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REVIEW

Congenital nephrogenic diabetes insipidus: the current state of affairs

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Abstract The anti-diuretic hormone arginine vasopressin (AVP) is released from the pituitary upon hypovolemia or hypernatremia, and regulates water reabsorption in the renal collecting duct principal cells. Binding of AVP to the arginine vasopressin receptor type 2 (AVPR2) in the basolateral membrane leads to translocation of aquaporin 2 (AQP2) water channels to the apical membrane of the collecting duct principal cells, inducing water permeability of the membrane. This results in water reabsorption from the pro-urine into the medullary interstitium following an osmotic gradient. Congenital nephrogenic diabetes insipidus (NDI) is a disorder associated with mutations in either the *AVPR2* or *AQP2* gene, causing the inability of patients to concentrate their pro-urine, which leads to a high risk of dehydration. This review focuses on the current knowledge regarding the cell biological aspects of congenital X-linked, autosomal-recessive and autosomal-dominant NDI while specifically addressing the latest developments in the field. Based on deepened mechanistic understanding, new therapeutic strategies are currently being explored, which we also discuss here.

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Introduction

Due to the importance of water homeostasis in the human body, disorders that interfere with proper urine concentrating ability can easily be life-threatening, especially in children. One such disease is nephrogenic diabetes insipidus (NDI). Here, we focus on the inherited form of this syndrome and describe the current knowledge concerning clinical symptoms, inheritance patterns, and the associated genes and their known disease-causing mutations. We then address the molecular and cellular mechanisms leading to the disease and finally discuss the latest developments aiming to find a cure for this disorder.

Congenital nephrogenic diabetes insipidus

The congenital form of nephrogenic diabetes insipidus (NDI) is a rare inherited disorder, characterized by insensitivity of the distal nephron to the antidiuretic effects of the neurohypophyseal hormone arginine vasopressin (AVP). As a consequence, the kidney loses its ability to concentrate urine, which may lead to severe dehydration and electrolyte imbalance (hypernatremia and hyperchloremia). Patients with NDI have normal birth weight and pregnancies are sometimes complicated by polyhydramnios. The urine concentrating defect in NDI is present from birth, and manifestations of the disorder generally emerge within the first weeks of life. With breast milk feedings, infants usually thrive and do not develop signs of dehydration. This is because human milk has a low salt and protein content,

and therefore a low renal osmolar load. With cows' milk formula feedings, the osmolar load on the kidney increases, resulting in an increased demand for free water. This is not provided by oral feeding and, therefore, hypernatremic dehydration appears. Irritability, poor feeding, and poor weight gain are usually the initial symptoms. Patients are eager to suck but may vomit during or shortly after the feeding. Dehydration is evidenced by dryness of the skin, loss of normal skin turgor, recessed eyeballs, increased periorbital folding, depression of the anterior fontanel, and a scaphoid abdomen. Intermittent high fever is a common complication of the dehydrated state, predominantly in very young children. Seizures can occur but are rare and most often seen during therapy, particularly if rehydration proceeds too rapidly. Constipation is a common symptom in children with NDI. Nocturia and nocturnal enuresis are complaints later in childhood.

Untreated, most patients fail to grow normally. In a retrospective study of 30 male NDI patients, most children grew below the 50th percentile, the majority having standard deviation (SD) scores lower than −1 [1]. Catch-up growth occurs at least in some patients after normalization of water and electrolyte balance, especially in those with adherence to treatment. Bone age is generally not delayed. Weight-for-height SD scores are initially low, followed by global normalization at school age [1]. Initial feeding problems and the ingestion of large amounts of low-caloric fluid resulting in a decreased appetite may play roles in failure to thrive seen in NDI. Furthermore, it is possible that repeated episodes of dehydration have some as yet undetermined negative effects on growth.

Mental retardation has long been considered an important complication of untreated NDI and assumed to be a sequel of recurrent episodes of severe brain dehydration and cerebral edema caused by overzealous attempts at rehydration [2, 3]. Additional evidence underscoring the assumption that NDI has adverse effects on the cerebrum is provided by several reports describing intracranial calcifications in NDI patients [4, 5]. Such lesions are generally considered to be the result of hemorrhage or necrosis. Most of the reported patients with cerebral calcifications were mentally retarded. Nowadays, mental retardation is rare due to earlier recognition and treatment of NDI. Reliable estimates of the current frequency of mental retardation under modern treatment are unknown, but in the largest psychometric study ever reported, only two of the 17 male NDI patients tested (aged 3–30 years) had a total intelligence quotient more than 2 SD below the norm. Fourteen patients had an intelligence score within or above the normal range and one patient had a general index score between −1 and −2 SD [6]. The psychological development of NDI patients is influenced by a persistent desire for drinking and the need for frequent voiding, which compete with playing and learning. Therefore, many NDI patients are characterized by hyperactivity, distractibility, short attention span, and restlessness.

Persistent polyuria can result in the development of megacystis, trabeculated bladder wall, hydroureter, and hydronephrosis. Large-capacity hypotonic bladder dysfunction might require clean intermittent catheterization [7]. Patients should be trained to void regularly in order to assure that the maximal urinary bladder capacity remains within normal range.

The primary congenital form of NDI has to be differentiated from central diabetes insipidus (due to lack of AVP) [8] and from the secondary or acquired forms, which are much more common. In our experience, the urinary osmolality obtained after dDAVP administration in secondary disorders is always higher than in congenital NDI. Several secondary causes are listed in Table 1 [9–19]. A systematic review including other less common, mainly drug-induced, secondary NDI forms is available elsewhere [9, 20].

Physiological significance of water homeostasis

In adults, intra- and extracellular water accounts for approximately 60% of the human body's total weight. This proportion is higher in infants (70–75%), and falls to 65% in toddlers and young children. The maintenance of water and electrolyte homeostasis is vital for a vast number of physiological processes. Accordingly, the excretion of water is a highly regulated process in order to enable the organism to adapt to varied water uptake and losses and varying body-salt concentrations. In normal circumstances, urinary water loss in children is approximately 1,000 ml/m^2/day. A significant proportion of the daily water is lost insensibly, as sweat via the skin and by exhaling (300 ml/m^2/day). The feces are usually a minor route of water output [21, 22].

