mead-Johnson, Evansville, IN) and 10% glucose mixed at a 1:1 ratio (2.825 g protein, 3.45 g fat, 12.65 g carbohydrate, trace minerals and vitamins per 100 mL). Mice in Group 1 and 3 were fed with a pelleted chow diet (Fig. 1). For correction of chronic hyponatremia, the osmotic minipumps were removed and the mice were fed with a pelleted chow diet for 4 weeks followed by liquid diet for 1 week before T-maze testing.

### *Biochemical analysis of blood and urine*

Blood was obtained by facial vein puncture with an animal lancet (MEDIpoint, Inc., Mineola, NY) before and 1, 2, 3 weeks after starting dDAVP injection. Mice were housed in metabolic cages (#TP85-M; Toyo-Riko

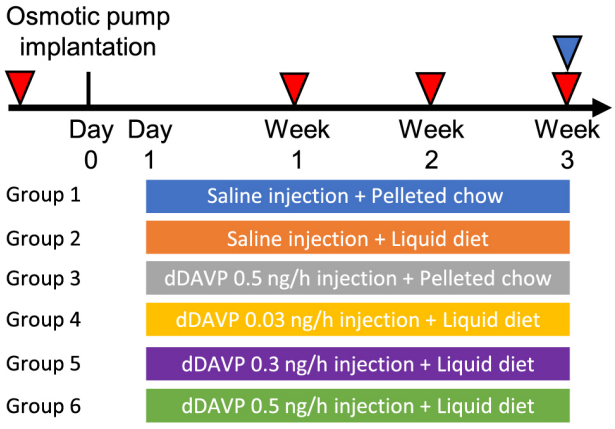


**Fig. 1** Experimental protocol.
Injection of saline or 1-deamino-8-D-arginine vasopressin (dDAVP) was started on day 0 and mice in Group 2, 4, 5, and 6 were fed a liquid diet from day 1. Blue triangle, urine collection; red triangle, blood sampling.

Co., Ltd., Tokyo, Japan) and 24-h urine output was collected over 3 weeks after starting dDAVP infusion. The serum sodium concentration in mice used for behavioral analysis was measured by Fingraph® (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Other biochemical analyses of blood and urine were performed at Oriental Yeast Co., Ltd.

### *Western blotting*

Whole kidney lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed by immunoblotting with the following primary antibodies: anti-aquaporin 2 (254–271) rabbit polyclonal antibody (1:500; Merck KGaA, Darmstadt, Germany) and monoclonal anti-β-actin antibody produced in mouse clone AC-74 (1:2,000; Merck KGaA). Bound antibodies were detected by using appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000–3,000; Dako Denmark A/S, Glostrup, Denmark) and Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont, UK). Western blots were quantified using ImageJ software. Optical density values were normalized to the β-actin signal.

### *Behavioral tests*

Most of the behavioral tests were performed as previously described [27], unless otherwise noted.

**Contextual and cued fear conditioning test**

A contextual and cued fear conditioning test was conducted as previously described [28, 29]. To assess fear related learning and memory, each mouse was placed in a test chamber (33 × 25 × 28 cm) with a stainless-steel grid floor (0.2 cm diameter, spaced 0.5 cm apart) on a white board (O'HARA & Co., Tokyo, Japan) illuminated at 100 ± 5 lx and allowed to explore freely for 2 min. A conditioned stimulus (CS; 55 dB white noise) was presented for 30 s, followed by a mild foot shock (2 s, 0.3 mA), which served as the unconditioned stimulus (US). Two more CS-US pairings were presented with a 2-min inter-stimulus interval. Each mouse underwent context testing, conducted 1 day after conditioning, in the same chamber for 300 s. Cued testing with an altered context was conducted 1 day after conditioning using a triangular box (33 × 29 × 32 cm) (O'HARA & Co., Tokyo, Japan), which was located in a different room. The test chamber was illuminated at 30 ± 5 lx. A tone stimulus for the cued test was applied for 180 s. During the test, images were captured at 1 frame per second. In the conditioning and context test of 2 control mice, a white board was not inserted under the stainless-steel grid floor. These data were included in the analysis, since it is thought to have little effect on the test.

**T-maze test**

To assess working memory, we employed a forced alternation task test without food deprivation using a T-maze apparatus (O'HARA & Co., Tokyo, Japan) [30]. Each trial consisted of first and second runs. On the first run of each trial, a mouse was forced to choose one of the arms of the T shaped-maze. After the mouse stayed for more than 10 s, sliding doors that separated the arms and the connecting passageways opened so that the mouse could return to the starting compartment through the connecting passageways. The mouse was then given a 3 s delay, followed by a free choice between the arms of the T shaped-maze. If the mouse chose the opposite direction from the previous forced run, it was counted as a correct response, and if the mouse chose the same direction as the previous run, it was counted as an incorrect response. Each mouse underwent 10 trials (1 session) per day, for 3 (before correction of hyponatremia) or 4 (after correction of hyponatremia) consecutive days. During the T-maze test after correction of hyponatremia, a delay (3, 30, 60, 120 s) was applied between forced and free choice runs after 4 sessions. Data acquisition, control of sliding doors, and data analysis were performed using ImageTM software.

**Data analysis**

All behavioral data were automatically collected using application software, which were derivatives of the ImageJ program optimized for each type of test by Tsuyoshi Miyakawa (available through O'HARA & Co., Tokyo, Japan).

### *Statistical analysis*

Results are expressed as the mean ± SEM. Statistical analyses were performed by paired *t*-test, unpaired *t*-test, one-way ANOVA followed by Tukey's test, and two-way repeated measures ANOVA, as indicated in the figure legends. *P* values less than 0.05 were considered to be significant.

