Activation of vasopressin neurons leads to phenotype progression in a mouse model for familial neurohypophysial diabetes insipidus

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AVP plays a pivotal role in the homeostasis of water balance, and a deficiency of AVP leads to diabetes insipidus (DI) characterized by polyuria (22). Familial neurohypophysial DI (FNDI) is a rare autosomal dominant disorder (23). More than 50 point mutations in FNDI have been reported in the AVP gene so far, with most mutations existing in the domain of neurophysin II (NPII) (11), which functions as a carrier protein of AVP (36). While mechanisms underlying progressive polyuria in FNDI have not yet been fully elucidated, we demonstrated recently (15) that aggregates had progressively accumulated in the ER of AVP neurons in parallel with the progressive polyuria in mice possessing a point mutation (Cys98stop) in the NPII gene, which causes FNDI in humans (26). These data suggest that the accumulations of aggregates in ER that were probably derived from AVP precursors are closely related to the pathogenesis of FNDI.

While the onset of polyuria is usually several months or years after birth (11), ages of the onset of DI vary even among FNDI patients with the same mutations (14, 24, 25, 33, 34, 38, 40), suggesting that environmental factors might also affect the phenotype. Si-Hoe et al. (39) reported that transgenic rats expressing mutant NPII (Cys98stop) showed no significant increases in urine volume until they had been subjected to intermittent dehydration for 6 wk, suggesting that progression of the phenotype in FNDI could be accelerated by stimulants for AVP synthesis and release.

To determine whether the activities of AVP neurons are related to the phenotype progression in the FNDI model, we have conducted two experiments in this study. In the first experiment, we administered desmopressin (dDAVP), a long-acting analog of AVP (13), to the FNDI mouse model to decrease AVP synthesis, and examined possible changes in the phenotype. In the second experiment, we examined whether the excess in Na intake might accelerate the phenotype in FNDI mice, as it was demonstrated that the Na intake stimulated AVP release (12, 42).

MATERIALS AND METHODS

Animals. One-month-old male FNDI model mice that were heterozygous for the mutant gene (Cys98stop) (15) and the age-matched control (C57BL6/J mice, Chubu Science Materials, Nagoya, Japan) were employed. They were maintained under controlled conditions (23.0 ± 0.5°C; lights on 0900-2100). All procedures were performed in accordance with the institutional guidelines for animal care at the Nagoya University Graduate School of Medicine and were approved by the Animal Experimentation Committee.

Effects of continuous administration of dDAVP on urine volume and water intake. For 30 days, 1-mo-old female mice were subcutaneously administered either 0.168 μg·kg−1·day−1 desmopressin ac-
etate (dDAVP; Kyowa Hakko, Tokyo, Japan) or a vehicle (0.2 M acetic acid) using Alzet osmotic minipumps (model 1002, pumping rate: 0.25 μl/h, duration: 14 days; Durect, Cupertino, CA). The minipumps were changed on days 10 and 20. Mice were housed in metabolic cages, and urine volume and water intake were monitored throughout the treatment as well as for 30 days after removal of the minipumps.

Effects of single administration of dDAVP on urine volume. Three-month-old female mice were subcutaneously administered either 0.168 μg/kg dDAVP dissolved in 0.2 M acetic acid (200 μl) or a vehicle at 0900, and changes in urine volume were monitored for 24 h.

Effects of continuous administration of dDAVP on hematocrit, blood Na, AVP mRNA expression, and aggregate formation in AVP cells. Two-month-old female mice were subcutaneously administered either 0.168 μg·kg⁻¹·day⁻¹ dDAVP or a vehicle with osmotic minipumps for 30 days. Blood and brain samples were collected on day 30.

0.2% or 2.0% Na diet. The FNDI or wild-type mice were divided into two groups; those given a 0.2% Na diet or 2.0% Na diet. The 0.2% Na diet rather than normal chow (0.6% Na) was chosen so as to facilitate the detection of differences in phenotypes between groups. Urine volume, water intake, food intake, urine Na, and urine AVP excretion were monitored until 7 mo of age using metabolic cages.