The average glomerular filtration rate (GFR) equals 180 l/day, which is the amount of water the renal glomerular capillaries convert to pro-urine by passing it on into the Bowman's capsule, so the total amount of urine ultimately excreted is less than 1% of the initially filtered volume. In the renal proximal tubule and the descending limb of Henle's loop, about 90% of the pro-urine is reabsorbed constitutively via aquaporin 1 water channels (AQP1). The remaining pro-urine is passed on via the distal convoluted tubule to the collecting duct, where further reuptake is regulated by blood osmolarity and blood volume.

Signaling at organism level

Various sensor systems have evolved to respond to changes in blood composition. Reduced blood volume (hypovolemia) is detected by endothelial baroreceptors, while increased blood electrolyte concentration (hypernatremia) is sensed by hypothalamic osmoreceptors. Both trigger the release of arginine-vasopressin (AVP, also: vasopressin, antidiuretic hormone, ADH) from the pituitary gland, where the hormone is stored after its synthesis in the hypothalamus [23].

Table 1 Major secondary causes of nephrogenic diabetes insipidus (NDI)

Cause	Mechanism	Comments	References
Prolonged lithium therapy (treatment of bipolar disorders)	Downregulation of AQP2, ENaC, UT-A1 and UT-B; loss of principal cells	Becomes irreversible by unknown mechanisms if left untreated	[9–12]
Hypokalemia	Reduction of AQP2 expression, reduction of major sodium transporters (NKCC2, NCC, ENaC)		[13, 14]
Hypercalcemia	Reduction of AQP2 and AQP3 expression, reduction of major sodium transporters (NaPi-2, NHE3, Na-K-ATPase, NKCC2)		[15, 16]
Low-protein diet	Reduction of AQP2 expression		[17]
Urinary tract obstruction	Decreased AQP1-4 expression, reduction of major sodium transporters (NHE3, Na-K-ATPase, NaPi-2, NKCC1, NCC), increased COX-2 levels, upregulated prostaglandin secretion	Reversible after removal of obstruction, slightly reduced urine conc. ability may be retained	[18, 19]

AQP, aquaporin; *ENaC*, epithelial sodium channels; *UT*, urea transporter

The receptor for AVP in the kidney, AVPR2, is a G-protein-coupled receptor (GPCR) expressed mainly at the basolateral site of collecting duct principal cells, where it functions as the key receptor for osmoregulation. AVPR2 has also been found on endothelial cells. There, the receptor is involved in the secretion of blood clotting and fibrinolytic factors into the bloodstream [24].

Signaling at cellular level

An overview of the AVP-dependent signaling network for the sustention of water homeostasis is shown in Fig. 1.

After release from the pituitary gland, AVP is transported via the bloodstream to the kidneys, where it binds to AVPR2 on the principal cells of the collecting duct. Upon binding of AVP, the receptor is activated and then stimulates GTP-loading of the small GTPase αG_S-subunit of its coupled trimeric G-protein, eventually leading to dissociation of the G-protein from the receptor. GTP-αG_S then can bind to the membrane-associated enzyme adenylate cyclase (AC), allosterically activating it, which results in an increased conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP in turn activates protein kinase A (PKA), leading to phosphorylation of cAMP responsive element binding protein 1 (CREB-1), Rho-GDP dissociation inhibitor (Rho-GDI) and aquaporin 2 (AQP2).

AQP2 is one of the 13 members of the aquaporin water channel family (AQP0-12), of which some are responsible for the reabsorption of water in the kidney [25]. After transcription, AQP2 is folded into its native monomeric conformation in the endoplasmic reticulum (ER) and homotetramerization takes place [26]. The tetramers are then forwarded to the Golgi apparatus, where two out of four monomers are complex *N*-glycosylated. These functional water channels are then stored in endosomal vesicles to be transported to the apical membrane [27]. Upon phosphorylation at Ser_{256} and Ser_{269}, the transport equilibrium between AQP2 shuttling to the apical membrane and re-internalization by clathrin-mediated endocytosis shifts and the AQP2 concentration in the apical membrane rises. Thereby, the membrane that is otherwise impenetrable for water is rendered permeable [28]. A mean of three out of four AQP2 monomers of an AQP2 homotetramer is required to be phosphorylated to induce this change in subcellular localization [28]. However, it has not yet been established whether two phosphorylated monomers could also induce translocation on the single-molecule level.

Apical membrane targeting is achieved by specific interaction between t-(target) and v-(vesicular) SNARE proteins. The apical membrane-specific t-SNARE protein is syntaxin-4 [29], which interacts specifically with the v-SNARE protein VAMP2, located on the cytoplasmic side of AQP2-containing vesicles [30–34]. The fusion complex is unwound and the v- and t-SNAREs are recycled by the AAA-type ATPase NSF. AQP2-containing vesicles belong to the early endosomal system [35].

Counterbalancing increased expression on the plasma membrane, AQP2 is constitutively internalized. Endocytosis is regulated by short-chain ubiquitylation at K270 in the AQP2 C-terminal tail. While the identity of the involved ubiquitin E3 ligase is at present still unknown, first studies have identified several candidates for further investigation [36].

To be available for recycling, AQP2-containing endosomes have to be redistributed to the perinuclear region. This process is mediated by minus-end-directed dynein-dependent transport along microtubules [37, 38]. Adaptor proteins in the endosomal membrane are recognized by the dynein-associated dynactin complex [35]. Specificity of the endocytotic AQP2 internalization is mediated by the Rab5 protein, an effector-binding factor involved in plasma membrane-to-early endosome transport [39]. From the endosomal system – early/late endosomes and/or multivesicular bodies (MVBs) – AQP2 is either recycled by the Rab11-dependent slow recycling pathway or marked for lysosomal degradation [40]. Prolonged K270 ubiquitylation induces