## Results

### *Induction of chronic hyponatremia in mice*

We induced chronic hyponatremia in mice by continuous injection of the vasopressin V2 receptor agonist dDAVP and liquid diet feeding. To evaluate the effect of dose dependency of dDAVP on serum sodium concentration, we infused different concentrations of dDAVP in mice (Fig. 1), *i.e.* Group 4; at a rate of 0.03 ng/h, Group 5; at a rate of 0.3 ng/h, Group 6; at a rate of 0.5 ng/h. Three groups served as controls (Fig. 1):, *i.e.*, Group 1; continuous saline injection and pelleted chow feeding, Group 2; continuous saline injection and liquid diet feeding, Group 3; continuous dDAVP injection at a rate of

0.5 ng/h and pelleted chow feeding. During 3 days after the start of dDAVP injection, mice in Group 5 and 6 drank little water and liquid diet, and afterwards, liquid diet consumption of Group 5 was similar to that in Group 2 and 4 (Supplemental Fig. 1). Liquid diet intake of Group 6 was slightly but significantly decreased compared to that of Group 4 (Supplemental Fig. 1). Body weight was significantly decreased in Group 6 compared with Group 1, 2, 3, and 4, and in Group 5 compared with Group 1 one week after starting dDAVP injection (Fig. 2A). Afterwards, body weight was comparable among the 6 groups. Urine volume was significantly increased in the liquid diet group (Group 2, 4, 5, 6) compared with the pelleted chow group (Group 1, 3) (Fig. 2B). Of note, urine volume in Group 6 was significantly decreased compared with Group 2, 4, and 5 one week after the start of dDAVP injection and was significantly increased compared to Group 2 and 4 three weeks after the start of dDAVP injection.

### *Serum and urine parameters of chronic SIADH mice*

The serum sodium concentration in Group 5 and 6 was significantly reduced compared with Group 1, 2, 3, and 4 one week after starting dDAVP injection (Fig. 3A). The serum sodium concentration in Group 4 was significantly lower than that of Group 1, 2 and 3. There was no significant difference in serum sodium levels between Group 5 and 6. The same tendency persisted for the remainder of the study. Plasma osmolality in Group 5 and 6 was also significantly reduced compared with Group 1, 2, 3, and 4 at all timepoints (Fig. 3B). Plasma osmolality in Group 4 was significantly reduced compared with Group 1 and 2 at week 1; with Group 1 at week 2, and with Group 1, 2, and 3 at week 3. Blood urea nitrogen (BUN) in the liquid diet group (Group 2, 4, 5 and 6) was significantly decreased compared with the pelleted chow groups (Group 1 and 3) (Fig. 3C). At week 3, BUN in Group 3 was significantly decreased compared with that of Group 1, and that of Group 2 was significantly increased compared with Group 4, 5, and 6. Serum creatinine concentrations were comparable among the 6 groups at week 1 and 2 (Fig. 3D). At week 3, the serum creatinine concentration in Group 1 was significantly increased compared with that of Group 3, 5, and 6. The serum uric acid concentration in Group 3 and 5 was significantly reduced compared with that of Group 1 and 2, and the concentration in Group 6 was significantly reduced compared with that of Group 1, 2, and 4 at week 3 (Fig. 3E). We measured urine parameters at week 3, when chronic hyponatremia was sustained stably and mice were thought to have adapted to chronic hyponatremia. Urine osmolality was approximately 2,690 ± 421.2 mOsm/kg $H_2O$ in the group that received saline injection and pelleted chow (data not shown), while it was approximately 517 ± 29.7 mOsm/kg $H_2O$ in the group that received dDAVP injection at a rate of 0.5 ng/h with liquid diet feeding. We analyzed urine of only the liquid diet groups (Group 2, 4, 5, and 6). Because there was a large difference in urine volume, urine osmolality, and sodium intake between the pelleted chow groups (Group 1 and 3; 4.51 ± 0.36 mg) and liquid diet group (24.4 ± 0.90 mg), a comparison of urine data between the solid diet and liquid diet group would not have been meaningful. In addition, the volume of urine from many of the mice in the pelleted chow groups was too low to enable analysis. The urine sodium concentration in Group 6 was significantly increased

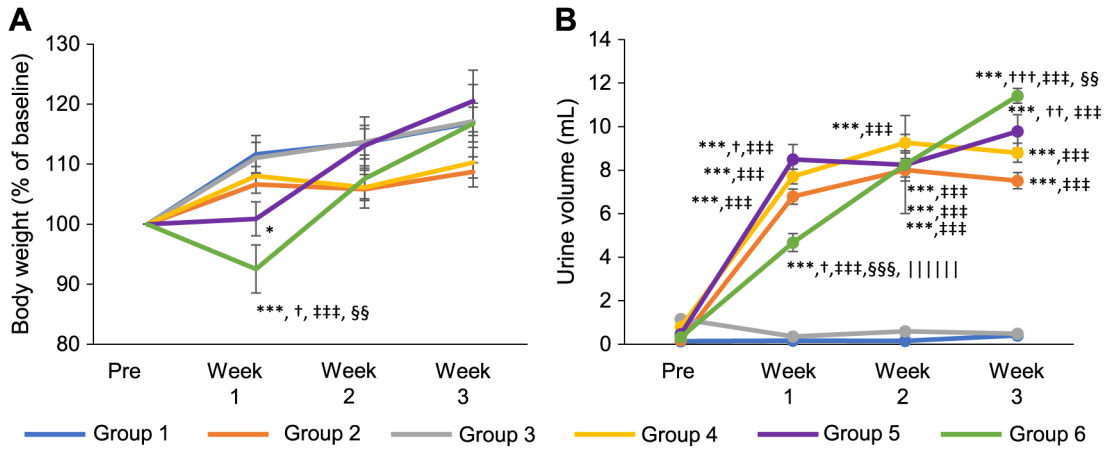


**Fig. 2** Body weight (A) and urine volume (B) of chronic hyponatremic mice.

$n$ = 5–8 for each group. One-way ANOVA followed by Tukey's test was used to determine statistical significance. *, $p < 0.05$ *vs.* Group 1; ***, $p < 0.001$ *vs.* Group 1; †, $p < 0.05$ *vs.* Group 2; ††, $p < 0.01$ *vs.* Group 2; †††, $p < 0.001$ *vs.* Group 2; ‡‡‡, $p < 0.001$ *vs.* Group 3; §§, $p < 0.01$ *vs.* Group 4; §§§, $p < 0.001$ *vs.* Group 4; ||||||, $p < 0.001$ *vs.* Group 5.

