Progressive polyuria without vasopressin neuron loss in a mouse model for familial neurohypophysial diabetes insipidus

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ARGININE VASOPRESSIN (AVP), an antidiuretic hormone, is synthesized in the magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus (4). It is released into the circulation mainly in response to osmotic stimuli (4).

Familial neurohypophysial diabetes insipidus (FNDI) is a rare autosomal dominant disorder that manifests itself in early childhood due to a progressive reduction in AVP release (11). Although the carriers are normal at birth, the symptoms appear several months or years later, despite the existence of one normal allele. While >50 point mutations in FNDI have been reported in the AVP gene so far, most mutations exist in the domain of NPII, which functions as a carrier protein of AVP from the hypothalamus to the pituitary with axonal transport (11).

The mechanisms underlying progressive polyuria in FNDI have been explored both in cell lines (10, 18, 23–25) and animal models (9, 15, 40, 44). Since mutant AVP precursors reportedly aggregate within the ER (12, 13, 23, 25, 31, 32, 44, 45), one plausible hypothesis is that the aggregated mutant proteins are toxic and cause a progressive loss of AVP neurons (17, 23, 25, 30, 40). Russell et al. (40) demonstrated that the number of immunoreactive cells for normal NPII was progressively diminished in knockin mice in which a Cys residue at position 98 of AVP gene was replaced with a stop codon (Cys98stop; previously called Cys67stop), and suggested that cell death is the primary cause of progressive polyuria in FNDI. It should be noted, however, that decreases in the number of immunoreactive cells could also be caused by mechanisms other than neuronal loss, such as decreases in protein expression and misfolding of the protein.

To better understand the mechanisms of progressive polyuria in FNDI, we have made and analyzed knockin mice of a mutation of NPII (Cys98stop), which causes FNDI in humans in this study. Our data demonstrated that, although the loss of AVP neurons was finally induced after an accumulation of aggregates in the ER, polyuria progressed substantially even in the absence of cell death.

MATERIALS AND METHODS

Generation of knockin mice of mutant NPII. Targeting vectors were constructed with an 8.4-kb fragment of 129/SvJ mouse genomic DNA containing AVP and oxytocin regions (kindly provided by Dr. Harold Gainer, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD). The PGK-neomycin resistance gene (PGK-fneo) flanked by two loxP sequences was inserted into the second intron, and the Cys98stop nonsense mutation (TGC to TGA) was introduced by PCR-based site-directed mutagenesis. Negative selection was achieved through the diphtheria toxin A II site was also created at the second intron to distinguish the mutant gene (Fig. 1A). The targeting vector was linearized and electroporated into 129/SvJ R1...
embryonic stem (ES) cells. After the homologous recombination, mutant AVP gene knockin ES cell lines were established by transiently introducing the Cre-expressed vector into ES cell lines, followed by removing the PGK-neomycin selection marker. Properly targeted ES cells were microinjected into C57BL/6J mouse blastocysts to generate chimeric mice. Male chimeras were then mated with female C57BL/6J mice (Chubu Science Materials, Nagoya, Japan) to obtain F1 offspring. F2 mice were obtained by intercrossing the F1 heterozygotes. All mice used in the study were backcrossed at least three generations into the C57BL/6J background. Mice were geno-
typed using genomic DNA purified from tail fragments, and all genotype mice were housed three or four per plastic cage 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 Nagoya University Graduate School of Medicine and were approved by the Animal Experimentation Committee.

Measurements of urine volume and water intake. Mice were housed in metabolic cages, and urine volume and water intake were measured. Urine osmolality was measured by an osmometer (Advanced Instrument, Norwood, MA).

Operation of ovariectomy and estrogen substitution. In separate experiments, female heterozygous mice were divided into three groups: those sham-operated (sham), those ovariectomized (OVX), and those OVX and given estrogen (OVX + E2). E2 replacement was performed by subcutaneously implanting a pellet containing 0.18 mg 17β-estradiol which is continuously released for 90 days (Innovative Research of America, Sarasota, FL). The operation was performed at the age of 1 mo, and changes in urine volume and urine osmolality were compared at the age of 3 mo.

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. They were embedded in Tissue-Tek (Sakura Finetechanical, Tokyo, Japan) and stored at −80°C until sectioning. Brains were cut at 14 µm on a cryostat at −20°C, thaw-mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan), and stored at −80°C until either in situ hybridization or immunohistochemistry.

