



Functional analyses of three different mutations in the *AVP-NP11* gene causing familial neurohypophyseal diabetes insipidus

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Abstract

Purpose Familial neurohypophyseal diabetes insipidus (FNDI), a rare disorder, which is clinically characterized by polyuria and polydipsia, results from mutations in the *arginine vasopressin-neurophysin II (AVP-NP11)* gene. The aim of this study was to perform functional analyses of three different mutations (p.G45C, 207_209delGGC, and p.G88V) defined in the *AVP-NP11* gene of patients diagnosed with FNDI, which are not included in the literature.

Methods For functional analysis studies, the relevant mutations were created using PCR-based site-directed mutagenesis and restriction fragment replacement strategy and expressed in Neuro2A cells. AVP secretion into the cell culture medium was determined by radioimmunoassay (RIA) analysis. Fluorescence imaging studies were conducted to determine the differences in the intracellular trafficking of wild-type (WT) and mutant AVP-NP11 precursors. Molecular dynamics (MD) simulations were performed to determine the changing of the conformational properties of domains for both WT and 207_209delGGC mutant structures and dynamics behavior of residues.

Results Reduced levels of AVP in the supernatant culture medium of p.G45C and p.G88V transfected cells compared to 207_209delGGC and WT cells were found. Fluorescence imaging studies showed that a substantial portion of the mutant p.G45C and p.G88V AVP-NP11 precursors appeared to be located in the endoplasmic reticulum (ER), whereas 207_209delGGC and WT AVP-NP11 precursors were distributed throughout the cytoplasm.

Conclusions The mutations p.G45C and p.G88V cause a failure in the intracellular trafficking of mutant AVP-NP11 precursors. However, 207_209delGGC mutation does not result in impaired cellular trafficking, probably due to not having any significant effect in processes such as the proper folding, gain of three-dimensional structure, or processing. These results will provide valuable information for understanding the influence of mutations on the function of the AVP precursor hormone and cellular trafficking. Therefore, this study will contribute to elucidate the mechanisms of the molecular pathology of *AVP-NP11* mutations.

Keywords Diabetes insipidus · AVP-NP11 · Mutation · Functional analysis · RIA

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Introduction

Congenital central diabetes insipidus, also commonly called “familial neurohypophyseal diabetes insipidus (FNDI)”, is a rare inherited disorder that appears to be largely autosomal dominant manner [1, 2]. It results from decreased production or release of the antidiuretic hormone arginine vasopressin (AVP). This hormone regulates total body water balance by stimulating the reabsorption of water from the renal collecting ducts [3]. FNDI is clinically characterized by polyuria, polydipsia, and thirst resulting from a deficiency of AVP. These symptoms usually appear several months or years after birth and then gradually progress [4, 5].

FNDI is caused by mutations in the *AVP-neurophysin II (NPII)* gene, which contains three exons and encodes the AVP-NPII preprohormone [6]. This AVP-NPII preprohormone consists of a signal peptide, AVP, NPII, and a C-terminal glycopeptide called copeptin [7, 8]. After the synthesis of preprohormone in the hypothalamic magnocellular neurons, it is converted to prohormone by removal of its signal peptide and the addition of a carbohydrate chain to the glycopeptide within the endoplasmic reticulum (ER) [4, 9, 10]. The prohormone is appropriately folded and its conformation is stabilized by the formation of disulfide bridges between the cysteine residues [11, 12]. Following folding in ER, the prohormone is transported to the Golgi apparatus where it is packaged into neurosecretory granules [13, 14]. During axonal transport from the hypothalamus to the posterior pituitary, the prohormone is cleaved into AVP itself, its carrier protein (NPII) and glycoprotein within neurosecretory granules [15]. NPII functions as preventing proteolysis of AVP, it is also important for the appropriate folding and trafficking process from the ER to the Golgi apparatus [16].

Until now, FNDI has been linked to more than 70 different mutations in the *AVP-NPII* gene [17]. Most of these mutations are located in the coding sequence of the NPII moiety and have an autosomal dominant mode of inheritance [18]. The mechanism by which these autosomal dominant mutations have a dominant-negative effect on the pathogenesis of FNDI is controversial [17]. However, most evidence suggests that mutant AVP-NPII prohormone is accumulated in the ER due to the impairment of the normal intracellular trafficking [7, 19]. Heterologous expression studies in either cell cultures or transgenic animals have supported that mutant AVP-NPII prohormone fails to exit the ER and is inefficiently processed into AVP and NPII [20–23].