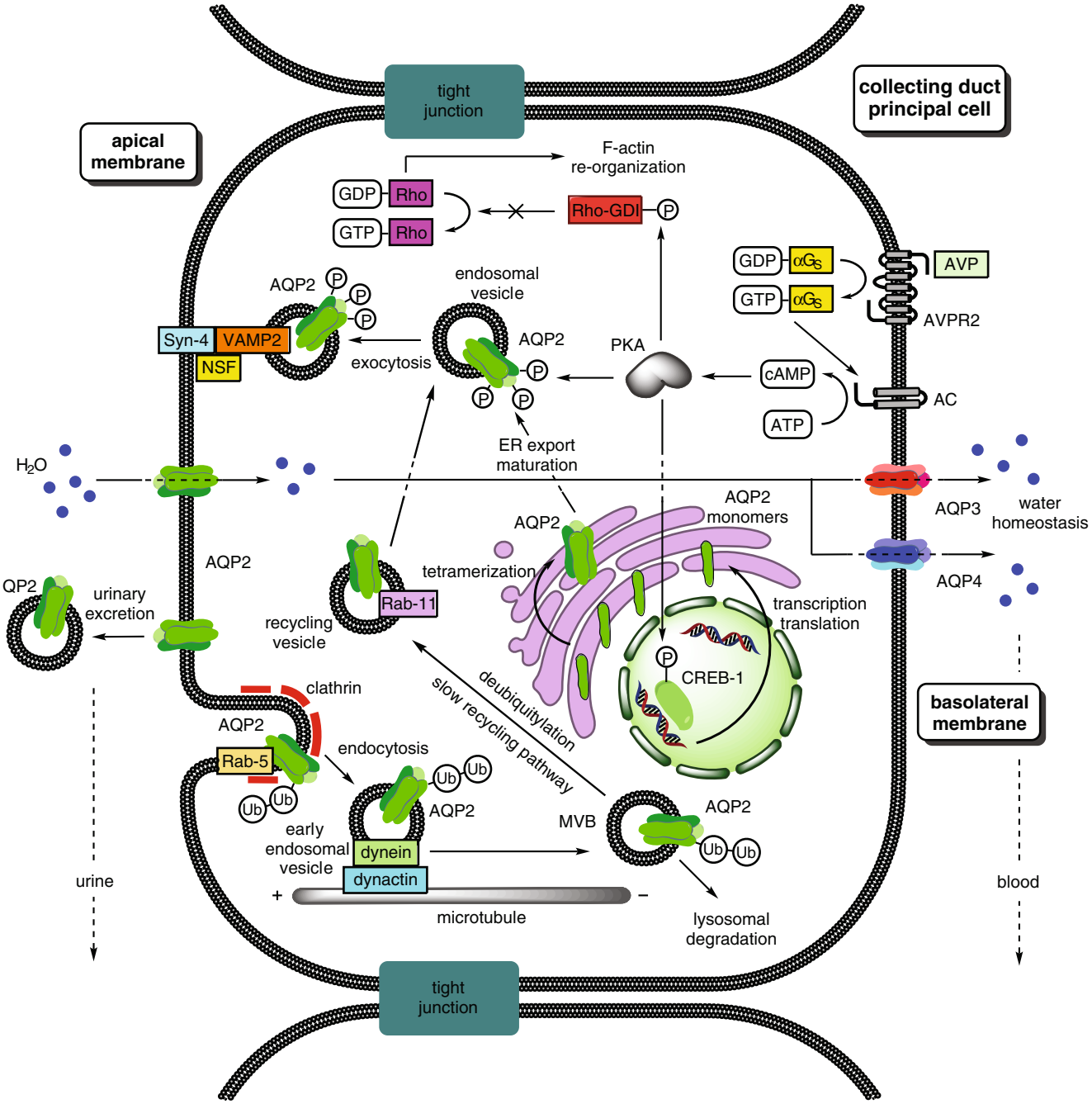

Fig. 1 Intracellular signal transduction pathway initiated by AVP-binding to AVPR2. Via activation of adenylate cyclase (AC) and cAMP-production stimulation, protein kinase A (PKA) is activated and phosphorylates its target proteins CREB-1, AQP2, and Rho-GDI. The transcription factor CREB-1-p stimulates AQP2 transcription, Rho-GDI-p initiates actin reorganization required for AQP2 transport and AQP2-p homotetramers are transported to the apical membrane. There they render the membrane permeable for water, which is reabsorbed from the passing pro-urine and transported back into the bloodstream by AQP3 and AQP4. Rab5-mediated AQP2 endocytosis by clathrin-coated vesicles is triggered by short-chain ubiquitylation and leads to termination of the response. Internalized AQP2 vesicles are transported to early and late endosomes as well as multivesicular bodies (MVBs) by (-)-end directed transport along microtubules for storage. From MVBs, they can then either be lysosomally degraded (prolonged ubiquitylation) or recycled via the Rab-11-dependent slow recycling pathway (requires deubiquitylation)

MVB trafficking and localization to internal vesicles of MVBs followed by lysosomal degradation, while deubiquitylation increases localization to early endosomes and the limiting membrane of MVBs and enables AQP2 recycling [41].

Rho-GDI inhibits dissociation of GDP from Rho small monomeric GTPase, thereby downregulating activation of Rho and probably leading to a reorganization of the actin cytoskeleton that is required for AQP2

transport to and accumulation at the apical membrane [42, 43].

Additionally, phosphorylation of the CREB-1 transcription factor stimulates synthesis of AQP2 by binding to the AQP2 gene promoter and activating its transcription, which increases cellular AQP2 levels [44]. Since transcriptional activation generally leads to a delayed response, this route contributes to longer-lasting AVP sensitization rather than immediate water permeability.

Driven by the osmotic gradient, which is built up in the kidney hypertonic medullary interstitium, water subsequently enters the collecting duct principal cells via the apical membrane, and is passed on into the blood stream by constitutionally expressed aquaporin type 3 and 4 water channels located in the basolateral membrane [45].

Activation of AVPR2 by AVP binding therefore leads to increased water reabsorption in the collecting duct of the kidneys, diluting the blood and restoring osmotic homeostasis.

Besides its function in stimulating water reabsorption via AQP2 water channels, AVP upregulates reabsorption of sodium from the urine passing the collecting duct via activation of epithelial sodium channels (ENaC), which leads to increased sodium retention [46]. This may be an important mechanism, although the major contribution to ENaC-mediated regulation of sodium reabsorption is exerted by the aldosterone signaling pathway of the renin-angiotensin-aldosterone system (RAAS) [47]. Additionally, AVP stimulates urea retention by activating the urea transporter A1 (UT-A1) [48].

Upon restoration of body water homeostasis, AVP levels in the blood decrease and consequently the trafficking equilibrium shifts to AQP2 re-internalization and redistribution to endosomal vesicles, whereby water reabsorption is reduced [49]. Due to this regulated fluid-absorption system, the body is able to adapt to periods of water shortage and excess water intake.

