Functional characterization of novel loss-of-function mutations in the vasopressin type 2 receptor gene causing nephrogenic diabetes insipidus

Iris Böselt¹, Despoina Tramma², Serafia Kalamitsou², Thomas Niemeyer³, Päivi Nykänen⁴, Klaus-Jürgen Gräf⁵, Heiko Krude⁶, Karen Sabrina Marenzi⁷, Stefania Di Candia⁷, Torsten Schöneberg¹ and Angela Schulz¹

¹Molecular Biochemistry, Institute of Biochemistry, Medical Faculty, University of Leipzig, Leipzig, Germany, ²Fourth Paediatric Department, Aristotle University of Thessaloniki, Thessaloniki, Greece, ³Clinic for Paediatrics and Youth Medicine, St Agnes-Hospital Bocholt, Bocholt, Germany, ⁴Department of Pediatrics, Mikkeli Central Hospital, Mikkeli, Finland, ⁵Endokrinologikum Berlin, Berlin, Germany, ⁶Institute of Experimental Pediatric Endocrinology, Charité-Universitätsmedizin Berlin, Berlin, Germany and ⁷Department of Pediatrics, Scientific Institute San Raffaele, University of Milan, Milan, Italy

Correspondence and offprint requests to: Torsten Schöneberg; E-mail: schoberg@medizin.uni-leipzig.de

Abstract

Background. X-linked nephrogenic diabetes insipidus (NDI) is a rare polyuric disorder caused by inactivating mutations in the arginine vasopressin receptor Type 2 (AVPR2) gene.

Methods. NDI patients from six unrelated families were subjected to mutational analysis of the AVPR2 gene. In-depth in vitro characterization of novel AVPR2 mutants by a combination of functional and immunological techniques.
provided further insight into molecular mechanisms causing receptor dysfunction.

Results. Mutational analysis revealed four novel (A89P, G107R, Q174R, W208X) and three recurrent (V277A, R337X, AR247-G250) mutations within the AVPR2 gene. One family carried the missense mutation R337X and a 12-bp deletion (ΔR247-G250), corresponding to a fragment in the third intracellular loop (ICL3), which was not genetically linked to R337X. The functionally tested missense mutations A89P, G107R and Q174R led to reduced receptor cell surface expression in transfected COS-7 cells, most probably due to misfolding and intracellular retention, and consequently to reduction or loss of agonist-mediated cyclic adenosine monophosphate formation. Deletion of R247-G250 had no effect on receptor function in vitro. Comparison with other mammalian AVPR2 orthologs showed that this part of the ICL3 is structurally not conserved and, therefore, less relevant for receptor function. In contrast, all missense mutations (A89P, G107R, Q174R, V277A) affect receptor positions that were fully preserved during mammalian evolution.

Conclusion. Our results provide valuable information about residues critical for AVPR2 folding, trafficking and function and proof that these mutations are responsible for causing NDI in the affected subjects.

Keywords: AVPR2; G protein-coupled receptor; NDI; nephrogenic diabetes insipidus; V2R

Introduction

G protein-coupled receptors (GPCR) constitute the largest family of membrane receptors and play pivotal roles in controlling almost every physiological function. They are important drug targets in treating numerous diseases but mutational alterations in GPCR can also be responsible for disorders. To date, >30 monogenetic diseases have been identified to be caused by mutations in GPCR [1, 2]. The X-linked nephrogenic diabetes insipidus (NDI) is a rare disorder (OMIM 304800) caused by loss-of-function mutations in the arginine vasopressin receptor Type 2 (AVPR2) gene [3]. AVPR2 is a Gs-coupled receptor mainly expressed in the renal principal cells of the collecting duct and is involved in water reabsorption. NDI is characterized by resistance of the kidneys to the antidiuretic effect of the neurohypophysial hormone arginine vasopressin (AVP). The kidney loses its ability to concentrate urine and produces abnormally large volumes of diluted urine. Consequently, this can lead to severe dehydration and electrolyte imbalance, primarily hypernatremia and hyperchloremia [4, 5]. In some families, however, NDI demonstrates an autosomal recessive mode of inheritance. In these patients, mutational analysis of the gene encoding for the AVP-sensitive water channel, aquaporin 2 (AQP2), revealed the molecular defect [6–8].