Measurements of urine osmolality. Urine osmolality was measured by an osmometer (Advanced Instrument, Norwood, MA).

Measurements of hematocrit. Blood was collected with heparin-coated microhematocrit capillary tubes. After centrifuging at 11,000 g for 5 min, the hematocrit levels were measured.

Measurements of blood Na. Blood (serum) Na levels were measured with an autoanalyzer (Hitachi, Tokyo, Japan).

Urine AVP measurement. Urine samples were directly diluted with assay buffer. All samples were assayed by a highly sensitive RIA kit (kindly provided by Mitsubishi Chemical, Tokyo, Japan). The sensitivity of the assay for AVP was 0.063 pg/tube, with < 0.01% cross-reactivity with oxytocin (28).

Brain collection for immunohistochemistry and in situ hybridization. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Abbott Laboratories, Abbott Park, IL) and transcardially perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). After fixation, brains were removed and immersed in cold fixative containing 4% paraformaldehyde in 0.1 mol/l phosphate buffer. All samples were assayed by a highly sensitive RIA kit (kindly provided by Mitsubishi Chemical, Tokyo, Japan). The sensitivity of the assay for AVP was 0.063 pg/tube, with < 0.01% cross-reactivity with oxytocin (28).

Brain collection for immunohistochemistry and in situ hybridization. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Abbott Laboratories, Abbott Park, IL) and transcardially perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). After fixation, brains were removed and immersed in the same fixative for 3 h at 4°C. The brains were kept in PBS containing 20% sucrose at 4°C for cryoprotection, then embedded in Tissue-Tek (Sakura Finetechical, Tokyo, Japan), and stored at −80°C until they were sectioned. Brains were cut at 14 μm on a cryostat at −20°C, and, according to the brain atlas (31), slices at −0.7 mm caudal from the bregma were thaw-mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan) and stored at −80°C until either in situ hybridization or immunohistochemistry.

Immunohistochemistry. Slides were washed in 0.1 mol/l PBS, followed by incubation in blocking solution (20% normal goat serum in PBS) for 30 min. All procedures were performed at room temperature (RT). The tissues were then incubated with anti-mutant antibody (15) at 1:1,000 solution in PBS with 0.3% Triton X-100 and 1% normal goat serum for 24 h at 4°C. The sections were rinsed with PBS for 15 min and incubated with either biotinylated goat anti-rabbit antibody at 1:200 in PBS for 3 h at RT. After being rinsed, tissues were incubated in avidin-biotin complex solution (1:100; Vector Laboratories) for 90 min at RT and then immersed in PBS containing 0.1% 3,3′-diaminobenzidine dihydrochloride (Sigma-Aldrich, St. Louis, MO). Antibody-binding sites were made visible by adding 0.004% hydrogen peroxide. The aggregates on both sides of SON in each mouse were counted, and the mean values were subjected to statistical analyses.

In situ hybridization. An AVP exonic probe was kindly provided by Dr. Harold Gainer (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD). Highly specific antisense probes were synthesized using 55 μCi ([35S]UTP and 171 μCi ([35S]CTP (PerkinElmer Life Sciences, Natick, MA), the Riboprobe Combination System (Promega, Madison, WI), 15 units Rnasin, 1 μg linearized template, and 15 units of SP6 RNA polymerase. After 60 min of incubation at 42°C, the cDNA template was digested with DNase for 10 min at 37°C. Radiolabeled RNA products were purified using quick-spin columns (Roche Diagnostics, Indianapolis, IN), precipitated with ethanol, and resuspended in 100 μl of 10 mM Tris·HCl, pH 7.5, containing 20 mM DTT. After thawing at RT, slides were fixed in 4% formaldehyde in PBS for 5 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-0.9% NaCl, pH 8, for 10 min at RT. Sections were then dehydrated in 70, 80, 95, and 100% ethanol, delipidated in chloroform, and hybridized overnight at 55°C with 2 × 10⁶ counts/min of 35S-labeled probes in 95 μl of hybridization buffer (50% formamide, 200 mM NaCl, 2.5 mM EDTA, 10% dextran sulfate, 250 μg/ml yeast tRNA, 50 mM DTT, and 1× Denhardt’s solution). After incubation, sections were subjected to consecutive washes in 4× standard saline citrate (SSC) for 15 min at RT and 50% formamide-250 mM NaCl containing DTT for 15 min at 60°C. After treatment with RNase A (20 μg/ml) for 30 min at 37°C, sections were washed with 2× SSC, 1× SSC, and 0.5× SSC for 5 min each at RT, followed by washes with 0.1× SSC to cool at RT and with 70% ethanol for 15 s. The slides were dipped in nuclear Kodak NTB2 emulsion (Kodak, Rochester, New York) and exposed for 12–24 h. To assist cellular localization of the hybridized signals, the emulsion-dipped sections were stained with cresyl violet. Any neuronal cross sections with grains of more than threefold in the background density were considered labeled.