Genetics and Molecular Mechanisms

Congenital NDI is genetically heterogeneous [50]. In most cases, the disorder is inherited in an X-linked recessive fashion and is caused by mutations in the vasopressin type-2 receptor gene (AVPR2). About 10% of patients show an autosomal-recessive inheritance as a result of mutations in the aquaporin-2 (AQP2) gene. A few families have been described with clear autosomal-dominant inheritance of NDI in which AQP2 mutations have been identified as well. The different genetic forms will be discussed in detail below.

X-linked recessive NDI

The X-linked form of NDI (XNDI) is found in about 90% of NDI patients and is caused by inactivating mutations in the AVPR2 gene [51–53]. Loss of function of AVPR2 disrupts water reabsorption in the collecting duct due to a loss of AVP signaling-dependent AQP2 expression and transport to the apical membrane.

Almost all XNDI patients are male, which is common for X-linked recessive diseases. In women carrying an AVPR2 mutation, phenotypic expression of NDI can be either totally absent, partially present, or complete. The most likely explanation for the existence of different phenotypes in NDI carriers is skewed X-inactivation [54, 55].

Arginine-Vasopressin Receptor Type 2 (AVPR2)

The AVPR2 gene is localized on the X-chromosome at locus Xq28. The gene consists of two introns and three exons and two isoforms are known that are generated by alternative splicing (v2a, v2b) [56]. The gene product is a seven-transmembrane domain receptor belonging to the G-protein-coupled receptor (GPCR) superfamily. The core protein consists of 371 amino acids with a total weight of 40 kDa (SWISS-PROT entry: v2r_human) [57]. The N-terminus is located on the extracellular side and the C-terminal domain resides in the cytoplasm, rendering AVPR2 a type IV-B transmembrane protein [58, 59]. Asn22, located in the N-terminal extracellular domain, is complex glycosylated. After transcription and mRNA processing, the primary protein is co-translationally inserted into the ER membrane with its extracellular domains located lumenally. Subsequently, folding of the extra- and intracellular domains is assisted by ER and cytosolic chaperones and *N*-glycosylation starts. Folding also involves formation of a disulfide bond (C_{112} and C_{192}) between the first and second extracellular loops [60–62], which is assisted by protein disulfide isomerase. Via the exocytotic pathway, the correctly folded and glycosylated receptor is transported to the Golgi apparatus, where the high-mannose glycan added in the ER is further processed into a complex glycan [63]. Additionally, it has been suggested that during maturation, AVPR2 also becomes *O*-glycosylated at one or more serines and/or threonines in the extracellular N-terminal tail, but definite sites remain to be identified [64, 65]. Next, the maturated receptor travels through the trans-Golgi network (TGN) to the basolateral cell membrane and is inserted there. The splice variant v2b has been implicated with a downregulatory dominant negative effect on v2a trafficking, leading to intracellular retention by hetero-oligomerization [66, 67]. The natural ligand of AVPR2 is AVP, but the receptor can also respond to a variety of other natural and synthetic molecules with varying affinities [68]. The most important alternative natural agonists are lysovasopressin (LVP) and oxytocin [69], the most widely used synthetic agonist is desmopressin (dDAVP) [70].

XNDI mutations

Currently, 221 mutations in the AVPR2 gene that cause XNDI and at least 21 variations that do not lead to disease are known (data combined from Spanakis et al. 2008 [71] and the Human Gene Mutation Database (HGMD [72, 73], accessed 07-09-2011). These non-disease-causing mutations are most likely polymorphisms that can be found in more than 1% of the population. The G12E mutation has for example also been found in non-affected individuals, suggesting that it belongs to this class of polymorphisms that do not exert a significant effect on proper functioning of the receptor [74].

The disease-causing mutations where extensively studied in 2008 by Spanakis et al. in 326 families [71]. They ordered the 211 mutations that were known by then into 15 different types, namely: missense, nonsense, frameshift caused by deletions ≤2 nucleotides, between ≥2 and <50 nucleotides, and ≥50 nucleotides, inframe deletions, frameshift due to ≤2 nucleotides and >2 nucleotides insertions, inframe insertions, duplications, splice site mutations, double and multiple mutations, compound heterozygotes (different mutations on both alleles) and complex gene rearrangements. Of all mutations, 48% are missense mutations. The second most found mutation in XNDI families was nonsense, with an occurrence of >13%. Small frameshift deletions are the third most common mutation found in XNDI families, they are responsible for over 10% of the XNDI-causing mutations.

The frequencies of these mutations are still valid since presently only ten new mutations have been found (small deletion: 222del1 [75], 303del1 [76]; large deletion: 11.2 kb incl. entire gene, described at genomic DNA level [77]; nonsense: W156X [78], W200X [79]; missense: L57R [80], Y128D [81], P173L [82], Q174H [83], M311V [84]).

AVPR2 variants and mutations seem to be present in all ethnical groups tested with no preference of one mutation/variant for any ethnic group over others.

Mutation classification and XNDI mechanisms

Mutant forms of the AVPR2 have been reported to be differentially processed, transported, and inserted into the plasma membrane, and can therefore be classified into four distinct groups, based on their functionality and intracellular localization (Table 2). This classification is based on the one developed for the cystic fibrosis-associated CFTR gene by Welsh et al. in 1993 [86] with only class IV adjusted to reflect AVPR2-relevant properties, and may easily be adapted to other proteins as well, including AQP2.

Assignation of any mutant to one of the classes described below is generally achieved through sequence analysis combined with functional mutant analysis either in vitro (cellular assays) or, more rarely, in vivo (mouse models). Functional analyses include expression levels, ligand binding, and subcellular localization assays.

Mutations are assigned to class I if they interfere with proper transcription, mRNA processing, and translation. Typical examples are promoter alterations, splice site mutations leading to exon skipping, intron retention or aberrant splicing, mRNA processing signal mutations causing unstable or untranslated mRNA, and frame-shift or nonsense mutations that prematurely terminate translation and give rise to shortened gene products. The truncated mutants are then rapidly degraded, and therefore cannot be expressed on the cellular membrane. This renders the cell insensitive to AVP. Examples of such class I mutants include the 733-738insG mutation, for which it has been shown that, though mRNA levels are unaffected, no protein is expressed. This indicates interference with translation or very rapid degradation of defective translation products [87]. Similarly, Ala et al. failed to detect translation products of the 700delC mutant upon expression in COS-7 cells [88]. The mutations W284X [89], W71X, and R337X [90] (premature stop codon) and 458delG [91] (frame-shift, 161X) are other examples of class I alterations. Mutants with premature stop codons in the late C-terminal tail might not be subject to increased mRNA degradation, but may be misfolded and retained in the ER. As such, they exhibit more class II-like features (see below).