compared with that of Group 4 (Fig. 4A). Unexpectedly, the urine sodium concentration of Group 2 was significantly increased compared with Group 4 and 5. Urine osmolality in Group 5 and 6 was significantly increased compared with Group 2 and 4 (Fig. 4B). There was no significant difference in daily sodium intake among 4 of the groups (Fig. 4C). However, daily urinary sodium excretion in Group 6 was significantly increased compared with Group 2, 4, and 5 (Fig. 4D). Therefore, sodium balance in Group 6 was significantly decreased compared with Group 2 and 4 (Fig. 4E).

### *Decreased expression of renal AQP2 in a chronic hyponatremic state*

It has been reported that escape from vasopressin-induced antidiuresis is attributable, at least in part, to a vasopressin-independent decrease in aquaporin-2 water channel expression in the renal collecting duct in the SIADH rat model [18, 31]. Therefore, we measured the levels of AQP2 protein in the whole kidney, a hallmark

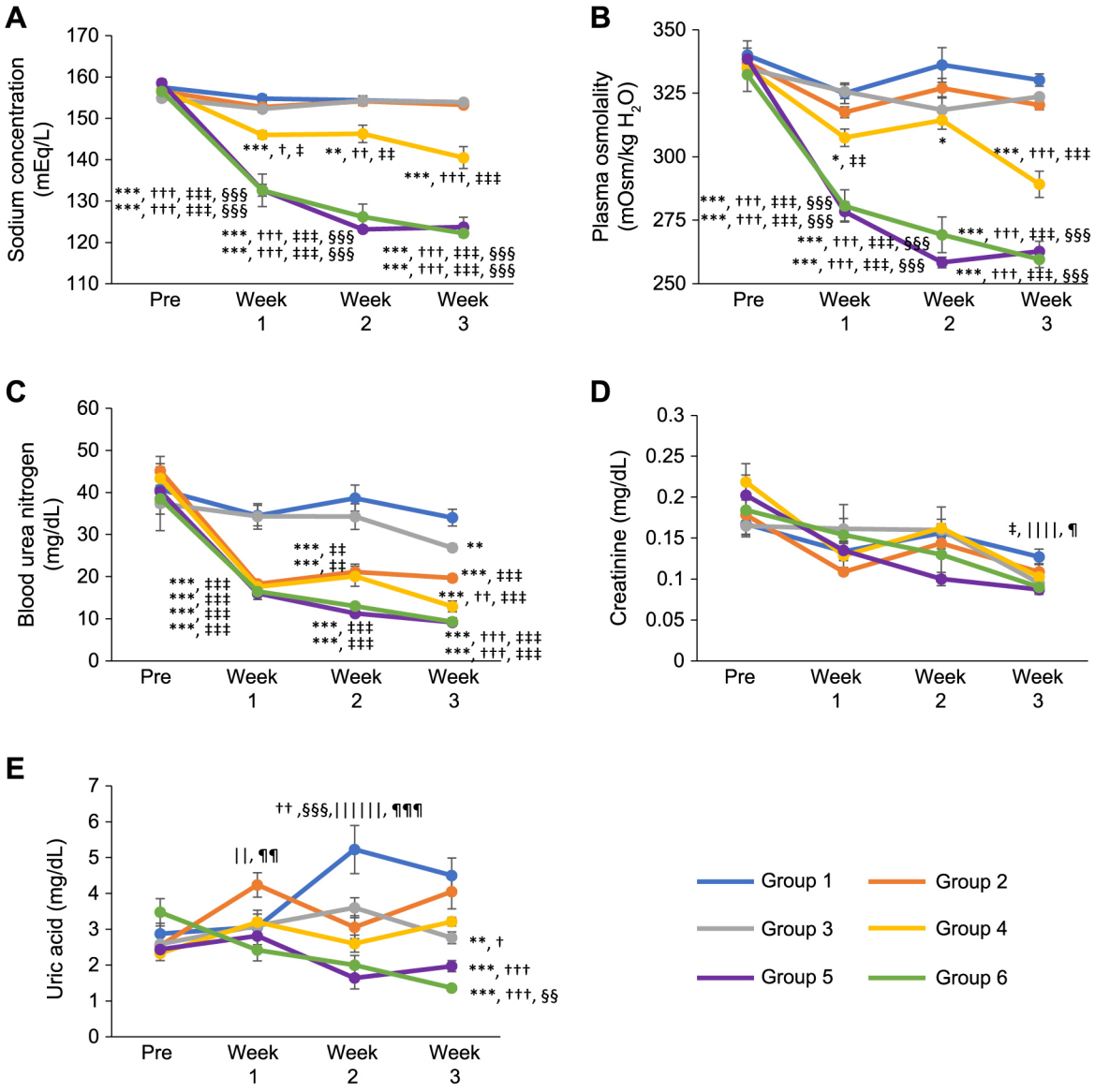


**Fig. 3** Biochemical analysis of blood from chronic hyponatremic mice.

(A) Serum sodium concentration. (B) Plasma osmolality. (C) Blood urea nitrogen. (D) Serum creatinine. (E) Serum uric acid. $n$ = 5–8 for each group. One-way ANOVA followed by Tukey's test was used to determine statistical significance. *, $p < 0.05$ *vs.* Group 1; **, $p < 0.01$ *vs.* Group 1; ***, $p < 0.001$ *vs.* Group 1; †, $p < 0.05$ *vs.* Group 2; ††, $p < 0.01$ *vs.* Group 2; †††, $p < 0.001$ *vs.* Group 2; ‡, $p < 0.05$ *vs.* Group 3; ‡‡, $p < 0.01$ *vs.* Group 3; ‡‡‡, $p < 0.001$ *vs.* Group 3; §§, $p < 0.01$ *vs.* Group 4; §§§, $p < 0.001$ *vs.* Group 4; ||, $p < 0.05$ *vs.* Group 5; |||, $p < 0.01$ *vs.* Group 5; ||||||, $p < 0.001$ *vs.* Group 5; ¶, $p < 0.05$ *vs.* Group 6; ¶¶, $p < 0.01$ *vs.* Group 6; ¶¶¶, $p < 0.001$ *vs.* Group 6.