Statistical analysis. All data are reported as means ± SE. Data were analyzed by one-way ANOVA, two-way ANOVA with repeated measures, or Student’s t-test. Post hoc comparisons were performed using Bonferroni’s analyses. Differences were considered statistically significant at P < 0.05. The number of mice was six in each group of wild-type and six to nine in each group of FNDI mice.

RESULTS

Effects of continuous administration of dDAVP on urine volume and water intake. Urine volume and water intake in the mice injected with a vehicle (control) were progressively increased (Fig. 1). On the other hand, urine volume and water intake in the mice injected with 0.168 μg·kg⁻¹·day⁻¹ dDAVP were significantly reduced compared with the control throughout treatment (Fig. 1). After the osmotic minipumps were removed on day 30, urine volume and water intake in the dDAVP group increased progressively, but remained significantly lower compared with the control until day 44 (Fig. 1).

Effects of single administration of dDAVP on urine volume. Urine volume was decreased in the mice injected with 0.168 μg/kg dDAVP compared with the control for 12 h (dDAVP: 1.4 ± 0.6% body wt, control: 7.7 ± 1.7% body wt, P < 0.05). However, 24 h after injection, there were no significant differences in urine volume between the dDAVP group and control (dDAVP: 32.7 ± 4.1% body wt, control: 34.8 ± 7.2% body wt).

Effects of continuous administration of dDAVP on hematocrit, blood Na, and AVP mRNA expression in the SON. Blood hematocrit was significantly lower in the mice injected with dDAVP for 30 days compared with the control (Fig. 2A). On the other hand, the blood Na levels did not differ between groups (Fig. 2B). The AVP mRNA expression levels in the SON were significantly reduced in the dDAVP group compared with the control (Fig. 2C). Representative photo-
graphs showing AVP mRNA expression in the SON are shown in Fig. 2D.

Effects of dDAVP administration on aggregate formation in AVP cells. While the size of the inclusion bodies in the SON were not different between groups (Fig. 3D), their numbers were significantly diminished in the dDAVP group compared with the control (Fig. 3C). Representative photographs depicting inclusion bodies in the SON are shown in Fig. 3, A and B. While some aggregates were not accompanied by immunostaining with NPII antibodies, this was probably because the massive aggregates occupied the cytoplasm in these neurons (15).

Fig. 2. Effects of continuous dDAVP administration on hematocrit, blood Na, and AVP mRNA expression in the supraoptic nucleus (SON). The mice were administered either dDAVP or a vehicle (control) for 30 days. Levels of hematocrit (A), blood Na (B), and AVP mRNA expression in the SON (C) on day 30. Representative photographs of AVP mRNA expression in the SON are shown (D). Results are expressed as means ± SE (n = 6 for control, n = 8 for dDAVP). *P < 0.01 vs. control. N.S., not significant.