In this study, we performed the functional analysis of three different mutations (p.G45C, 207_209delGGC, p.G88V) defined in the *AVP-NPII* gene by using Neuro2A cell line to examine whether these mutations result in impaired cellular trafficking. The p.G45C and 207_209delGGC mutations were identified for the first time in the previous studies by our group [24, 25]. While p.G45C is a heterozygous missense mutation at codon 45, which causes the substitution of GGC (Gly) by TGC (Cys), the 207_209delGGC mutation is a heterozygous three base pair deletion (GGC) in exon 2 at codon 69–70 that only leads to a deletion of the amino acid alanine from the protein sequence without a frameshift. The third mutation, p.G88V, was previously reported for the first time by Melo et al. [26] and also in our previous study [27]. This mutation is a heterozygous missense mutation at codon 88, which causes the substitution of GGC (Gly) by GTC (Val). All three mutations in this study are located within the NPII moiety in exon 2 of the *AVP-NPII* gene and associated with the dominant inheritance of FNDI. Detailed clinical data of the patients were mentioned in previous studies of our group [24, 25, 27].

Materials and methods

Generation of mutant AVP-NPII constructs

Mutant AVP-NPII constructs were generated with a PCR-based site-directed mutagenesis and restriction fragment replacement strategy on the mammalian expression vector named as a pL vector. This vector is a modified version of the pcD-ps vector with a removed poly-A fragment 3' of the MCS-cloning region. All constructed mutant vectors, named as pL-AVP-G45C, pL-AVP-207_209delGGC, and pL-AVP-G88V, were sequenced to verify the presence of the mutations introduced and to exclude PCR-derived nucleotide changes.

For fluorescence imaging studies, wild type (WT) protein and all mutants were fused to the sequence encoding the enhanced green fluorescent protein (EGFP) right after the AVP-NPII coding sequence and before the stop codon. After restriction digestion of the vectors, the Gibson assembly method was used to generate constructs with EGFP.

Cell culture and transfection

Mouse neuroblastoma Neuro2A cells were obtained from Cell Lines Service (Eppelheim, Germany, RRID: CVCL_0470) and were cultured in Eagle's minimum essential medium (EMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 µg/mL streptomycin, 100 U/mL penicillin (all from Biowest®, Riverside, USA). The cells were maintained at 37 °C in a 5% CO₂ incubator. Transfections of the cells with WT, mutant or empty pL vectors, the latter as a control, were performed using Lipofectamine 2000 (Invitrogen®, Carlsbad, USA). Neuro2A cells were cultured in 6-well plates (16 × 10⁴ cells/well) and 1.6 µg plasmid DNA to 1.6 µl of Lipofectamine 2000 reagent were used to transfect cells for AVP radioimmunoassay (RIA) analysis. For fluorescence imaging studies, cells were cultured in 48-well plates (4 × 10⁴ cells/well) and 0.4 µg plasmid DNA to 0.4 µl of Lipofectamine 2000 reagent were used to transfect each well of cells. Pure EMEM (without serum and supplement) was used during transfection and the medium was replaced with complete EMEM 4–6 h after the transfection.

AVP radioimmunoassay (RIA)

For measurement of AVP levels from cell culture supernatants, Neuro2A cells were cultured for ~48 h after transfection until the highest transfection efficiency was observed. Then, the culture medium was collected and centrifuged at 1000 × g for 20 min at 2–8 °C followed by an

extraction procedure. AVP levels were measured by a radioimmunoassay (Vasopressin-RIA, KIPERB319, DIA-source ImmunoAssays S.A, Belgium). Total protein was extracted from the cells using TRIzol-G (Applichem Inc, Germany) and its concentration was determined by bicinchoninic acid (BCA) protein assay (Takara Bio, Tokyo, Japan). The AVP values were detected as pg/ml and converted in pg per milligram of total cell lysate protein per well to adjust AVP measurements for differences in cell culture size. All samples were assayed in triplicate representing three independently transfected wells with separate batches of cells on separate days. The sensitivity of the assay was 0.5 pmol/L.