Extraction and measurement of pituitary AVP. Pituitary glands were removed immediately after death and then kept in acetone at −20°C. Each pituitary gland was homogenized and stirred in 0.1 N HCl at 4°C for 24 h. After centrifugation, the supernatants were diluted and assayed for AVP contents. AVP was measured with a highly sensitive radioimmunoassay kit (AVP-RIA kit, kindly provided by Mitsubishi Kagaku Iatron, Tokyo, Japan). The sensitivity of the assay for AVP was 0.063 pg/tube (0.17 pg/ml), with < 0.01% cross-reactivity with oxytocin (34).

Antibodies. Antibodies for normal NPII (PS41) and AVP (VA-4) were kindly provided by Dr. Harold Gainer. The epitopes of PS41 were reportedly the amino acid positions 75–86 of NPII (2, 3), and VA-4 were raised against AVP (1), suggesting that PS41 only reacts with normal but not mutant AVP precursors, while VA-4 reacts with normal as well as mutant AVP precursors. Anti-Cys98stop antibody was created by immunizing rabbits with the peptide KPCGSGGRCAV corresponding to the COOH-terminal sequence of the mutated NPII. Rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, MA) was used to detect apoptosis. We used horse anti-mouse and goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) as the secondary antibodies in the biotin-conjugated form.

Immunohistochemistry. Slides were washed in 0.1 mol/l PBS, followed by incubation in blocking solution (20% normal goat or horse serum in PBS) for 30 min. Before incubation in blocking solution, some sections were heated in a citohe buffer solution in a microwave oven at 600W for 3× 5 min. The tissues were then incubated with either VA-4 at 1:1,000, PS41 at 1:50, anti-mutant antibody at 1:1,000, or anti-cleaved caspase-3 antibody at 1:200 solution in PBS with 0.3% Triton X-100 and 1% normal goat or horse serum for 24 h at 4°C. The sections were rinsed with PBS for 15 min and incubated with either biotinylated goat anti-rabbit or horse antimouse antibody at 1:200 in PBS for 3 h at room temperature. After being rinsed, tissues were incubated in avidin-biotin complex solution (1:100; Vector Laboratories) for 90 min at room temperature, and were 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.

In situ hybridization. The rat AVP exonic probe was kindly provided by Dr. Harold Gainer. Highly specific antisense probes were synthesized using 55 µCi [35S]UTP and 171 µCi [32P]CTP (PerkinElmer Life Sciences, Natick, MA), the Riboprobe Combination System (Promega, Madison, WI), 15 units ribonuclease inhibitor, 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 room temperature, 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 room temperature. 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 room temperature 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 room temperature, followed by washes with 0.1× SSC to cool at room temperature 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.

Counting cell numbers in SON. To assist cellular localization of the hybridized signals, the emulsion-dipped sections hybridized with AVP mRNA probes were stained with cresyl violet. The best-matched slice at −0.7 mm caudal from the bregma, according to the brain atlas (35), was chosen in each mouse for the analysis. Any neuronal cross sections with grains of more than threefold the background density were considered labeled. The number of cells located within 200 µm of the lateral tip of the optic chiasma, which were defined as cells in SON and possibly included not only AVP but also oxytocin and glial cells, were also counted. The counting of cells on both sides of SON in each mouse was performed by two observers who were blinded as to whether the slices were wild-type or mutant mice, and the mean values were subject to statistical analyses.

Measurement of number and diameter of aggregates. The number and diameter of the aggregates in the SON were measured using an image analyzing system equipped with a computer-based charge-coupled device camera (model BX51, Olympus, Tokyo, Japan).

Electronmicroscopy. Animals were fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde 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. Frontal 100-µm sections were cut with a Vibratome (Lancer, Sherwood Medical, St. Louis, MO), and stored in 0.1 mol/l PBS, followed by postfixation with 2% osmium tetroxide in the same buffer. Each section was then dehydrated twice in 50, 70, 90, 95, and 100% ethanol each twice, treated with propylene oxide, and embedded in epon812 (Nissin EM, Tokyo, Japan). Resin was polymerized at 60°C for 2–3 days. After ultrathin sections were cut with a diamond knife on a Reichert Ultracut ultramicrotome, they were counterstained with uranyl acetate and lead citrate, and examined with an electron microscope (model JEM-1400EX; JEOL, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The DNA fragmentation characteristic of apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay using the ApopTag Plus Peroxidase in situ Apoptosis Detection Kit (Chemicon, Billerica, MA).
**RESULTS**