Class II mutations retain the correct translational frame and therefore full-length protein is expressed. Mature receptors can either be functional or non-functional; however, class II mutants are structurally disrupted and therefore are not able to fold into their native conformation. The ER quality control system accordingly recognizes the aberrant

Table 2 Classification of AVPR2 mutants

Class	Mechanism	Mutation type	Examples	References
I	Interference with transcription, mRNA processing, translation	Missense, splice site, frame-shift, early nonsense	W71X, 458delG	[90, 91]
II	Aberrant folding and intracellular (ER) retention	Missense, in-frame del/ins, last exon nonsense	S167T, others [71]	[85]
IIIa	Loss of G-protein-binding site	Missense	D85N, P322S	[95]
IIIb	Loss of AVP-binding site	Missense	delR202	[88]
IV	Defects in intracellular trafficking	Missense	R137H	[96]

folding, and translational products are retained in the ER and incompletely processed. Finally, the quality control mechanism activates the ER-associated degradation (ERAD) pathway leading to proteasomal degradation of the mutant receptors. Prolonged interaction with calnexin, a molecular ER chaperone known to be involved in protein folding and ER quality control, has been reported for some AVPR2 mutants [92]. This suggests the involvement of calnexin in AVPR2 quality control and that dissociation from this chaperone is one of the hurdles for AVPR2 to take before continuing its transport to the Golgi complex. A single example of retention during receptor maturation in post-ER compartments such as the Golgi apparatus has also been found [93]. Accumulation of the Y205C mutant was detected in spite of low expression levels in the ER-Golgi intermediate compartment (ERGIC) by co-staining with ERGIC-53, indicating that ERGIC localization was not an artifact of overloading ER quality control. The amount of retention and degradation varies within this class, since different mutations affect protein folding to a different extent, sometimes allowing partial transport of at least partially active receptors to the plasma membrane. Class II mutations are typically insertions or deletions of one or more nucleotide triplets and missense mutations, slightly altering the amino acid sequence, but retaining the reading frame. Class II mutations form the most important cause of NDI [71].

Class III mutations do not lead to misfolded receptors, but interfere with proper interaction with their natural ligands, thereby causing reduced/abolished signaling [94].

Class III mutations can be subdivided into two minor groups. IIIa mutations interfere with binding of or signal transduction to the coupled trimeric G-protein, especially the αG_S-subunit, and thereby inhibit or reduce activation of the G-protein. Mutations in this class also are missense mutations and in-frame deletions/insertions, which generally are located in transmembrane or intracellular domains. Examples are the D85N and P322S mutations [95].

If AVP binding is disrupted or reduced by the mutation, the mutant belongs to class IIIb. Similar to class IIIa mutations, these substitutions include missense and small inframe insertions or deletions and result in full-length expressed protein. Class IIIb variations are found in extracellular domains and transmembrane regions. Mutated positions are thought to be located within or close to the AVP binding site, such as delR202 [88].

The last type of XNDI causing defects, class IV, is assigned to all mutations that neither interfere with protein synthesis and maturation, nor with ligand binding, but affect other aspects of protein function. We here assign class IV to mutants that differ from wild-type protein in their intracellular localization due to altering signal sequences vital for correct intracellular trafficking. The best characterized example is the R137H missense point mutation, which is located in the DRY/H motive, highly conserved in GPCRs. The effect of this mutation is constitutive endocytotic internalization of AVPR2 [96], leading to reduced expression of the receptor in the plasma membrane and thereby reduced adenylate cyclase-dependent cAMP signaling upon AVP stimulation.

Some mutants exhibit properties of two or more classes of mutations, indicating that single mutations can affect multiple features of the receptor. For example, a mutant that is partially retained in the ER may still be partially transported to the plasma membrane, where it then shows a reduced affinity for either AVP or its coupled G-protein, thus having characteristics of class II and IIIa or IIIb, respectively.

Autosomal NDI forms

Autosomal-recessive NDI (ARNDI)

Approximately 10% of NDI patients have the autosomal-recessive form of NDI. Similar to AVPR2 inactivating mutations, autosomal-recessive alterations in AQP2 disrupt proper synthesis, functioning, or localization of the gene product, rendering renal collecting duct principal cells irresponsive to AVP stimulation [97].

Aquaporin 2 (AQP2)

The AQP2 gene involved in autosomal NDI is located at chromosome 12q13. It is part of an aquaporin water channel gene cluster, where it maps together with AQP0, AQP5, and AQP6. The gene codes for the 271 amino acid AQP2 protein, which consists of six transmembrane domains connected by five loops and intracellularly located N- and C- termini (type IV-A TM protein) [98]. Tetramerization takes place during and/or after folding of monomers in the ER. Subsequently, high-mannose glycans are attached to Asn_{123} of one or two monomers of a homotetramer [99] and ER export is initiated. During maturation in the Golgi network, the sugar moieties of the high-mannose glycosylated monomers of each AQP2 tetramer are further processed to complex glycans [100]. Upon mutation of the *N*-glycosylation acceptor site (AQP2-N123Q) plasma membrane expression of AQP2 was found in *X. laevis* oocytes and accumulation in the Golgi complex of mammalian cells was shown [26]. These results suggest that glycosylation of AQP2 is not required for passing ER quality control and ER export, but is essential for post-Golgi trafficking in mammalian cells.

AQP2 contains a consensus site for PKA phosphorylation at Ser_{256} [101] and phosphorylation sites controlled by unknown kinases at Ser_{261}, Ser_{264}, and Ser_{269} [102], all located in the cytoplasmic C-terminal tail. Upon phosphorylation of Ser_{256}, phosphorylation of Ser_{269} is triggered [103,

104] and AQP2 tetramers are translocated to and inserted in the apical membrane, where they render the membrane water permeable. However, recent mass spectrometry data from Xie et al. [105] suggest otherwise: Ser_{269} was found to be the key regulator in shifting the equilibrium between apical membrane targeting and re-internalization. High basal Ser_{256} phosphorylation levels were found even in the absence of AVP stimulation and suggested to be required for AVP-dependent induction of Ser_{269} phosphorylation, which in turn downregulates AQP2 internalization. In addition to regulation of Ser_{256} and Ser_{269}, Ser_{261} is dephosphorylated [106] and phosphorylation levels at Ser_{264} increase [103] upon AVP treatment. However, these last two events seem not to be necessary for translocation of AQP2 to the apical membrane. A review discussing the various, occasionally contradictory, lines of evidence currently present concerning phosphorylation (as well as other post-translational modifications) of AQP2 has recently been published [107].