Fluorescence imaging studies

For the studies of fluorescence imaging, Neuro2A cells were transfected with the EGFP-tagged constructs. Approximately 48 h after transfection, the cells were fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 30 min and then washed with 1 X PBS. For visualization of ER in the cells, cells were treated with one μ M ER-TrackerTMRed (BODIPY[®] TR Glibenclamide; Thermo Fisher Scientific, Paisley, UK) and incubated for 30 min at 37 °C. After incubation, the cells were permeabilized on ice with 250 μ L 1 X PBST (0.05% Tween-20 in 1 X PBS) for 10 min. Cells were washed with 1X PBS and incubated with 4',6-Diamidino-2-phenyl-lindoldihydrochloride (DAPI; Thermo Fisher Scientific) for 5 min to label nuclei. The following two washes with 1X PBS, cells were visualized using a fluorescence microscope (EVOS FLoid Cell Imaging Station, Thermo Fisher Scientific, USA). While expressed WT and mutant AVP-NP2 proteins were detected as green fluorescent molecules due to the EGFP-tagged constructs, ER was stained red in the cell.

Statistical analysis

The levels of AVP secreted by the cells transfected with pL-AVP-WT and mutant vectors were normal distributed and statistically compared by one-way ANOVA and Tukey's multiple comparison test. *P* values <0.05 were considered statistically significant.

Molecular dynamics (MD) simulation study

The vasopressin-neurophysin 2-copeptin structures (WT type and mutant) were modeled with the ROBETTA server, all MD simulations were done with GRONingen Machine for Chemical Simulations (GROMACS)-2019 software [28] with OPLS-AA force field [29] during 10 ns. MD trajectory analyzes by BIOVIA Discovery Studio 2021 software [30] and USCF CHIMERA (Version 1.14) [31].

Results

AVP radioimmunoassay

To investigate whether the mutations affect vasopressin expression and excretion, AVP secretion of Neuro2A cells transiently transfected with WT and mutant AVP-NP2 cDNAs was examined. AVP levels in the supernatant culture medium were detected by RIA. As shown in Fig. 1, the level (mean \pm SD) of AVP secreted by the cells transfected with pL-AVP-WT was significantly greater (7.7 ± 0.6 pg/mg cell protein) than the levels secreted by the cells transfected with pL-AVP-G45C (3.6 ± 1.6) and pL-AVP-G88V (2.9 ± 1.4). The 207_209delGGC (6.6 ± 0.7) mutation was not seen to affect AVP secretion out of the cell into the culture medium. A statistically significant difference was found between AVP levels in the cell culture medium of Neuro2A cells transfected by pL-AVP-WT and pL-AVP-G88V (*p* < 0.05).

Fluorescence imaging studies

In order to compare intracellular trafficking of WT and mutant AVP-NP2 precursors and/or their processed products, the intracellular localizations of EGFP-tagged AVP-NP2 precursors were determined in transiently transfected Neuro2A cells (Fig. 2).

In Neuro2A cells transfected with mutant either p.G45C-EGFP or p.G88V-EGFP cDNA, most of the

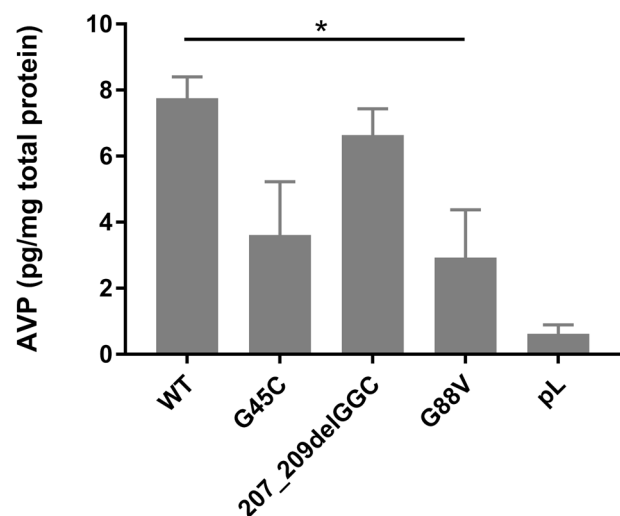


Fig. 1 Radioimmunoassay (RIA) measurements of AVP secreted into the cell culture medium of Neuro2A cells. The AVP values indicated as picograms of AVP per mg cell protein. The culture medium from cells transfected with an empty pL vector was analyzed as a control. The data represent the mean \pm SD of three independent experiments and compared by one-way ANOVA and Tukey's multiple comparison test. There was statistically significant difference between AVP levels in the cell culture medium of Neuro2A cells transfected by pL-AVP-WT and pL-AVP-G88V (**p* < 0.05)