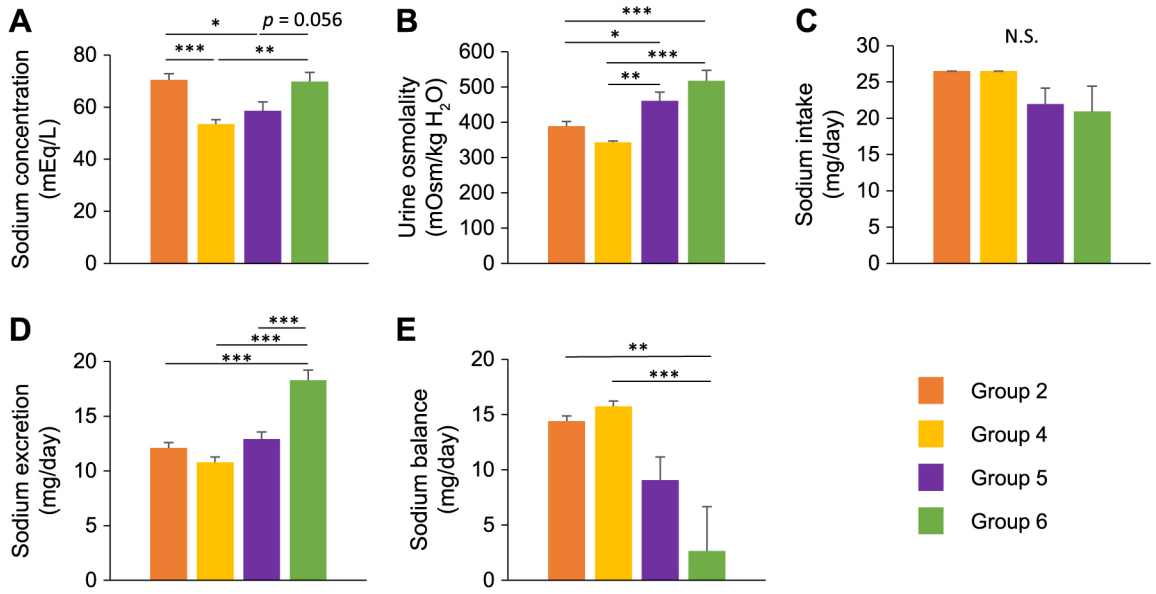


**Fig. 4** Biochemical analysis of urine and sodium balance in chronic hyponatremic mice 3 weeks after starting dDAVP infusion.
(A) Urine sodium concentration. (B) Urine osmolality. (C) Sodium intake. (D) Sodium excretion. (E) Sodium balance. $n = 5–8$ for each group. One-way ANOVA followed by Tukey's test was used to determine statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

of vasopressin escape [18, 31], of each group of mice 3 weeks after starting dDAVP injection. The renal AQP2 expression level increased in a dose-dependent manner in the dDAVP-infused groups which were all fed the same liquid diet (Group 2, 4, 5, 6; Fig. 5A and B). As shown in Fig. 5C and D, renal AQP2 expression in Group 2 was significantly decreased compared with that of Group 1, possibly owing to decreased endogenous AVP secretion. Exogenous dDAVP injection at a rate of 0.5 ng/h significantly increased the expression of AQP2 (Fig. 5C and D, comparison of Group 1 and 3). Renal AQP2 expression in Group 6 was significantly decreased compared with that of Group 3, despite the same rate of dDAVP infusion. These results indicate that vasopressin escape also occurred in our chronic hyponatremic mouse model, as previously reported in a rat model.

### *Cognitive function is impaired in the chronic hyponatremic mouse model*

We have previously shown that chronic hyponatremia impairs memory in a rat model [26]. Therefore, we tested whether memory is also impaired in our chronic hyponatremic mouse model. We used Group 4 mice as controls and Group 5 mice as the hyponatremic group because all of them were infused with dDAVP and fed a liquid diet, and the serum sodium concentration in Group 5 was significantly reduced compared with that of Group 4. We performed a contextual and cued fear conditioning test to assess fear-conditioned memory 7 weeks after starting dDAVP injection (Fig. 6). Neither the response to foot shocks, percentage of the time of freezing caused by the foot-shock, nor the distance traveled during electrical foot shocks were significantly different between Group 4 and 5 (Fig. 6A, B and E), indicating that chronic hyponatremia had no effect on the sensitivity to the electric foot-shock. On the following day, the mice were returned to the conditioning chamber and allowed to explore the chamber without either the CS or UCS (context test). The percentage of the freezing time and distance traveled were not significantly different between Group 4 and 5 during the total test period ($p = 0.33$ for the percentage of freezing and $p = 0.060$ for the distance traveled) (Fig. 6C and F). However, during the first 2 minutes of observation, there was a significant difference in the distance traveled between Group 4 and 5 (Fig. 6C and F), suggesting that chronic hyponatremia impairs contextual fear memory, as previously reported [26]. The cued test was conducted after the context test. There was no significant difference in the percentage of freezing time and distance traveled during pre-CS ($p = 0.44$ for the percentage of freezing and $p = 0.62$ for the distance traveled) and CS ($p = 0.14$ for the percentage of freezing and $p = 0.051$ for the distance traveled), but Group 5 showed a significantly reduced percentage of freezing time and distance traveled only during the middle 1 min of the CS (Fig. 6D and G).