The mechanism of selectivity for water of aquaporins in general has been resolved in the homologous AQP1 protein [108] and has further been strengthened by molecular dynamics modeling approaches [109, 110]. The water pore is formed between the first and sixth transmembrane domains and is lined by the intracellular B-loop and the extracellular E-loop. The residues F_{56}, R_{195}, H_{186}, and C_{189} located in the ar/R (aromatic/arginine-rich) region determine the water specificity of AQP1 [111, 112], while F_{24}, N_{76}, and N_{192} are the residues of the central asparagine/proline/alanine (NPA) region that ensure proton exclusion [113]. In the case of AQP2, the crucial residues correspond to F_{48}, R_{187}, H_{172}, and C_{181} (ar/R region) and F_{23}, N_{75}, and N_{184} (NPA region).

ARNDI mutations and mechanisms

There are 40 mutations known to give rise to ARNDI. These include 32 missense and two nonsense mutations, two 1-bp deletions, one 2-bp deletion, and three splice site mutations [HGMD [72, 73], accessed 07-09-2011]. In ARNDI, most alterations are found between the first and the last transmembrane domain of AQP2. Whereas frameshift and early stopcodon variants lead to shortened translation products that are rapidly degraded, the large group of missense mutations predominantly causes proteins to be folded aberrantly. As for AVPR2, for those mutants this results in ER retention due to extended interaction times with ER chaperones and eventually proteasomal degradation. However, some mutants retain intrinsic functionality, and would be able to show at least partial activity when expressed in the apical membrane by means of forced transport or overexpression [99].

The elucidation of the molecular mechanisms governing water homeostasis defects in autosomal-recessive NDI patients has mainly been achieved by extensive research in cellular expression systems such as *X. laevis* oocytes for water permeability assays [114] and plasma membrane expression levels, and polarized MDCK cells for subcellular localization studies. More recently, mouse models have contributed to the confirmation of results obtained earlier in vitro and their comparability to in vivo situations [115, 116].

Investigation of subcellular localization has revealed ER accumulation of multiple ARNDI mutants by different techniques. Colocalization studies with ER-resident proteins such as the chaperone Grp94 have been used [117]. Immunocytochemistry analysis has revealed dispersed cytosolic staining in unpolarized oocytes and mice kidney cells [99, 114], and ER localization has also been demonstrated in polarized MDCK cell lines [116]. Thus, all these findings are consistent with the ER retention theory.

For most missense mutants, partial single-channel water permeability has been shown in *X. laevis* oocytes by a standard cell swelling assay [9, 118], indicating that the native conformation is disturbed only slightly. This suggests that the observed disease phenotype is generally due to aberrant subcellular localization of AQP2 rather than loss of function. As we will discuss later, this opens therapeutic options directed to restoring mutant trafficking.

Glycosylation of AQP2 is usually assessed by immunoblotting, and has revealed the occurrence of four different AQP2 forms: unglycosylated (29 kDa), ER-retained high-mannose (32 kDa), ER-exported complex glycosylated (40–45 kDa) and degradation fragments (27 kDa) [99].

Doubt has been cast by Marr et al. [99] on the earlier finding that some AQP2 mutants in NDI were correctly transported, but exhibited disrupted functionality [119]. While formerly it was found that the AQP2 mutants T125M and G175R were transported to the plasma membrane normally, but still showed no water permeability increase after cRNA injection into *X. laevis* oocytes, this could not be confirmed by Marr et al. They proposed as a possible explanation for residual plasma membrane expression the saturating effect of high expression levels of aberrantly folded AQP2 on mutant protein degradation. Therefore, the main mechanism by which AQP2 mutants cause ARNDI seems to be ER retardation and they can thus generally be classified as class II mutants. However, it cannot be excluded that reduced permeability of AQP2 mutants at least contributes to NDI phenotypes. Definitive confirmation of water channel functionality and transport requires studies in vivo or at least in mammalian cell lines without overexpression of mutant protein [120].

Whatever the case may be regarding the causativeness of reduced water channel function in NDI, there is no doubt that different mutants retain different levels of functionality when expressed in the plasma membrane [99, 114]. The characterization of this feature for each mutant is of high therapeutic importance, since it determines whether molecular chaperones (discussed later) are a valid option.

The generation of an in vivo model to study the effect of the ARNDI mutations has long been hampered by the fact that mice in contrast to humans seem not to be able to survive if AQP2 functionality is completely lost. Mice harboring homozygous T126M mutations died in early infancy [121]. In contrast, the more recent F204V mouse model [116] was able to survive to adulthood. This has been accounted to a residual transport of F204V mutant AQP2 homotetramers to the apical membrane in collecting duct principal cells, indicating that specific mutants may retain some tetramerization and transporting capability even if excessively ER-retained. A possible explanation is provided by the fact that F204V residually matured (complex glycosylated in contrast to high-mannose only) [116], which is required for plasma membrane expression, but is not essential for tetramerization [26]. It still needs to be determined whether residual ER export, glycosylation, tetramerization, and plasma membrane expression also take place for AQP2 mutants in NDI patients.

In the F204V mouse model, all common symptoms observed in humans as well as cellular behavior of expression systems (unresponsiveness to dDAVP, ER retention) could be reproduced, indicating its general similarity to a human situation.

In heterozygous mice, Lloyd et al. additionally showed that AQP2-F204V can homotetramerize as well as form heterotetramers with wt-AQP2 [116]. This is in contrast to previous findings on the R187C mutant, which could do neither [26, 99].

A single exception to the general mechanism of ER retention of AQP2 mutants in recessive NDI has been found with the AQP2-P262L mutant. Due to mechanistic overlap, we will discuss the molecular basis of ARNDI caused by this mutant below at the end of the section on autosomal-dominant NDI.