More than 200 different disease-causing AVPR2 mutations have been identified in X-linked NDI families; yielding ~50% missense mutations, 27% small deletions/insertions, 9% non-sense mutations and 8% large or complex deletions [1, 9, 10]. In addition to mutational analysis of NDI patients, detailed functional analysis of the altered AVPR2 proteins is an important part of molecular characterization. Mutations in the AVPR2 gene result in receptor dysfunction at different functional levels: (i) gene deletion and complex genomic rearrangements [11–13], (ii) improper messenger RNA splicing [12, 14, 15], (iii) improper protein folding and intracellular trafficking [6, 16, 17] and (iv) alterations in ligand-binding properties [16, 18, 19]. Functional characterization becomes more relevant because several misfolded and premature stop codon truncated AVPR2 can be at least partially rescued by pharmacological chaperones and drugs influencing the ribosomal fidelity, respectively [20–24].

Herein, we report six patients with NDI carrying four novel missense mutations (A89P, G107R, Q174R, W208X) and three recurrent mutations (V277A, R337X/AR247-G250) in the AVPR2 gene. Functional analysis of the novel AVPR2 mutants revealed that these missense mutations probably led to intracellular receptor retention.

Materials and methods

Study subjects

Six NDI patients and some closely related family members from six different families were investigated. The patients were from different ethnic backgrounds. Patient 1 was of Iranian, Patient 2 of Italian and Patients 3 and 5 of German descents. Patient 4 (female) was from Finland and Patient 6 had Greek origins. The parents of all families were apparently not related. Patients had a history of fever, polyuria, polydipsia and periods of constipation. The diagnosis of NDI was based on clinical symptoms (Table 1). All chemical laboratory tests were performed using standard clinical laboratory assays. Institutional approval and written informed consent from all patients and the family members were obtained. All clinical, laboratory and genetic investigations were conducted according to Declaration of Helsinki principles.

Sample preparation and AVPR2 gene analysis

Genomic DNA was prepared from whole blood samples by conventional methods (DNeasy Kit; QIAGEN, Hilden, Germany). The AVPR2 gene was amplified by polymerase chain reaction (PCR) using 0.1 μg of genomic DNA as template. One primer pair was used to amplify the entire coding region of AVPR2 including the two introns: V2-sense 5'-TCACC-TCCAGGCCCCCTCAGAACACCT-3' and V2-antisense 5' -CCTAAGG CAGAGCACCACACAG' 3'. In each case, two independent AVPR2 amplifications were performed to verify correct sequence analysis. All PCRs were performed with Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt, Germany) essentially following manufacturer’s protocol (exception: addition of 5% dimethyl sulphoxide to PCR) under the following conditions: (35 cycles) 10 s 98°C, 30 s 60°C, 1 min 72°C. PCR samples were separated by 1% agarose gel electrophoresis and DNA was extracted. Specific PCR fragments were sequenced with a dye-terminator cycle sequencing kit on an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA). In case of heterozygosity, allelic separation was performed by subcloning (pCR2.1-TOPO vector; Invitrogen, La Jolla, CA) and subsequent sequencing. For Patient 6 and family members, genetic analysis of the aquaporin 2 (AQP2) gene was performed as described in [6, 8].