We also performed the T-maze test to assess the effect of chronic hyponatremia on working memory 5 weeks

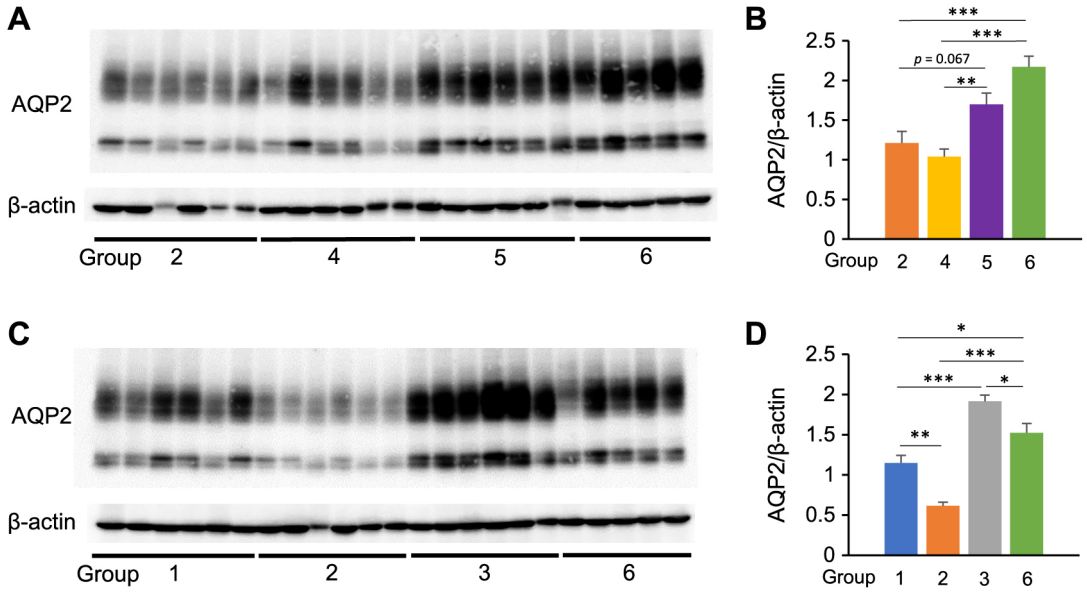


**Fig. 5** AQP2 protein levels in the kidney of chronic hyponatremic mice.
(A and C) Immunoblots of Group 2, 4, 5, and 6 (A) and Group 1, 2, 3, and 6 (C) mice. (B and D) Quantification of the data presented in A (B) and C (D). $n$ = 5–6 for each group. One-way ANOVA followed by Tukey's test was used to determine for statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

after starting dDAVP injection. We employed a protocol for a forced alternation task test without food deprivation. Group 5 had a significantly decreased percentage of correct responses in sessions 1 through 3 compared with Group 4 (Fig. 7A) indicating that chronic hyponatremia impairs working memory. There were no significant differences in latency to complete a session nor in distance traveled (Fig. 7B and C).

Finally, we investigated whether correcting chronic hyponatremia reverses working memory impairment. We corrected chronic hyponatremia by removing the dDAVP infusion pumps and feeding the mice pelleted chow for 4 weeks, and the mice were then fed liquid diet for 1 week. After the correction, there were no significant differences in serum sodium concentration between Group 4 and 5 (151.01 ± 0.68 mEq/L in Group 4 and 152.93 ± 0.67 mEq/L in Group 5, $p$ = 0.064). Group 5 mice showed a slightly but significantly decreased percentage of correct responses in sessions 1 through 4 compared with Group 4 mice (Fig. 7D). There were no significant differences in latency to complete a session nor in distance traveled (Fig. 7E and F). To increase the difficulty of the task, delay periods (3, 30, 60, or 120 s) were then applied between forced and free choice runs. Under these conditions, though a two-way repeated measures ANOVA failed to show a significant difference in the percentage of correct responses between Group 4 and 5 ($p$ = 0.12), Group 5 mice showed a significantly decreased percentage of correct responses during 3-s and 30-s delay (Fig. 7G). Next, we compared the mean percentage of correct responses of Group 5 mice before and after correcting chronic hyponatremia (Fig. 7H). After the correction, Group 5 mice showed a significantly increased percentage of correct responses *versus* before the correction. These data indicate that correcting chronic hyponatremia significantly, but not completely, restores memory impairment.

## Discussion

We developed a model of chronic SIADH in mice in which chronic hyponatremia was stably sustained, and the body weights of chronic hyponatremic mice were comparable with those of control mice without hyponatremia. Using the chronic SIADH model mice, we found that vasopressin escape (*i.e.* significant reduction of AQP2 expression in the kidney) occurred in the chronically adapted hyponatremic state. In addition, results of behavioral analysis using contextual and cued fear conditioning testing and T-maze testing showed that chronic hyponatremia induced memory impairment in mice.

Body weight was comparable among the 6 groups in the second and third weeks after starting dDAVP injection, suggesting that mice had adapted to hyponatremia and grew up healthy. The decrease in body weight that was observed in the group that received dDAVP infusion at a rate of 0.3 and 0.5 ng/h during the first week as compared with other groups was probably due to the signifi-

cant decrease in serum sodium and plasma osmolality during the same period. Decreased appetite and a chronic catabolic state several days after water load and dDAVP or AVP infusion were reported in previous studies using other models of experimental hyponatremia [11, 32]. In our mouse model, it is possible that hyponatremic mice were catabolic until 1 week after starting dDAVP infusion. However, hyponatremic mice and normonatremic mice subsequently had similar nutritional states. To control the nutritional state more strictly, paired feeding should be employed in a future experiment.