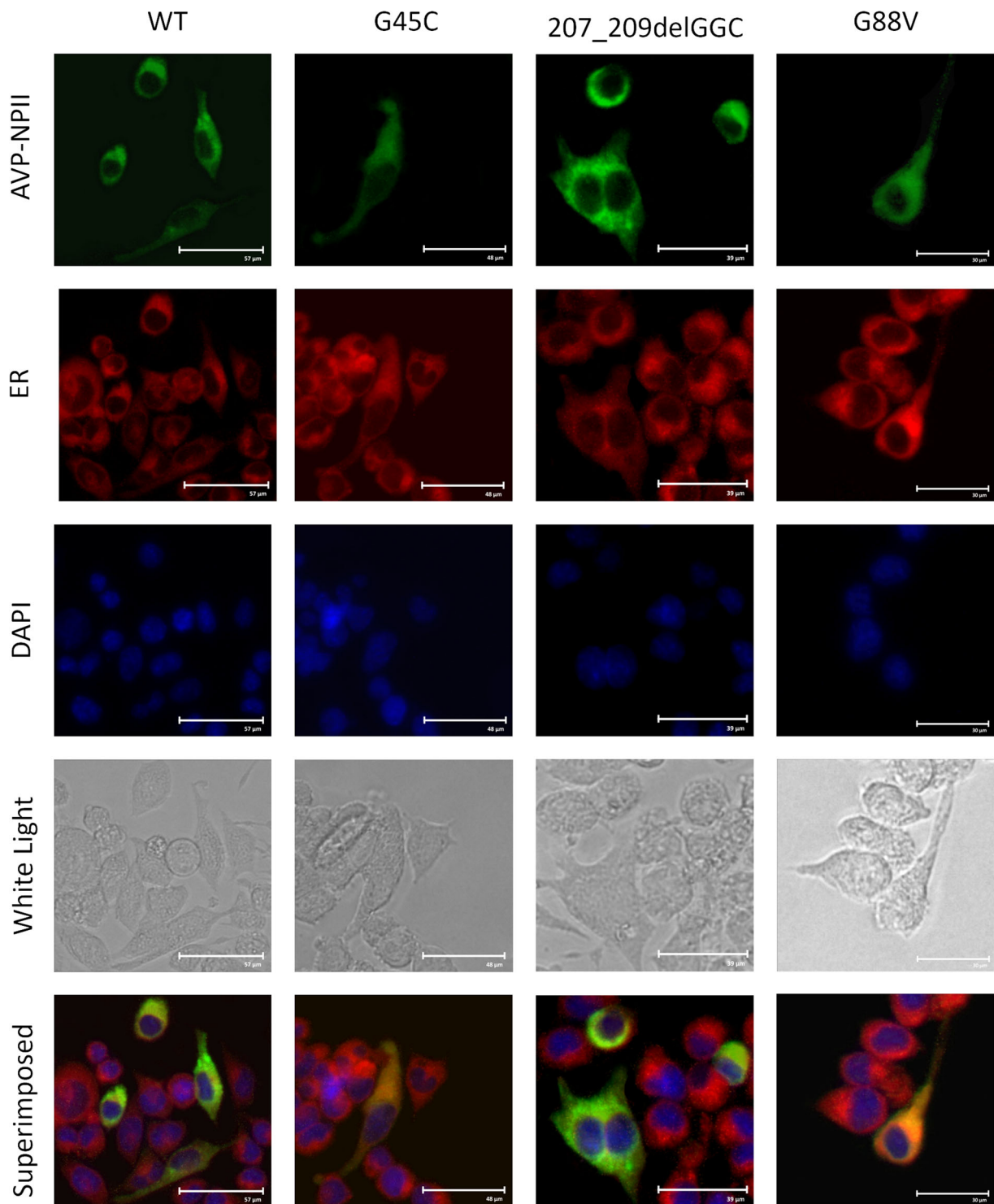


Fig. 2 The intracellular localization of WT and mutant AVP-NP11 proteins in transiently transfected Neuro2A cells by fluorescence microscopy. The cells were maintained until 48 h after transfection, fixed and permeabilized. While expressed WT and mutant AVP-NP11 proteins were detected as green fluorescent molecules due to the

EGFP-tagged constructs, ER was stained as red color in the cell. Co-localized proteins (yellow or orange color) were detected by superimposing pictures of the same sections resulting in merging of the green and red color. Nuclei were stained with DAPI as blue color. White light imaging of the cells is also shown in the figure

AVP-NP11 precursors appeared to be located around the nucleus that overlapped with the ER. In contrast, in the cells transfected with WT-EGFP or mutant 207_209del

GGC-EGFP cDNA, AVP-NP11 precursors were not co-localized with the ER and mainly dispersed throughout the cytoplasm.