Generation of mutant AVPR2

AVPR2 mutations (A89P, G107R, Q174R and AR247-G250) were introduced into the human AVPR2 expression plasmid, V2-pcDNA [25], using a PCR-based site-directed mutagenesis and restriction fragment replacement strategy. In addition, wild-type and mutant AVPR2 were epitope-tagged with an N-terminal haemagglutinin (HA)-tag (inserted after the initiating methionine codon) and a C-terminal FLAG-tag (inserted before stop codon) by a PCR-based overlapping fragment mutagenesis approach. The identity and correctness of all PCR- or restriction-derived sequences was confirmed by DNA sequencing. To monitor the transfection efficiency and for control...
**Table 1. Clinical parameters and mutational analysis of NDI patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>AVPR2 mutation</th>
<th>Parental carrier</th>
<th>Age at NDI diagnosis</th>
<th>Serum-Na&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serum-CI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Urine volume</th>
<th>Urinary osmolality</th>
<th>Plasma APA level</th>
<th>Other clinical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A89P (g.626G &gt; C)</td>
<td>n.d.</td>
<td>57 years</td>
<td>146 mmol/L</td>
<td>n.d.</td>
<td>12 L/day</td>
<td>n.d.</td>
<td>7.96 ng/mL</td>
<td>Diabetes mellitus type 2, mild hypothyroidism</td>
</tr>
<tr>
<td>2</td>
<td>G107R (g.680G &gt; C)</td>
<td>n.d.</td>
<td>6 months</td>
<td>181 mmol/L</td>
<td>n.d.</td>
<td>1.5 L/day</td>
<td>n.d.</td>
<td>Pre-term birth</td>
<td>Perinatal asphyxia, Dehydration</td>
</tr>
<tr>
<td>3</td>
<td>Q174R (g.882A &gt; G)</td>
<td>Maternal</td>
<td>1 month</td>
<td>157 mmol/L</td>
<td>120 mmol/L</td>
<td>74 mosmol/kg</td>
<td>n.d.</td>
<td>24 ng/mL</td>
<td>n.d.</td>
</tr>
<tr>
<td>4 (female)</td>
<td>W208X (g.985G &gt; A)</td>
<td>Maternal</td>
<td>15 months</td>
<td>140 mmol/L</td>
<td>98 mmol/L</td>
<td>227 mosmol/kg</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>V277A (g.1191T &gt; C)</td>
<td>Maternal</td>
<td>15 months</td>
<td>140 mmol/L</td>
<td>107 mmol/L</td>
<td>24 ng/mL</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R337X (g.1476C &gt; T)</td>
<td>Maternal</td>
<td>2.5 months</td>
<td>165 mmol/L</td>
<td>130 mmol/L</td>
<td>3.5 L/day</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup>AVPR2 mutations, clinical parameters and additional clinical findings of the patients investigated in this study are listed. The values shown are from the untreated patients when NDI was first diagnosed at an age as indicated. Untreated patients with NDI typically excrete >50 mL urine/kg body weight/day and show urine osmolality >290 mosmol/kg and serum Na<sup>+</sup> >150 mmol/L. n.d., not determined.

<sup>b</sup>Patient 6 was also genotyped for aquaporin 2, no protein changing mutations were found.

**Immunoﬂuorescence studies**

Immunoﬂuorescence studies were carried out to examine the sub-cellular distribution of the wild-type and mutant AVPR2. Seventy-two hours after transfection, HEK293 cells were ﬁxed and probed with an anti-HA monoclonal antibody (12CA5; Roche Applied Science, 1:1000 in PBS-T), plates were incubated for 1 h at room temperature with a peroxidase-labelled monoclonal anti-HA antibody (3F10; Roche Applied Science, 1:1000 in PBS-T). Bound antibody was detected and quantiﬁed as described above.