Summary autosomal-recessive NDI

Cell expression systems and mouse models have led to several theories about pathogenesis in ARNDI, some of them generally accepted, others extensively discussed. The unresponsiveness of kidney collecting duct principal cell to increased vasopressin levels is caused by the disruption of AQP2 accumulation in the apical membrane upon AVP stimulation and therefore remaining water impermeability. Except for AQP2-P262L, this is a consequence of the AQP2 depletion of endosomal storage vesicles due to failed ER export. It is commonly accepted that ER retention and subsequent degradation of AQP2 mutants is the major causative event in ARNDI caused by missense AQP2 proteins [95, 117]. While folding and maturation of AQP2 normally is facilitated by the interaction with ER chaperones, in mutants, most probably, the native conformation is destabilized due to the mutation. Followed by aberrant folding, this induces prolonged interaction with ER quality control chaperones and eventually targeting for proteasomal degradation [99, 122].

The role of glycosylation in AQP2 maturation is much less clear. While non-glycosylated AQP2 appears to be fully functional, compelling evidence has been found for the essentiality of complex glycosylation for translocation to the apical membrane: AQP2 mutants missing the N_{123} glycosylation site are trapped in the Golgi network and do not reach the apical membrane [26]. The underlying mechanism has not been elucidated yet.

Mouse models now provide tools to study possible therapeutic options and their safety in a more appropriate setting than cellular expression systems. However, it has been shown that mice in part react differently to AQP2 dysfunctionality than man does: Homozygous mutant mice are not viable for the mutants tested to the day, except for F204V [116, 121]. In contrast, NDI usually is not fatal in infants. Early lethality in mice could be a mere reflection of their high natural urine osmolarity (4,000 mOsm compared to 1,200 mOsm in humans), the high body surface/volume ratio, thin skin and high respiration rate, requiring a high fluid intake/reabsorption per kilogram body weight needed for pup survival, while survival of human newborns is a consequence of the extensive care for weak offspring in humans compared to mice. Still, a mechanistic difference cannot be excluded. Consequently, conclusions have to be looked upon with caution and critical evaluation is required.

Autosomal-dominant NDI (ADNDI)

Autosomal-dominant inheritance is the least prominent form of NDI and is responsible for <1% of NDI cases. It also involves mutations in the AQP2 gene. In contrast to autosomal-recessive NDI AQP2, dominant mutants show perfect functionality, but are not correctly transported to the apical membrane, resulting in a similar impairment of urine concentrating ability.

Yet, ADNDI patients show generally higher urine osmolalities and a residual ability to increase the osmolality of their urine slightly upon dehydration [25]. Thus, water reabsorption capacity is decreased significantly, but not completely disrupted. This usually leads to a milder phenotype than that of recessive or X-linked NDI.

ADNDI mutations and mechanisms

Currently, eight mutations causing dominant NDI inheritance are known [123–128], each of which was found within a single family. These are three missense mutations, one single-nucleotide insertion, and four small deletions (≤10 nucleotides). All mutations associated with ADNDI to the day are located in the part of exon 4 of the *AQP2* gene that

encodes the cytoplasmic C-terminus of the AQP2 protein. This component of AQP2 is not part of the functional water pore, but contains important sorting signals that govern intracellular transport of the protein [95, 125]. AQP2 tetramers containing one or more mutant forms are unable to respond properly to signals that normally induce transport to the apical membrane. Therefore, all autosomal-dominant mutants can be assigned to class IV.

The dominant phenotype of these mutations is due to their localization in the C-terminal tail of AQP2. Variations in this part of the protein do not seem to influence correct protein folding and normal maturation can occur. After ER export, both homo- and heterotetramers are formed, but tetramers containing one or more mutant AQP2 monomers show aberrant intracellular localization or insensitivity to AVP signaling-induced re-localization to the apical membrane. However, residual trafficking to the apical membrane can be detected. This is supposedly due to the fact that one-sixteenth of all tetramers formed in dominant NDI are wt-AQP2-only tetramers [129, 130].

In 2009, Moon et al. [131] reported another heterozygous mutation located in the 6th transmembrane domain of AQP2 (S216F), possibly being a novel ADNDI mutation. However, several facts cast doubt on the involvement of S216F in the dominant form of NDI. Firstly, mutation of S216 has been found earlier to lead to ARNDI (S216P, [132]). Moreover, ADNDI mutations are usually located in the C-terminal domain of AQP2 while ARNDI mutations tend to be in transmembrane domains. Lastly, the patient's parents were unaffected and could not be genotyped and thus absence of this mutation could not be proven. Therefore, we propose functional analyses of the S216F mutant in order to establish its mechanism and type of inheritance with sufficient certainty.

The loss of appropriate AQP2 heterotetramer trafficking as a downstream effect of AVP signaling is caused by several mechanisms.

As described above, transport of endosomally stored AQP2 tetramers requires phosphorylation of a mean of three out of the four monomers at S_{256}, which is part of the PKA phosphorylation consensus sequence R_{253}-R_{254}-X_{255}-S_{256}. The first mechanism by which dominant-type mutations cause irresponsiveness of AQP2 tetramers to AVP signaling involves disruption of this phosphorylation site. Through the G_{761} (cDNA) to A or T transitions, two of the known ADNDI mutations, the arginine residue at position 254 is changed into leucine or glutamine [123, 128].

Experimental evidence that these mutations indeed impair phosphorylation of AQP2 and thereby induce the NDI phenotype, has been brought about by De Mattia et al. [123] for R254L and Savelkoul et al. [128] for R254Q. In *X. laevis* oocytes and polarized MDCK cells, both mutants exhibited full functionality of AQP2 tetramers, but decreased plasma membrane expression and retention in early endosomes, even after treatment with the potent PKA stimulating reagent forskolin [123] . The artificial S256A mutant, in which the serine to be phosphorylated was replaced by alanine, showed comparable results, indicating that indeed impaired phosphorylation of S_{256} accounts for disrupted transport to the plasma membrane. Furthermore, co-precipitation of wt- and mutant AQP2 was shown, suggesting the correctness of the dominant NDI model in which AQP2 mutants capture wild-type water channels by heterotetramerization [126, 133]. The importance of phosphorylation at position 256 as a signal for apical membrane transport has further been strengthened by the fact that phosphorylation of nearby serines (S_{261}, S_{264}, and S_{269}) could not induce AQP2 translocation [101, 134], whereas the artificial AQP2-R254Q-S256D mutant, which is constitutively phosphorylated, showed increased AQP2 levels in the plasma membrane, presumably due to increased AQP2 translocation [103, 123, 135, 136]. Conversely, S_{269} phosphorylation has been implicated with AQP2 accumulation in the plasma membrane due to decreased endocytosis [103]. This example nicely visualizes how the combination of enduring research with various experimental methods and in vitro expression systems helped to identify complex biochemical matters leading to NDI.