Molecular dynamics (MD) simulation study

To determine the changing of the conformational properties of domains for both WT and 207–209delGGC mutant structures and dynamics behavior of residues, MD simulations were performed (Figs. 3 and 4). According to the simulation results of WT and mutant structures, it was observed that the RMSD value of both structures became stable around 0.3 nm at the end of the simulation (Supplementary Fig. 1). When the WT and 207–209delGGC mutant structures of the protein are examined, it is seen that the residues of Arg31, Ala32, Met33 and Ser34 turn into alpha-helix form due to the mutation effect (Fig. 4). As a result of MD simulations, it was observed that these structures remained stable. Due to the alpha-helix form, the bond relationship between the AVP peptide and NPII-Neurophysin 2 has changed. This relationship was examined by focusing on the Ala32 residue, where both subgroups were linked. Considering WT, it is seen that the Ala32 residue makes Pi-Alkyl bonds with Pro26 and Phe10

(Fig. 3). After maturation, these bonds are easily broken and the subunits can be separated from each other. However, when the mutant structure is examined, it is seen that a different pattern emerges. The Ala32 residue forms hydrogen bonds with Asp35 and Gly29, Alkyl bonds with Pro81, and van der Waals bonds with Met33 (Fig. 4). Here, hydrogen bonds play a critical role. The alpha helix form in the mutant structure prevents the peptide from reaching the 3D conformation it should take after separation and the structural change in the mutant structure. Therefore, based on these data, we can associate the loss of function of the AVP peptide with the hydrogen bonds made by the Ala32 residue and the peptide.

Discussion

In this study, functional analysis of three different mutations defined in the *AVP-NP11* gene of patients with diabetes insipidus was performed. Using assays of fluorescence

Fig. 3 3D (a) and 2D (b) representation of the bonds between the Ala32 residue and the surrounding residues in the WT structure. It makes Pi-Alkyl bonds with Ala32, Pro26 and Phe10. These bonds do not prevent the subunits from separating because of their weakness. c In the 3D structure of the WT structure, the Ala32 residue is not in alpha-helix form

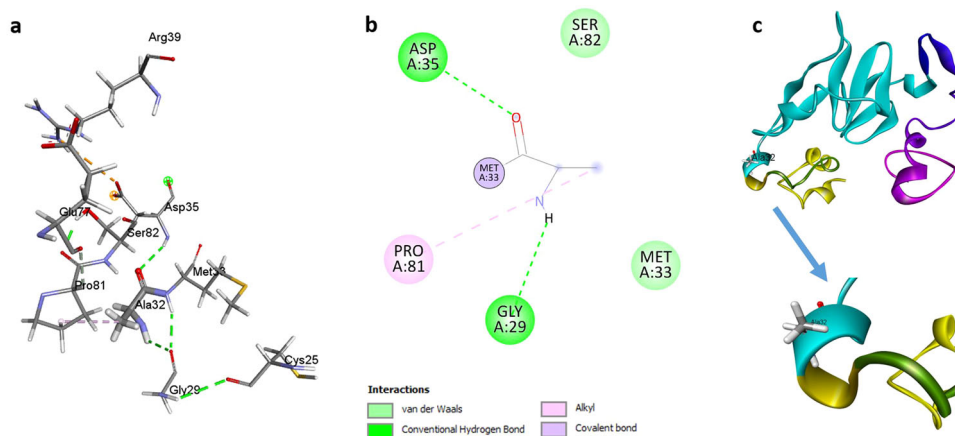
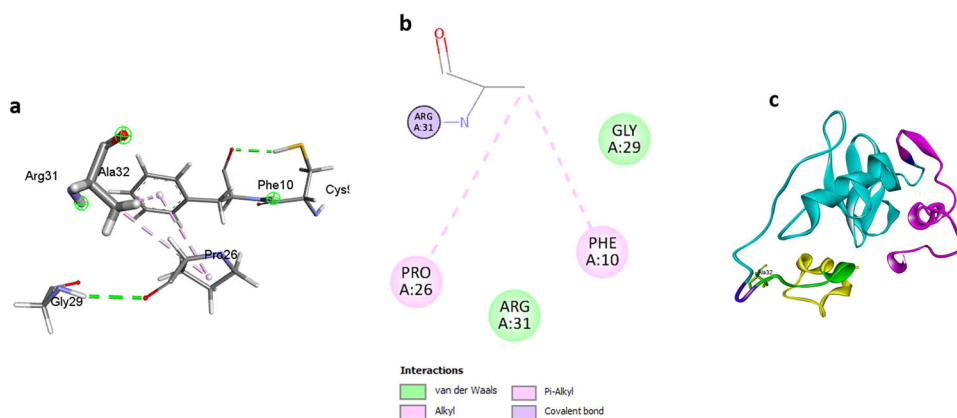