**Results**

**Mutational analysis**

Genomic DNA was prepared from blood samples of each patient and the complete coding region of the AVPR2 gene including the two introns was ampliﬁed by PCR. Subsequent sequence analysis revealed four novel missense mutations (Patient 1: A89P, Patient 2: G107R, Patient 3: Q174R, Patient 4: W208X) and two recurrent mutations (Patient 5: V277A, Patient 6: R337X) affecting the peptide sequence of AVPR2 (Figure 1, Table 1). In the case of Patient 6, both the mother and sister were polydipsic, showed slightly increased urine volume and decreased urinary osmolality and, therefore, were genotyped. The mother of Patient 6 carried two different mutations in the AVPR2, R337X and a 12-bp deletion ΔR247-G250. Allelic separation showed that the mutations were located on different alleles. The sister was heterozygous for the 12-bp deletion mutation. Additionally, mutational analysis of Family 6 was extended to the AQP2...
gene. All three family members shared a recurrent, silent mutation in exon 2 [S167 (TCC > TCT), rs426496] and three common single-nucleotide polymorphisms (rs410837, rs371777 and rs403201) in the third intron of the AQP2 gene. The patient and his mother had an additional heterozygous mutation (rs3741559) in the first intron, close to the splice donor site. However, no functionally relevant mutation was identified in the AQP2 gene.

**Functional characterization of mutant AVPR2**

For functional characterization, novel mutations (A89P, G107R, Q174R) were introduced into AVPR2 and mutant constructs were heterologously expressed in COS-7 cells. Following AVP stimulation, COS-7 cells expressing the wild-type AVPR2 responded with a 14-fold increase in intracellular cAMP levels (EC50: 0.52 ± 0.09 nM). In contrast, cells transfected with Q174R showed no detectable cAMP formation upon stimulation with AVP (Figure 2, Table 2). AVPR2 constructs carrying A89P and G107R mutation showed reduced efficacies, Emax values: 20 and 68% of wild-type and potencies, EC50: 88.0 ± 19.0 nM and 156.2 ± 57.0 nM, respectively (Figure 2, Table 2). The functional consequences of W208X, V277A and R337X were obvious (truncation) or have been previously investigated [27] and, therefore, were not further characterized in this study.

Because the mother of Patient 6 (carrier of the 12-bp deletion ΔR247-G250) showed mild symptoms of NDI but previous studies assumed wild-type function of this AVPR2...
variant [12, 28], functional studies were performed also with this mutant receptor. COS-7 cells expressing \( \text{AVPR2} \) showed AVP-mediated signal transduction properties comparable to wild-type receptor function (EC\(_{50} = 0.85 \pm 0.23 \text{nM}, \ E_{\text{max}} = 97.3 \pm 12.1 \% \) of wild-type \( E_{\text{max}} \)).

To further assess molecular mechanisms underlying the impaired or complete loss of receptor function of AVPR2 missense mutants, an indirect cellular ELISA was performed to estimate cell surface expression of the epitope-tagged receptor variants [16]. Additionally, the total cellular receptor expression was determined using a sandwich ELISA [6, 29]. All AVPR2 missense mutants showed significantly reduced cell surface expression levels (Table 2). Only the deletion mutant \( \text{AVPR2} \) was delivered to the plasma membrane properly and showed expression levels equivalent to the wild-type receptor (Table 2). All AVPR2 mutants had similar levels of total cellular expression compared to the wild-type receptor (Table 2). This indicated that A89P, G107R and Q174R were properly synthesized but intracellularly retained.

<table>
<thead>
<tr>
<th>Transfected construct</th>
<th>cAMP accumulation</th>
<th>Cell surface expression</th>
<th>Total cellular expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E_{\text{max}} ) (% wt)</td>
<td>EC(_{50}) (nM)</td>
<td>(% wt)</td>
</tr>
<tr>
<td>AVPR2 (wt)</td>
<td>100 (20)</td>
<td>0.52 ± 0.09 (20)</td>
<td>100 (22)</td>
</tr>
<tr>
<td>A89P</td>
<td>18.8 ± 2.8 (4)</td>
<td>78.0 ± 19.0 (4)</td>
<td>13.6 ± 3.2 (4)</td>
</tr>
<tr>
<td>G107R</td>
<td>67.7 ± 5.5 (6)</td>
<td>156.2 ± 57.0 (5)</td>
<td>52.3 ± 6.4 (8)</td>
</tr>
<tr>
<td>Q174R</td>
<td>0 (^b)</td>
<td>n.d.</td>
<td>17.9 ± 2.6 (5)</td>
</tr>
<tr>
<td>( \Delta 247-G250 )</td>
<td>97.3 ± 12.1 (4)</td>
<td>0.85 ± 0.23 (4)</td>
<td>114.4 ± 2.6 (8)</td>
</tr>
</tbody>
</table>