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Fig. 1. Effects of continuous administrations of desmopressin arginine vasopressin (dDAVP) on urine volume and water intake. Time course changes in urine volume (A) and water intake (B) are shown. From days 1 to 30, mice were administered either dDAVP (black circles) or a vehicle (control, white circles) with osmotic minipumps. On day 30, the minipumps were removed (arrowheads). Results are expressed as means ± SE (n = 9 for control, n = 6 for dDAVP). BW, body weight; *P < 0.05 vs. control.
Effects of Na intake on food intake and body weight. There were no significant differences in food intake or body weight between the 2.0% Na and 0.2% Na groups at any time point examined in both wild-type and FNDI mice (Fig. 4).

Effects of Na intake on urine Na excretion. Urinary Na excretion was significantly increased in the 2.0% Na group compared with the 0.2% Na group in both the wild-type and FNDI mice throughout the experiments (Fig. 5, A and B).

Effects of Na intake on urine volume and water intake. Urine volume and water intake were significantly increased in the 2.0% Na compared with the 0.2% Na group throughout the experiments in both the wild-type and FNDI mice (Fig. 5, A and B).
In addition, we noted a significant effect of the treatment-time interaction on urine volume \( F(1,16) = 6.547, P < 0.05 \) and water intake \( F(1,16) = 7.312, P < 0.05 \) in the FNDI mice (Fig. 5, D and F). Post hoc analyses revealed that urinary volume and water intake were significantly increased in the FNDI mice at 7 mo compared with those at 2 mo in the 2.0% Na diet group, but not in the 0.2% Na group (Fig. 5, D and F).

On the other hand, there were no significant effects of treatment-time interaction on urine volume or water intake in wild-type mice (Fig. 5, C and E).

**Effects of Na intake on urine AVP excretion.** The urine AVP excretion was significantly increased in wild-type mice of the 2.0% Na compared with the 0.2% Na group throughout the experiment (Fig. 5G). On the other hand, urinary AVP excretion was significantly increased in the FNDI mice of the 2.0% Na group compared with that in the 0.2% Na group only at 2 and 3 mo (Fig. 5H).

**Analysis of AVP cells in the SON.** No significant differences were observed in the number of AVP cells counted with emulsion-dipped slides, between the 0.2% Na and 2.0% Na
groups in the FNDI mice (data not shown). While the size of the inclusion bodies was not significantly different between groups (Fig. 6B), the number of inclusion bodies increased significantly in the 2.0% Na compared with the 0.2% Na group (Fig. 6A). Representative photographs depicting inclusion bodies in the SON are shown in Fig. 6, A and B.

DISCUSSION

In the first of the two experiments we conducted in this study to see whether the activities of AVP neurons would affect the progression of phenotypes in the FNDI mice, we examined the effects of dDAVP administration in the female mice, since they showed more progressive polyuria than males (15) and were therefore more suitable for revealing the effects of dDAVP during a period of a few months. In the second experiment, we examined chronic effects of Na intake on the phenotype progression in the male FNDI mice in which progression of polyuria is less clear. Our data demonstrated that 1) the administration of dDAVP suppressed both AVP mRNA expression and aggregate formation in the AVP cells as well as the progression of polyuria in female FNDI mice, and that 2) an excess of Na intake, which was demonstrated to be a stimulant for AVP release, accelerated the polyuria and the aggregate formation in the male FNDI mice. These data suggest that the activation of AVP neurons accelerated the phenotype, and that the aggregate formation is closely related to the progression of polyuria in FNDI.

The dose of dDAVP used in this study was comparable to that administered to patients with DI (41). While dDAVP treatment did not affect blood Na levels, it significantly lowered hematocrit levels in the FNDI mice, suggesting that dehydration had been improved by the treatment. Decreases in AVP mRNA expression in the SON were likely caused by the change in hydration, since chronic dehydration is known to increase the AVP mRNA expression (5, 8, 21). Thus, it is likely that those mice administered dDAVP synthesized fewer AVP precursors than the control mice during treatment. Our data also showed that both the urine volume and water intake were significantly reduced in the dDAVP group compared with the control, even after treatment was terminated. This could not be attributed to the residual dDAVP, since we confirmed that the antidiuretic effects of dDAVP at an amount equivalent to that injected daily with minipumps (0.168 μg/kg) disappeared in 24 h in separate experiments. These findings suggest that dehydration could delay the progression of polyuria in FNDI. While dDAVP treatment did not affect blood Na levels in this study, it is reported that dDAVP decreased blood Na levels in water-loaded rats (37). Thus, it would be interesting to see in future studies whether dDAVP treatment in combination with water load would further delay the progression of DI in the FNDI mice.