Fig. 4 The mutant Ala32 residue makes hydrogen bonds with Asp35 and Gly29 residues. 3D (a) and 2D (b) representations show the Alkyl bond with Pro81 and the van der Waals bond structures with Met33. These bonds prevent the peptide from separating from the main structure. c In the three-dimensional conformation of the C-mutant

structure, the residues Arg31, Ala32, Met33 and Ser34 were transformed into the alpha-helix structure. This structural form prevents the peptide from having the necessary conformation to function even if it is separated from the main structure

imaging and measuring AVP secretion of transfected Neuro2A cells, we show for the first time that the mutations p.G45C and p.G88V cause a failure in the transport of the mutant AVP-NP_{II} proteins from ER and consequently a marked reduction of AVP secretion to the cell culture medium. Our results confirm previous findings concerning the impaired intracellular transport of mutant AVP-NP_{II} prohormones and abnormal retention of these prohormones in the ER [21, 32]. Eubanks et al. indicated that various mutations had structural consequences, which decrease the conformational stability and folding efficiency of the prohormones [32]. Glycine and cysteine residues in the prohormone are quite important to form a stable three-dimensional structure and in the maintenance of this conformational stability of the precursor hormone [25, 33]. Glycine residues confer flexibility and rigidity to the protein backbone by producing turns in the polypeptide chain, whereas cysteine residues form intrachain disulfide bridges during the folding of prohormone in ER. In addition, Ito et al. [34] and computer-assisted prediction of the secondary structure of NP_{II} molecule by the method of Garnier et al. [35] reported that amino acid substitution at codon 88 could influence AVP binding or lead to a conformational change of NP_{II} molecule due to Gly at codon 88 (NP57G) is a conserved amino acid among most vertebrate species [36]. According to these findings, it was concluded that due to the lack of glycine residue in the mutant proteins formed by p.G45C and p.G88V mutations and the addition of an extra cysteine residue into the structure of mutant precursor by p.G45C mutation, impaired folding and stabilization of the precursor hormone might occur in this study.

Besides p.G45C and p.G88V mutations, we performed the functional analysis of the other mutation, 207_209delGGC, and no significant difference could be observed in the cells transfected with the pL-AVP-207_209delGGC mutant compared to cells transfected with pL-AVP-WT. These results suggested that the deletion had no effect on conformational stability and folding of the AVP-NP_{II} precursor and so intracellular transport of it from ER to the cell culture medium closely resembles the WT AVP-NP_{II}. To date, five smaller deletions have been identified in the *AVP-NP_{II}* gene except for this deletion [37]. In the literature, only one of these deletions has a functional analysis study [38]. A 3-base pair deletion resulted in the deletion of phenylalanine at position 3 of the AVP moiety and preservation of the rest of the coding sequence in a frame as in the present study. The authors observed that immunoassayable AVP was secreted into the cell culture medium from Neuro2A cells expressing a WT AVP-NP_{II} cDNA, whereas the cells expressing the mutant construct or untransfected cells had no immunoassayable AVP with RIA. They proposed that the deletion of Phe 3 in the AVP moiety had a critical role for the proper binding of

AVP to NP_{II} and disruption of that interaction lead to the accumulation of improperly folded protein products in the cell [38]. Although the exons on which the deletions occur on the AVP-NP_{II} gene are different, it is thought that obtaining similar results is generally due to impaired folding followed by the intracellular trafficking of the mutant precursor hormones are similar. In addition, the cytoplasmic localization of the 207-209delGGC mutant protein might fit better with the autophagy hypothesis which is vital for the clearance of aggregates and protein quality control [39]. Misfolded mutant protein or dimerization of the mutant protein with WT protein might lead to autophagy. These structures could be sequestered into autophagosomes rather than packaging into neurosecretory granules and fused with lysosomes in the cytoplasm. Therefore, 207–209delGGC mutant protein could be seen mainly dispersed throughout the cytoplasm.