**Genotyping of knockin mice and analysis of NPII expression in SON.** Southern blot analyses of the genomic DNA digested with *Hincl* showed a 3.5-kb fragment in wild-type mice, a 2.4-kb fragment corresponding to the replaced gene in homozygous mutant mice, and both fragments in heterozygous mice (Fig. 1, A and B). The genotype distribution from the breeding of F2 heterozygous mice was 17 wild-type, 41 heterozygous, and 19 homozygous mutant mice, in agreement with the 1:2:1 Mendelian distribution expected in the absence of prenatal mortality. Although the homozygous mice appeared normal at birth, all of them died within 1 wk. Immunohistochemical analyses with antibody for mutant NPII at the age of 1 mo for wild-type and heterozygous mice and at the age of 5 days for homozygous mice revealed that mutant protein is expressed in the SON (Fig. 1, G and J) as well as in the PVN and suprachiasmatic nuclei (data not shown) in both wild-type and heterozygous mice. The analysis also revealed that, while normal NPII is expressed in both the cell bodies and axons in wild-type mice, it is mainly expressed in the cell bodies with diminished axonal staining in heterozygous mice (Fig. 1, E and H). It is also confirmed that normal NPII was not expressed in homozygous mice (Fig. 1f). As the skin of homozygous mice appeared dry within a few days after birth, it is suggested that they could not survive probably due to severe dehydration at least among wild-type and heterozygous pups, although it was not possible to measure urine volume at this age. The following experiments were performed with wild-type and heterozygous mice.

**Changes in urine volume, water intake, food intake, body weight, urine osmolality, and pituitary AVP content.** Urine volume in heterozygous mice significantly increased at 1 mo of age (male: 2.1-fold; female: 2.1-fold) and values in wild-type mice, Fig. 2A), and increased progressively until 12 mo (male: 5.9-fold; female: 9.3-fold) in wild-type mice (Fig. 2A). The increases in urine volume were accompanied by increases in water intake (Fig. 2B). There were no differences in food intake or body weight between wild-type and heterozygous mice in both genders (data not shown). Urine osmolality was significantly reduced at 1 mo in heterozygous compared with wild-type mice, and it progressively declined until 12 mo in both male and female mice (Fig. 2C). Pituitary AVP content was significantly decreased at 1 mo in heterozygous mice compared with wild-type mice, and it also progressively declined until 12 mo (Fig. 2D). Of note, female heterozygous mice showed greater urine volume and water intake, lower urine osmolality, and less pituitary AVP content than their male counterparts at 3, 6, and 12 mo (Fig. 2).

**Effects of OVX and E$_{2}$ substitution on water balance.** Urine volume and water intake decreased significantly and urine osmolality rose significantly in the OVX group compared with the sham-operated (sham) group at the age of 3 mo, while E$_{2}$ substitution reversed the effects of OVX on urine volume and urine osmolality (Fig. 3).

**Analysis of AVP cells in SON with immunohistochemistry.** Due to the relatively weak staining of cell bodies with PS41 (normal NPII antibody, Fig. 1C) and VA-4 (AVP antibody, data not shown), cell counting with immunohistochemistry in wild-type mice was difficult. While cell counting with mutant NPII antibody was possible in heterozygous mice at 1 mo, it was also difficult at 6 or 12 mo since round inclusions, which were not immunostained with either VA-4, PS41, or mutant NPII antibody, appeared in the SON (Fig. 4, A–F) and PVN (data not shown). These inclusions were not immunostained even after the sections were subjected to microwaving (data not shown).
shown). The inclusion bodies were not detected in the suprachiasmatic nuclei (data not shown), indicating that they were mainly expressed in the magnocellular neurons. Both the number and size of the inclusions increased with age in male heterozygous mice (Fig. 4, *G* and *H*). On the other hand, while their size increased with age in the female mice as well (Fig. 4 *H*), there were fewer at 12 mo than at 6 mo (Fig. 4 *G*).