\(^b\)No significant increase in cAMP above basal levels.

Fig. 3. Sub-cellular localization of wild-type and mutant AVPR2 in HEK293 cells. HEK293 cells grown on glass cover slips were transfected with epitope-tagged wild-type (wt) and mutant AVPR2 constructs. After 72 h, immunofluorescence studies were carried out with intact (non-permeabilized) and permeabilized cells. Cells were treated with a monoclonal anti-HA antibody and then incubated with an fluorescein isothiocyanate-labelled secondary antibody (green). Nuclei were stained with DAPI (blue). For specific staining of the ER, the plasmid pDsRed2-ER was co-transfected (red). The intracellular co-localization of the ER and the respective receptor constructs is shown as yellow–orange overlay. Confocal immunofluorescence pictures are representative of three independent experiments.

Table 2. Functional characterization of mutant AVPR2 receptors

<table>
<thead>
<tr>
<th>Transfected construct</th>
<th>cAMP accumulation</th>
<th>Cell surface expression</th>
<th>Total cellular expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E_{\text{max}} ) (% wt)</td>
<td>EC(_{50}) (nM)</td>
<td>(% wt)</td>
</tr>
<tr>
<td>AVPR2 (wt)</td>
<td>100 (20)</td>
<td>0.52 ± 0.09 (20)</td>
<td>100 (22)</td>
</tr>
<tr>
<td>A89P</td>
<td>18.8 ± 2.8 (4)</td>
<td>78.0 ± 19.0 (4)</td>
<td>13.6 ± 3.2 (4)</td>
</tr>
<tr>
<td>G107R</td>
<td>67.7 ± 5.5 (6)</td>
<td>156.2 ± 57.0 (5)</td>
<td>52.3 ± 6.4 (8)</td>
</tr>
<tr>
<td>Q174R</td>
<td>0 (^b)</td>
<td>n.d.</td>
<td>17.9 ± 2.6 (5)</td>
</tr>
<tr>
<td>( \Delta 247-G250 )</td>
<td>97.3 ± 12.1 (4)</td>
<td>0.85 ± 0.23 (4)</td>
<td>114.4 ± 2.6 (8)</td>
</tr>
</tbody>
</table>

\(^b\)For functional studies, COS-7 cells were transiently transfected with wild-type (wt) or mutant AVPR2 constructs. Non-radioactive cAMP assays were carried out as described, and \( E_{\text{max}} \) and EC\(_{50}\) values were obtained from AVP concentration–response curves (1 fM–10 \( \mu M \) AVP) using GraphPad Prism. Data are given as mean ± SEM of independent experiments, each carried out in duplicate. The number of independent experiments is indicated in parentheses. For each experiment, cAMP levels of 10 \( \mu M \) AVP-stimulated wt AVPR2 (32.75 ± 5.71 amol/cell) served as reference \( E_{\text{max}} \) value and was set 100%. For expression studies, cell surface and sandwich ELISA were used to measure cell surface and total cellular expression levels, respectively. Specific optical density (OD) readings (OD value of double HA/FLAG-tagged AVPR2 constructs minus OD value of GFP-transfected cells) are given as percentage of double HA/FLAG-tagged wt AVPR2. For the cell surface ELISA, the non-specific OD value (GFP) was 0.011 ± 0.006 (set 0%) and the OD value of wt AVPR2 was 0.791 ± 0.127 (set 100%). OD readings of 0.033 ± 0.007 (set 0%) and 0.714 ± 0.103 (set 100%) were found in sandwich ELISA for the negative control GFP and the wt AVPR2, respectively. The number of independent experiments, each carried out in triplicate, is given in parentheses. n.d., not determinable with sufficient accuracy.