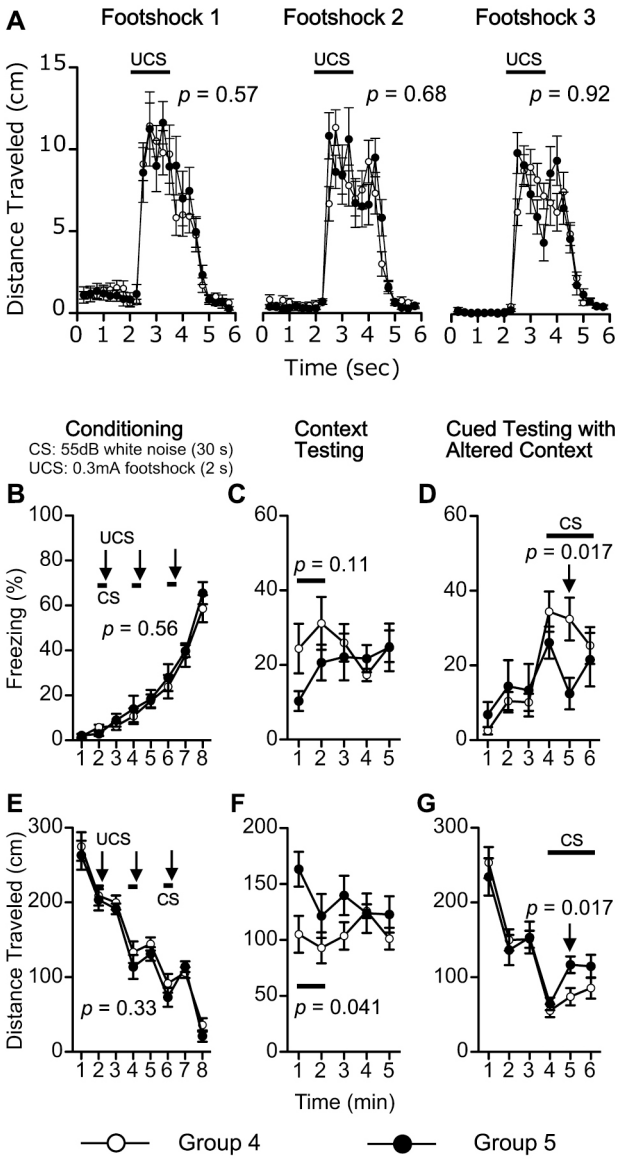


**Fig. 6** Assessment of fear-conditioned memory in chronic hyponatremic mice.

(A) Distance traveled before, during and after each foot shock in the conditioning phase. (B) Percentage of freezing response during the conditioning phase. (C and D) Percentage of freezing response on the context (C) and cued (D) tests at 1 day after conditioning. (E) Distance traveled during the conditioning phase. (F and G) Distance traveled in the context (F) and cued (G) tests at 1 day after conditioning. $n$ = 16 in Group 4 and $n$ = 11 in Group 5. Unpaired $t$-test was used to determine statistical significance of percentage of freezing response and distance traveled during each minute in the cued test 1 day after conditioning. Other statistical analyses was performed using two-way repeated measures ANOVAs.

As to the blood biochemical data, serum sodium and plasma osmolality levels were significantly decreased in the dDAVP infusion groups, and the effect was observed dose-dependently at infusion rates of 0.03, 0.3, and 0.5 ng/h. In addition, low serum sodium levels and low plasma osmolality were sustained at week 3. Uric acid levels were decreased in the groups that received dDAVP infusion at 0.3 and 0.5 ng/h as compared with the other groups. Urine osmolality was increased in the groups that were infused at rates of 0.3 and 0.5 ng/h. The blood and urine data for groups that received dDAVP infusion at 0.3 and 0.5 ng/h group were compatible with the phenotype of human SIADH. Taken together with the body weight data, the blood and urine data strongly suggest that we developed a model of chronic SIADH in mice that hyponatremia sustained stably for 3 weeks. In the present study, serum sodium levels were approximately 122 mEq/L in the group infused with dDAVP at 0.5 ng/h and fed liquid diet, while the level was approximately 154 mEq/L in the group infused with dDAVP at 0.5 ng/h and fed pelleted chow, which were similar to the levels in controls (*i.e.*, saline injection and pelleted chow). Importantly, these data support the validity of fluid restriction therapy, a gold standard therapy, for hyponatremia due to SIADH. Despite high levels of circulating vasopressin, water restriction can prevent or improve hyponatremia, while too much water intake may worsen hyponatremia. There are several limitations to the present study. For example, it is curious that the urine sodium concentration in the group fed a liquid diet and not infused with dDAVP group was higher than that of group fed liquid diet that received dDAVP infusion at 0.03 ng/h. This is probably duo to the difficulty in collecting urine and measuring urine sodium levels in mice.

Chronic infusion of AVP has been reported to increase AQP2 expression in the collecting duct in Brattleboro rats and Sprague-Dawley rats [33, 34], resulting in water reabsorption. We showed that AQP2 expression in the kidney was increased in a dose-dependent manner by infusion of dDAVP at rates of 0.03–0.5 ng/h at week 3 after starting dDAVP injection. To our knowledge, this is the first demonstration of a dDAVP dose-dependent increase in AQP2 expression due to chronic hyponatremia in an animal model of chronic SIADH. It is well known that vasopressin escape is an important phenomenon in human and rats for limiting severe hyponatremia