**AVP mRNA expression in SON.** The analysis of cells expressing AVP mRNA in the SON demonstrated that the number of AVP cells did not differ between genotypes in male mice at any time point examined (Fig. 5 *A*). The histogram showing the grain numbers per cell in the male mice at 12 mo is demonstrated in Fig. 5 *G*. In female mice, however, while the numbers of AVP cells did not differ between genotypes at 1 and 6 mo, they were significantly diminished at 12 mo in heterozygous compared with wild-type mice (Fig. 5 *B*). The neuronal loss in the SON of 12-mo-old female heterozygous mice was confirmed by counting the cell numbers stained with cresyl violet in the SON (wild-type female mice: 234 ± H11006 9, heterozygous female mice: 193 ± H11006 14 cells/SON, P = 0.05), which possibly involve oxytocin and glial cells as well. The number of AVP mRNA grains per cell was significantly reduced in heterozygous compared with wild-type mice at all time points examined (Fig. 5, *C–F*). The number of grains in heterozygous mice were 50% of the values in the wild-type mice at 1 and 6 mo, and there were no significant differences between genders. At 12 mo, however, the percentage of the grain numbers in the heterozygous mice was significantly lower in the females (39.3 ± 4.0%) compared with that in the males (58.1 ± 3.7%).

**Analysis of AVP cells in SON by electron microscopy.** To characterize the inclusions in the AVP neurons in heterozygous mice detected with immunohistochemistry, we performed an electron microscopy analysis. In 1-mo-old heterozygous mice,
aggregates were present in the ER lumen (Fig. 6, A and B), which were not detected in wild-type mice. Some aggregates were surrounded by multiple membranes in the cytoplasm (Fig. 6C). Of note, aggregates were also found in the nucleus (Fig. 6D) in 36.6 ± 4.6% AVP cells (n = 10, total 44 cells were examined) of 1-mo-old heterozygous mice, when AVP cells were defined by the presence of aggregates in their cell bodies. In contrast, such aggregates were not observed in the SON of wild-type mice (data not shown). In 12-mo-old heterozygous mice, the lumens of ER in the AVP cells were almost entirely occupied by the aggregates (Fig. 6E). In some cells, there were massive aggregates surrounded by multiple membranes (Fig. 6F) that appeared to exist in an enlarged lumen of the ER (Fig. 6G). In others, round-shaped inclusions occupied the cytoplasm (Fig. 6H). In contrast to 1-mo-old mice, aggregates of 12-mo-old mice were found in the nucleus of only 2.6 ± 1.7% AVP cells (n = 8, total 46 cells were examined), and the statistical analysis revealed a significant difference in the ratio between 1-mo-old and 12-mo-old mice.

**Cell death.** Neither active caspase-3 nor terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive neurons were observed in 12-mo-old female heterozygous mice (data not shown). Furthermore, the morphological characteristics of apoptosis, including chromatin condensation or nuclear fragmentation (7), were not detected by the electron microscopy analysis (Fig. 6).

**DISCUSSION**

In this study, we have made and analyzed knockin mice of the mutant NPII that causes FNDI in humans. The heterozygous mice demonstrated progressive polyuria accompanied by...
decreases in urine osmolality and pituitary AVP contents. While such phenotypes in our model are similar to previous studies using rodent models for FNDI (9, 15, 40, 44), our study clarified that 1) there were differences in the phenotype between genders; 2) AVP mRNA levels were decreased in heterozygous mice compared with that in wild-type mice; 3) inclusion bodies, which presumably consist of AVP mutant precursors, increased in the SON in parallel with increases in urine volume; and 4) although neuronal loss in the heterozygous mice was induced over time, polyuria progressed substantially even in the absence of AVP cell death.

In a previous study, Russell et al. (40) also analyzed knockin mice harboring Cys98stop, the same mutation used in the current study, and reported that the loss of AVP neurons in the SON and PVN was the primary cause of progressive polyuria in the mice, based on counting the number of cells immunostained with normal NPII antibody in the SON and PVN. In this study, immunoreactivities for normal NPII, as well as mutant NPII and AVP, also seemed to diminish in the older heterozygous mice, but this was mainly due to the appearance of inclusions that were not immunostained with any antibody. We therefore employed in situ hybridization to count AVP cells in the present study, and clearly demonstrated that the number of AVP cells in the SON did not decrease until the mice showed substantial polyuria. The findings that polyuria progressed in the absence of neuronal loss indicated that cellular dysfunction rather than cell death is the primary cause of the disease. While it remains to be clarified whether such mechanisms are limited to FNDI due to Cys98stop, cellular dysfunction rather than cell death has also been implicated in diseases such as amyotrophic lateral sclerosis (6, 36), Huntington’s disease (48), and Alzheimer’s disease (21).