\([\text{12, 28}]\)
To evaluate whether the reduced number of cell surface receptors is responsible for the loss of efficacy and potency of the A89P and G107R mutations, expression of the wild-type receptor was reduced to cell surface expression levels comparable to those observed for mutant receptors. Thus, following transient transfection of COS-7 cells with different amounts of wild-type DNA (serial 1:5 dilution down to 1/3125 of regular amount), cell surface expression levels and AVP-stimulated cAMP formation were determined. As expected, expression levels decreased with decreasing amounts of transfected DNA (data not shown). The efficacy of wild-type AVPR2 correlated with the cell surface expression \((P < 0.0001)\). However, no correlation of the receptor potency (EC50 values) and receptor expression level was seen \((P = 0.4167)\). Figure 2 shows exemplarily the AVP concentration–response curve for the 1:125 dilution of wild-type DNA, which displayed similar expression levels (49.0 ± 8.0%) as the G107R mutant. Hence, the observed reduction in potency of A89P and G107R is not caused by a reduced number of surface receptors but is most likely due to a reduced affinity for AVP.

We performed immunofluorescence experiments with the epitope-tagged wild-type and mutant AVPR2 constructs on intact and permeabilized HEK293 cells to analyse whether the reduced cell surface expression combined with an unchanged total cellular expression is caused by intracellular retention, as a result of inappropriate folding or by constitutive internalization [30]. As expected, cells expressing the wild-type AVPR2 and the deletion mutant ΔR247-G250 displayed intensive staining of the cell surface (Figure 3). For A89P and Q174R (showing basically no surface expression in the ELISA) as well as for the G107R mutant (displaying ~50% of wild-type expression in the cell surface ELISA after all), no specific immunofluorescence of the plasma membrane was found (Figure 3). Immunofluorescence studies on permeabilized cells revealed co-localization of A89P, G107R and Q174R with the ER (Figure 3). Internalization vesicles, as signs of constitutive internalization [30], were not observed. Therefore, full (A89P, Q174R) or partial (G107R) intracellular retention of mutants was most likely due to improper folding and/or trafficking to the plasma membrane.

**Discussion**

Genetic analysis of the AVPR2 and AQP2 genes is currently the standard in diagnosis of NDI and for genetic counselling of the families. Additionally, the functional relevance of mutations found is studied in an *in vitro* expression system followed by functional testing of the mutant receptors. Molecular screening of six NDI patients and their families revealed four novel mutations and three recurrent mutations affecting the AVPR2 coding sequence. Second messenger assays and immunological studies of the novel missense mutations (A89P, G107R, Q174R) confirmed the clinical diagnosis of NDI.

Sequencing of the AVPR2 gene of NDI Patient 1 led to identification of a novel missense mutation A89P within the highly conserved second transmembrane domain (TMD2). Evolutionary comparison of this amino acid position revealed complete conservation of Position 89 in >80 mammalian AVPR2 orthologs (Figure 1). Moreover, this position is fully preserved within all available sequences of mammalian vasopressin/oxytocin receptor family members and highlights the functional importance of the alanine residue for the maintenance of receptor structure and function. But,
despite the high conservation of Position 89, the mutational substitution by proline may also explain complete loss-of-function. Prolines are known to kink and, therefore, disturb the α-helical structure. It is reasonable to assume that A89P leads to misfolding of TMD2 and consequently to the observed intracellular retention of the receptor protein. Similarly, the NDI-causing mutation A98P, also located in TMD2, induces complete intracellular retention of the mutant receptor protein [31]. Interestingly, molecular diagnosis of NDI in Patient 1 occurred at an age of 57. This patient did not receive any medication despite living in the hot climate of Iran and lifelong polyuria.