Since the identification of the first mutation (G88S) that caused the autosomal dominant form of FN_{DI} in 1991 [34], many mutations have been identified [13, 19, 40–42]. Various studies concerning both cell lines and animal models have also been conducted to clarify the effects of these mutations on the pathogenesis of the disease and the mechanisms involved in this pathogenesis [4, 21, 23, 43–46]. According to the results obtained from these studies, different hypotheses for the mechanism of the autosomal dominant form of FN_{DI} pathogenesis were reported. The most important hypothesis is that the mutations cause impaired folding or dimerization of the mutant precursor hormones, altered protein processing followed by defective intracellular trafficking of these mutant precursors and retention of them in the ER to form cytotoxic protein aggregates [4, 21, 43]. These toxic aggregates lead to loss of cell function and eventually progressive neuronal cell death [21, 23, 43]. In another hypothesis, the processing and secretion of WT AVP-NP_{II} precursors are blocked due to forming heterodimers with mutant AVP-NP_{II} precursors, which is called as “dominant-negative effect” [8, 44]. The exact mechanism of the FN_{DI} mutations on pathogenesis is controversial and besides, in vitro molecular studies, models that more closely mimic conditions in vivo are needed to better understand this effect.

In conclusion, we conducted functional studies of three different mutations defined in the *AVP-NP_{II}* gene of patients with FN_{DI} for the first time. Our results demonstrate that the functional analysis of the p.G45C and p.G88V mutations result in reduced AVP levels in the cell culture medium of Neuro2A cells compared to the WT AVP-NP_{II} probably due to ER retention. Therefore, we can suggest that these mutations lead to the production of mutant AVP-NP_{II} precursors that fail to stabilize the conformational structure, process into AVP and NP_{II}, fold and/or dimerize properly in the ER. The impaired trafficking of

AVP may cause its retention in the ER instead of its secretion to the cell medium. Furthermore, AVP levels were reduced in media cultured with these mutations, but were not absent. This suggests some ability to properly traffic and enzymatically process the prohormone, likely due to the short-term nature (48 h) of the cell cultures, i.e., before the toxicity of the mutant protein retained in the ER was fully expressed. Similarly, the normal AVP concentration in the medium from cells cultured with the 207–209delGGC mutant may reflect a slower toxicity process that takes more time to be fully expressed. This slower toxicity of mutant proteins or autophagy processes could also be the cause of the similar cytoplasmic localizations of the 207–209delGGC mutant with the WT protein.

In addition, according to the MD simulation studies, we observed the different bond interactions between AVP peptide and NPII in WT and 207_209delGGC mutant structures. The changing of the bond interaction and conformational properties of domains prevents the separating of the AVP, NPII or copeptin from each other after maturation. This can result in the loss of function of the AVP peptide which normally binds its receptor to stimulate the reabsorption of water from the renal collecting ducts in healthy individuals. When the 207_209delGGC mutation occurs, AVP peptide could not stimulate reabsorption of water due to not separating from the NPII and/or copeptin. This can explain why urine volume is too much in DI patient with 207_209delGGC mutation. Taken together with these data, more research is required to fully elucidate the maturation mechanism of this small deletion mutation.

This study comprising the functional analyses of these mutations is quite important due to FNDI is a rare hereditary disease and a low number of patients. It is critical to elucidate molecular mechanisms leading to the disease pathogenesis especially in rare hereditary diseases like as DI. For this purpose, functional analysis studies of mutant proteins are carried out and the results of these studies could be used for developing new treatment strategies which requires further research.

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Author contributions The study conception and design were contributed by M.O.T., T.K., B.E.T., and H.M. Formal analysis and investigation were performed by M.O.T., T.K., and B.E.T. MD simulation study was performed by M.A.U. The first draft of the manuscript was written by M.O.T. and T.K. The editing of the manuscript and funding acquisition were performed by Hatice Mergen (Supervisor).

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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