In this study, we demonstrated that female heterozygous mice had more apparent phenotypes than males, such as progressive polyuria and time course changes in AVP cell numbers as well as in AVP mRNA grains. As OVX resulted in a decrease in urine output and E2 replacement reversed it in OVX mice, it is suggested that E2 could accelerate the progression of polyuria in female mice. Although the effects of E2 on AVP release seem to depend on the experimental condition (14, 46, 49, 50), such studies have not been performed extensively in mice. Furthermore, while estrogen receptor-β is expressed in the SON in rats and humans (20, 22), it is not in mice, at least under ad libitum water intake conditions (43). Given that E2 could alter the effects of AVP on the renal water retention as well as osmoreceptor function (47), the effects of E2 on the progression of the phenotype in heterozygous mice may not reflect direct action on the AVP neurons. The accelerated effects of E2 on progression of polyuria also suggest that other than genetic factors could affect the phenotype in FNDI. This may explain why the ages of onset vary among patients even with the same mutations in FNDI (39, 41, 45). On the other hand, there are no studies showing the difference in the phenotype between genders in FNDI. This may be explained by the fact that polyuria in humans reportedly appeared before adolescence in most cases (16, 29, 30, 45), with some exceptions (39).

Our data also showed that AVP mRNA expression levels were decreased to ~50% at any time point examined in heterozygous mice compared with those in wild-type mice. Although it was not possible to distinguish normal AVP mRNA from mutant mRNA with in situ hybridization in this study, the nonsense-mediated mRNA decay system, which degrades transcripts containing premature termination codons, might be involved in the mechanism (26). Alternatively, mutant as well as normal AVP mRNA expression might be decreased by other mechanisms. While it is possible to consider decreasing AVP mRNA to be one of the cellular protective mechanisms as mutant AVP precursor accumulation would be decreased, the downregulation would be responsible for depletion of pituitary AVP content as well, which would lead to inability to maintain the required levels of plasma AVP levels in water balance. Further studies are required to clarify the significance of downregulation of AVP gene in FNDI as
well as the mechanisms by which AVP mRNA expression is decreased.

As mutant AVP precursors were shown to be trapped in the ER in previous studies (12, 13, 23, 25, 31, 32, 44, 45), it is plausible that the aggregates in the ER shown in this study were formed by such precursors. However, the aggregates were not immunostained with antibody for mutant NPII, normal NPII, or AVP even after microwaving. These data suggest that misfolded AVP precursors with the epitopes masked were refractory even to this treatment or that the epitopes were degraded in the aggregates. Our data also demonstrated that the staining of normal NPII in the axons, as well as the pituitary AVP content, drastically decreased in the heterozygous mice; the pituitary AVP content was ~21% of that in wild-type at 1 mo, and progressively decreased until the 12th mo. These decreases cannot be explained by haploinsufficiency, and suggest that normal AVP precursors were also trapped in the ER, which could be the primary cause of polyuria of FNDI.

Our electron microscopic analysis demonstrated that aggregates existed not only in the ER but also in the nucleus of 1-mo-old heterozygous mice. The fact that such aggregates were not observed in wild-type mice would suggest that the formation of aggregates was related to the production of mutant AVP precursors. We also found multiple membrane structures that surrounded aggregates in the cytoplasm, suggesting preautophagic signs (27). Autophagy is a process that delivers regions of cytosol to lysosomes for degradation and has been implicated in neurodegenerative conditions such as Huntington’s (37, 38) and Alzheimer’s diseases (33, 51). The possible involvement of autophagy in FNDI was also suggested in previous studies in which transgenic rats expressing cys(67)–NPII, or AVP even after microwaving. These data suggest that the aggregates in the ER shown in this study of the mechanisms by which the aggregates are formed

Perspectives and Significance

We showed that polyuria progressed substantially in the absence of AVP neuronal loss in the knockin mice of mutant NPII that causes FNDI in humans, suggesting that cell death is not the primary cause of that disease. The aggregates accumulated in the ER might be involved in the dysfunction of AVP neurons that lead to progressive polyuria in FNDI. Further study of the mechanisms by which the aggregates are formed in the ER is important for the future.

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