Likewise, the other newly identified missense mutations, G107R and Q174R, both affect amino acid residues that are fully conserved within mammalian AVPR2 sequences (Figure 1) and within sequences of other vasopressin/oxytocin receptors indicating a pivotal role of these residues in receptor folding and/or function. Substitution of glycine at Position 107 with an arginine causes a change from a neutral to a positively charged amino acid. Another NDI-causing missense mutation affecting the same amino acid position, G107E (g.681G>A), was previously identified and described [32]. This mutation resulting in a change of polarity from a small neutral residue to an acidic residue was predicted to cause moderate impairment of protein function. Here, COS-7 cells expressing the mutant G107R receptor showed partially impaired AVP signalling with half maximum response compared to wild-type AVPR2 and a shift in the EC₅₀ value to >200-fold higher AVP concentrations. Immunological studies revealed a partial reduction of cell surface expression caused by retention in the ER, which explains the decreased sensitivity of the transfected cells to AVP. G107 is located in the first extracellular loop (ECL1). Several residues in this extracellular receptor loop were proposed to be involved in ligand binding and specificity [6, 33, 34]. Moreover, structural changes in the ECL1 were shown to be responsible for differences in cell surface expression [35]. Therefore, it is reasonable that the highly conserved G107 is important for proper folding and trafficking of AVPR2.

The missense mutation Q174R found in Patient 3 is located in TMD4 (Figure 1), one of the lesser evolutionary conserved TMD [10]. This AVPR2 mutant is characterized by a complete loss of receptor function with no detectable stimulation of cAMP formation and basically, no receptor expression at the cell surface. Previously, a different missense mutation at Position 174 resulting in a substitution by proline may also explain complete loss-of-function [12, 27]. However, R337X can be functionally rescued by pharmacological chaperones and by aminoglycoside-induced stop codon over-read [23, 37]. Clinical investigation of the family members revealed mild symptoms of NDI for both the mother and sister of Patient 6. The mother was compound heterozygous for R337X and ∆R247-G250. NDI symptoms of the mother can be explained by skewed X-inactivation [23, 26, 38, 39]. However, the sister of Patient 6 carried only ∆R247-G250, and the AQP2 alleles showed no functionally relevant mutations. We and others have demonstrated that ∆R247-G250 has no negative effects on ligand binding and cAMP accumulation in in vitro assays [12, 18, 28]. Furthermore, the sequence of the deleted region in ICL3 and its length are evolutionary not conserved (see Figure 4) [10]. The cause of the mild polyuria observed in the sister of Patient 6 remains unclear.

Skewed X chromosome inactivation is most likely the cause of NDI in the female Patient 4 carrying W208X. This mutation leads to a premature stop of the polypeptide chain at the N-terminal end of TMD4. Function of this truncated protein is very unlikely as shown for a very similar and closely located mutation W200X, which did not signal and interfere with proper receptor trafficking [23].

In conclusion, following molecular analysis of six NDI patients four novel (A89P, G107R, Q174R, W208X) and three recurrent (V277A, R337X/∆R247-G250) mutations in the AVPR2 gene were found responsible for clinical symptoms. Our study again demonstrated that the combination of functional and evolutionary data is beneficial for interpretation of clinically relevant mutations and provides useful information for structure–function relationship in GPCR.

Acknowledgements. We are very grateful to Susann Lautenschläger und Petra Krumohlz for technical assistance. Deutsche Forschungsgemeinschaft, Bundesministerium für Bildung und Forschung (IFB) and the State of Saxony (LIFE).

Conflict of interest statement. None declared.

References