About 80% of circulating AVP has been reported to be excreted into urine, and therefore, daily excretion of AVP in the urine reflects daily secretion of AVP from the posterior pituitary (18, 44). Our findings that urine AVP increased in the high-Na diet group in wild-type mice suggest that AVP release is increased by intake of high Na, consistent with a previous study in humans (20). Plasma osmolality is sensed at the organum vasculosum lamina terminalis (3, 35) as well as AVP neurons (29), and AVP release is tightly regulated by plasma osmolality (1, 36). Thus, it is possible that excess Na intake stimulated AVP release by increasing plasma osmolality. In this context, however, previous reports demonstrated that the amount of sodium intake did not significantly affect plasma AVP (4, 12, 16) or Na levels (43). One possible explanation for these studies might be that plasma AVP and Na levels might vary quickly, making it difficult to detect changes. In addition to the central action, it might also be possible that ingested Na stimulated AVP release as well as the sense of thirst through osmoreceptors located in the peripheral tissues (7, 27, 42).

Fig. 6. Immunohistochemical analyses of AVP neurons in SON. Hypothalamic sections from 7-mo-old mice were stained with antibodies for mutant NPII. Round-shaped inclusion bodies not immunostained are observed in both groups (arrowheads). Number of inclusion bodies in SON (A) and size of inclusion bodies in SON (B) are shown. Results are expressed as means ± SE (n = 9). Representative photographs of SON in 0.2% Na (C) and 2.0% Na groups (D) are shown. Scale bar: 20 μm. *P < 0.05 vs. 0.2% Na group.
In contrast to the wild-type mice, urine AVP was increased in the 2.0% Na diet group compared with the 0.2% Na group only for 2 mo in the FNDI mice. As gradual decreases in urine AVP were accompanied by increases in urine volume, it is suggested that polyuria due to AVP deficiency progressed significantly with the high-Na diet. From the clinical point of view, it might be difficult to maintain the water balance in infants with DI, and chronic dehydration in childhood might lead to neurocognitive impairment (10, 19). Thus, it is important to delay the onset or progression of polyuria in FNDI, and our data suggest that the restriction of Na intake might be beneficial.

In a previous study, we showed that aggregates had accumulated in the ER of AVP neurons in the SON of FNDI mice, and that both their size and number increased in parallel with increases in urine volume. In this study, we demonstrated that dDAVP treatment decreased the inclusion bodies, whereas a high-Na diet increased them. As such aggregates were not observed in wild-type mice (15), it is likely that the aggregates were formed by mutant AVP precursors. On the other hand, the aggregates were not immunostained with mutant NPII antibodies, a result reconfirmed in the present study. While it is possible that the epitope was masked or degraded, given that many other proteins are also synthesized in association with AVP, the detailed mechanisms by which aggregates were accumulated in the ER warrant further studies. Furthermore, it remains to be clarified whether aggregate formation leads to cellular dysfunction or is protective of the AVP neurons, as has been suggested in other neurodegenerative diseases such as Parkinson’s disease (9, 30, 32) and prion diseases (17). We should also keep in mind that the findings in our mouse model do not necessarily apply to all the other mutations in FNDI.

Perspectives and Significance

We demonstrated that the activation of AVP neurons accelerated the progression of polyuria as well as aggregate formation in the FNDI mouse model. While the detailed mechanisms by which the aggregates are formed remain to be clarified, the results of present study suggest that the aggregates are formed in relation to AVP synthesis and that they are closely related to the progression of polyuria in FNDI. Our data also suggest that restricting Na intake might be effective in delaying the onset and progression of FNDI.

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DISCLOSURES

The authors have nothing to declare.

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