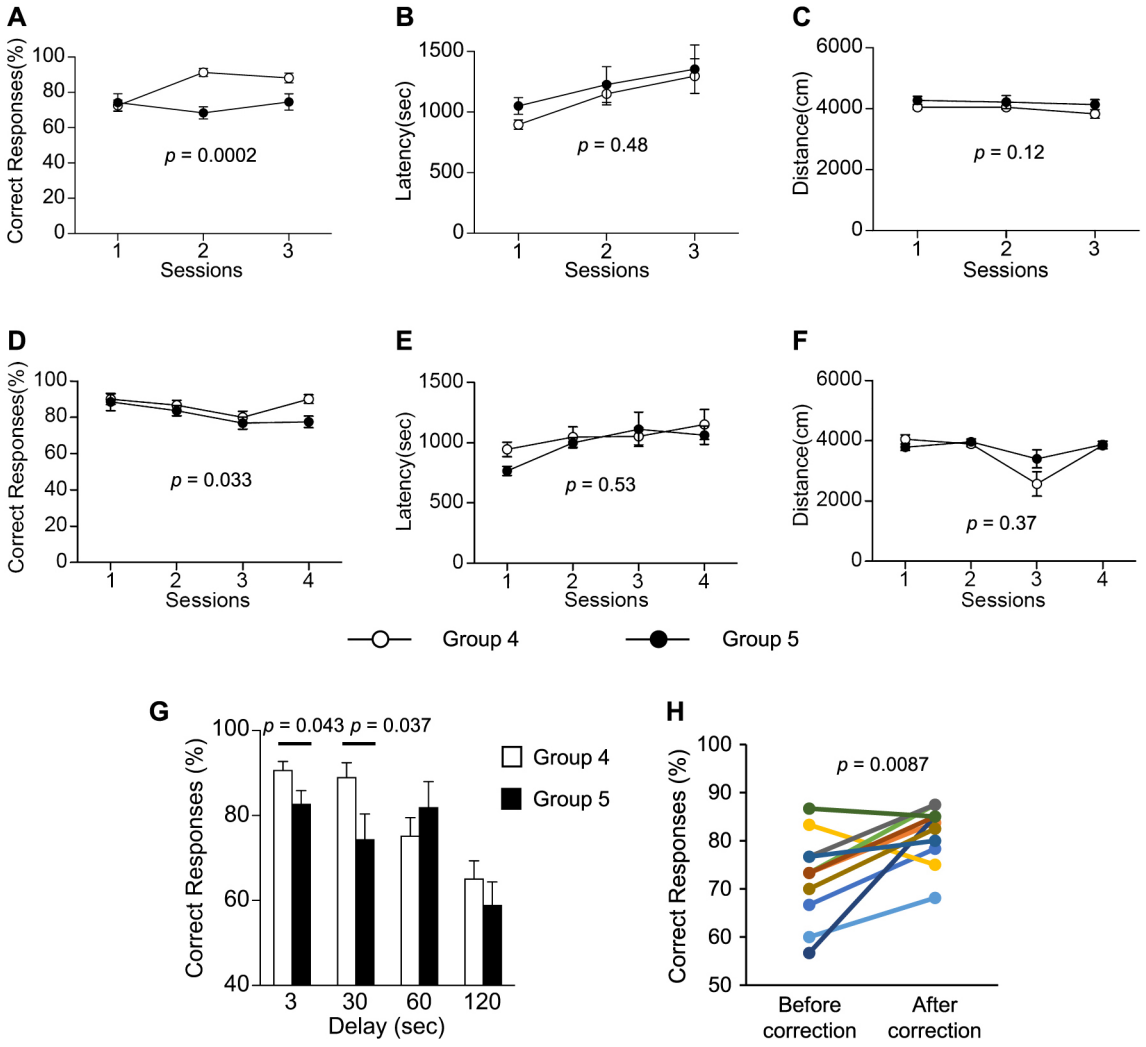


**Fig. 7** Assessment of working memory in chronic hyponatremic mice before and after correcting chronic hyponatremia.
(A–C) T-maze test of chronic hyponatremic mice before correcting chronic hyponatremia. $n$ = 16 in Group 4 and $n$ = 12 in Group 5. (A) Percentage of correct responses. (B) Latency to complete a session. (C) Distance traveled. (D–G) T-maze test of chronic hyponatremic mice after correcting chronic hyponatremia. $n$ = 15 in Group 4 and $n$ = 11 in Group 5. (D) Percentage of correct responses. (E) Latency to complete a session. (F) Distance traveled. (G) Percentage of correct responses in the T-maze test with delay periods between forced and free choice runs. Unpaired $t$-test was used to determine statistical significance of correct responses of mice in the same delay period. (H) Comparison of percentage of correct responses in Group 5 mice before and after correcting chronic hyponatremia. Paired $t$-test was used to determine statistical significance. (A–G) Two-way repeated measures ANOVA was used to test for statistical significance unless otherwise noted.

to prevent further water retention and hyponatremia [16-20, 35]. Several lines of evidence have shown that AQP2 levels are significantly decreased, despite high dDAVP levels from continuous infusion, a few days after liquid diet feeding, which correlates with the onset of escape from the antidiuretic action (*i.e.* vasopressin escape) [18, 21, 22, 31, 36]. In the present study, chronic hyponatremic mice, in which serum sodium levels were approximately 122 mEq/L at week 3 after starting dDAVP infusion at 0.5 ng/h, showed decreased AQP2 expression in the kidney relative to mice that were fed pelleted chow and received the same dose of infused dDAVP. Thus, this is the first study to report a decrease in AQP2 expression in the kidney, a hallmark of vasopressin escape, in a mouse model of chronic SIADH. We did not observe AQP2 expression at the onset of escape from antidiuresis a few days later after water loading with dDAVP infusion. Precise measurement of urine volume is more difficult in mice than in rats, and thus analysis of the onset of escape from vasopressin-induced antidiuresis is also more difficult. However, vasopressin escape in the chronic hyponatremic state is important in preventing further serious reduction in serum sodium levels. An important finding in our study was the

significant increase in daily urinary sodium excretion and the significant decrease in sodium balance in chronic hyponatremic mice as compared with mice that did not receive or received lower-dose dDAVP (Fig. 4D, E). It has been reported that natriuresis occurs following free-water retention in animal models of vasopressin escape [37, 38]. Natriuresis is thought to relate to the hyponatremia. The regulation of aldosterone and sodium cotransporter/channel activity in relation to natriuresis has been reported in a model of vasopressin escape in rats [39, 40], but not in mice. The molecular mechanism of the natriuresis is not yet fully understood. Our SIADH mice model may be useful for studying the mechanism of vasopressin escape in chronic SIADH.

In the present study using a mouse model of chronic SIADH, contextual fear memory impairment was observed until 2 minutes after being placed in conditioning cage 1 day after foot shock, and thereafter seemed to disappear in context texting within the contextual fear conditioning paradigm. Similarly, we previously reported that rats in a chronic hyponatremic state showed fewer freezing responses until 2 minutes after being placed in conditioning cage 1 day after foot shock [26], indicating that chronic hyponatremia impairs fear-related associative memory, especially in the initial setting. Other groups have reported significantly reduced step-through latency in a passive avoidance test in hyponatremic rats [41]. In addition, to our knowledge, this is the first report involving the use of the T-maze test in an SIADH animal model. We found that chronic hyponatremia impaired spatial working memory. We previously reported that chronic hyponatremic rats showed impaired recognition memory in a novel-object recognition test [26]. In terms of reversibility, it is important to assess whether the correction of hyponatremia may reverse the memory impairments. We previously reported that rats whose serum sodium levels were corrected to 131 mEq/L from 120 mEq/L following treatment with the vasopressin V2R antagonist tolvaptan showed no significant differences in memory compared with control normonatremic rats [26]. The study, however, was not considered to be a bona fide correction study [42]. The present investigation showed that the memory impairment initially assessed in the hyponatremic state was then restored in the corrected normonatremic state by using same animals, indicating that the correction of hyponatremia improved the memory impairment that accompanied hyponatremia. In terms of the methodology of correcting hyponatremia, in the present study hyponatremia was corrected by removing the dDAVP-loaded osmotic minipumps. In contrast, in a previous study using a rat model of chronic SIADH, hyponatremia was corrected with the vasopressin V2 receptor antagonist tolvaptan [26]. In addition, water restriction is a gold standard therapy for SIADH in clinical settings. Therefore, further studies are needed to evaluate how the method used to correct hyponatremia affects the improvement of neurocognitive impairment.

It is possible that dDAVP itself could influence the behavior. However, we previously worked on the issue of direct effects of dDAVP on memory in a rat model [26]. Normonatremic dDAVP-infused rats were induced by injecting dDAVP and feeding them a high-salt liquid diet. These normonatremic dDAVP-infused rats showed no significant changes in memory as assessed by novel-object recognition test and contextual fear conditioning test, when compared with control normonatremic rats without dDAVP infusion that were fed with liquid diet. These results indicated that injected dDAVP did not affect the behaviors. In the present study, we used Group 4 mice as normonatremic controls and Group 5 mice as the hyponatremic group for behavioral analysis. Mice in Group 4 and 5 were infused with dDAVP at a rate of 0.03 ng/h or 0.5 ng/h, respectively. In addition, most of the central actions of vasopressin are mediated *via* a V1 receptor. dDAVP is a V2 receptor-selective agonist. Taken together, dDAVP infusion itself is thought not to influence memory impairment, while chronic hyponatremia causes memory impairment.

Many lines of evidence show that vasopressin enhances learning/memory [43-45]. Recently, it was reported that activation of endogenous AVP neurons *via* the DREADD system decreased food intake [46]. Therefore, following vasopressin infusion, different behaviors could be caused through the V1 receptor [47, 48]. Previously, vasopressin was injected in animals to generate chronic hyponatremia due to SIADH. However, presumably due to the difficulty in inducing chronically stable and sustained hyponatremia with injection of vasopressin, dDAVP has been generally used instead to create chronic hyponatremic animals due to SIADH. Therefore, if vasopressin is used to induce hyponatremia, mice could exhibit behaviors different from those of hyponatremic mice infused with dDAVP. This point awaits clarification in the future.

As to the mechanism of memory impairment in chronic hyponatremia, we previously reported that long-term potentiation, the main neural mechanism involved in memory storage [49], was decreased in chronic hyponatremic rats [26]. During the adaptation of brain cells to hyponatremia, a process known as volume regulatory decrease, intracellular solutes including electrolytes and organic osmolytes are extruded [25]. Glutamate, an important excitatory neurotransmitter, is one such organic osmolyte [50, 51]. Using *in vivo* microdialysis, we previously showed that chronic hyponatremia increased extracellular glutamate levels in the hippocampus

and that low sodium levels decreased glutamate uptake by astrocytes in cultured cells. Since an excessive extracellular glutamate concentration has been demonstrated to impair LTP [52, 53], it is possible that increased extracellular glutamate levels in the hippocampus due to decreased glutamate uptake by astrocytes may lower LTP expression, resulting in memory impairment associated with chronic hyponatremia [26]. In addition, we reported that memantine, a noncompetitive N-methyl-D-aspartate receptor antagonist, improved electrophysiological dysfunction, including impaired basal synaptic transmission and long-term potentiation [54]. Therefore, memantine may be a candidate therapy for hyponatremia-associated memory impairment. In the present study, LTP expression and glutamate levels in the hippocampus was not evaluated. Further studies are needed to clarify the mechanism of hyponatremia-associated memory impairment in mice.

Though we observed both vasopressin escape and memory impairment in a chronic SIADH mouse model in this study, it is assumed that there is no direct relationship between vasopressin escape and memory impairment. However, to develop a model of chronic SIADH in mice for the investigation of the effect of chronic hyponatremia on behavior, we think that the phenomenon of vasopressin escape to limit potentially fatal severe hyponatremia would be required. In the present study, we were the first to report that vasopressin escape occurred 3 weeks after starting dDAVP injection in mice when the animals adapted to chronic hyponatremia and grew up normally. If vasopressin escape did not occur, we would not be able to investigate the effect of stably chronic hyponatremia on memory over a long period of time beyond 3 weeks.

In conclusion, our findings suggest that vasopressin escape (*i.e.*, the reduction of AQP2 expression in the kidney) occurred in the chronically adapted hyponatremic state, and that chronic hyponatremia induced memory impairment in mice. Together with our previous finding that chronic hyponatremia induces memory impairment in rats, the present results suggest that clinicians should pay attention to neurocognitive impairment in patients with chronic hyponatremia and careful correction of hyponatremia may improve QOL.

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## Author Disclosure Statement

The authors have nothing to disclose.

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