Another mechanism identified is the misrouting of phosphorylated AQP2 tetramers by the introduction of two basolateral sorting signals. An adenosine insertion (c779-780insA) leads to a -1-frameshift that changes the last 12 amino acids of the AQP2 C-terminal tail and additionally shifts the stop codon downstream, thereby lengthening the C-tail by 14 amino acids. Moreover, it introduces two independent basolateral sorting signals, namely a leucine-based sequence surrounding the new L_{261} and an YXXØ-motif at Y_{269}QGL (X indicating random and Ø large hydrophobic residues). Accordingly, tetramers containing this mutant have been shown to translocate to the basolateral instead of apical membrane upon phosphorylation [124].

The E258K mutant has been shown to be unresponsive to AVP stimulation because the disruption of the opposite charges of E_{258} and $R_{252–254}$. The repulsion between the resulting positive charges was suggested to disrupt a structural element maintained by the naturally opposite charges that seems to be required for translocation upon phosphorylation. Instead, tetramers containing mutant AQP2 were found to be retained in the Golgi apparatus in *X. laevis* oocytes [127] and enriched in MVBs in MDCK cells [137, 138].

The mechanism by which the remaining four AQP2 mutants found in ADNDI, AQP2-812-818del, AQP2-721delG, AQP2-727delG, and AQP2-763-772del, lead to misrouting has not yet been fully uncovered. All induce a +1 frameshift that shifts the stop codon downstream leading to lengthened AQP2 (34–35 kDa compared to 29 kDa wild-type) with 61 similar C-terminal residues [125]. While the

(intra-)cellular localization of 727delG was independent of forskolin treatment [126], the 763-772del mutant has been found to translocate to the basolateral membrane upon stimulation with forskolin [133], despite the disappearance of the S_{256} phosphorylation site. The reason for basolateral misrouting remains to be established, since no experimental proof for the suggestions that the elongation of the C-terminal tail might introduce new basolateral sorting signals or function as such itself [125] has yet been provided. The dependence of this sorting on treatment with forskolin for 763-772del suggests the involvement of other phosphorylation sites, either upstream of the insertions or in the lengthened C-tail itself. It remains unclear, why 727delG showed no change in subcellular localization upon addition of forskolin and how this relates to the observed changes for the other three deletion mutants.

The subcellular localizations of the two remaining mutants, 727delG and 812-818del, and the effect of stimulation of phosphorylation with forskolin on their trafficking have not yet been investigated. Especially the latter mutant would be of major interest, since it contains, besides the added 61 amino acids of the other deletion mutants, the entire wt C-terminal tail as well. Therefore, the natural S_{256} PKA phosphorylation site is still present.

Only for c727delG, co-localization with the late endosomal/lysosomal marker Lamp1 has been shown [126]. The absence of the other mutants in these compartments remains to be confirmed by co-localization studies with Lamp1 and the early endosomal marker EEA1 [133, 139].

ADNDI mouse models

The clinical symptoms caused by the c763-772del mutant have further been investigated in the first in vivo model, a knock-in mouse harboring this mutation [140]. Typical (AD) NDI symptoms such as polyuria and low-urine osmolality but also the milder phenotype than that of a recessive NDI mouse model, could be reproduced, indicating a general applicability of this model to research questions in humans. The small increase of urine osmolality upon dDAVP administration or fluid deprivation found in human patients was also observed in the mutant mice. Moreover, the presence of residual wt-AQP2 homotetramers in apical membranes and mutant/wt heterotetramers in basolateral membranes of kidney collecting duct cells, as suggested by earlier in vitro-studies, could be confirmed in vivo.

Summary of autosomal-dominant NDI

Autosomal-dominant NDI is caused by sorting signal-based retention or basolateral misrouting of mutant-containing AQP2 tetramers. All mutations hitherto known are located in the C-terminal tail, indicating the importance of this part of AQP2 for cellular trafficking [99, 101, 130, 141], which has also been shown in other proteins of the AQP family [142]. In wt tetramers, the phosphorylation site at S_{256} serves as an inducible apical sorting signal. It may be inactivated, overruled by basolateral sorting signals, or reprogrammed to induce basolateral sorting, all causing intracellular misrouting. Formation of heterotetramers with wt-AQP2 has been shown for all eight mutants known [123–127], providing an explanation for their dominant behavior. Residual 4wt-homotetramers are explanatory of the relatively milder phenotype when compared to recessive forms of NDI. Additionally, mutant-containing tetramers retain water channel functionality and thus, if indeed, phosphorylation of three out of the four monomers within one tetramer is sufficient for apical shuttling [28], 3 wt/1 mut-heterotetramers also contribute to the relatively milder phenotype.

Mainly studies in *X. laevis* oocytes and polar MDCK cells, but more recently, also several heterozygous mouse models [143–145] have contributed to the still deepening knowledge about the cellular mechanisms involved in ADNDI.

Penetrance of sorting signals in autosomal-dominant and recessive NDI

Interestingly, though located in the AQP2 C-terminal domain, the P262L mutation has been found to cause recessive NDI. The leucine-based basolateral sorting signal introduced by AQP2-P262L seems not to exert any effect following heterotetramerizing with wt AQP2, suggesting that it is overruled by the stronger natural apical sorting signal of wt-AQP2. Only upon introduction of another recessive mutation on the other allele (R187C, A190T), AQP2-P262L fails to form heterotetramers due to the ER retention of R187C and A190T. Its homotetramers are mainly retained in intracellular vesicles, while a small fraction is redirected to the basolateral membrane upon forskolin-induced phosphorylation [146].

Remarkably, the observations made in vitro regarding the subcellular localization and inheritance pattern of P262L are comparable to the results of an accidentally discovered AQP2-S256L NDI mouse model. This mutant lacks the S_{256} PKA phosphorylation site, but, in contrast to the human R254L mutant, which also shows loss of phosphorylation, causes recessive instead of dominant NDI [143]. Altogether, these findings indicate that the destination of AQP2 tetramers is determined by the relative strengths of all apical and basolateral sorting signals present [147].

Genetically unresolved NDI cases

For approximately 5% of all NDI patients, it has not yet been possible to identify the causative mutations. To the day, it has not been investigated if these mutations might be located in promoter regions of the associated genes.

Alterations in these spots could result in lower mRNA expression levels and therefore are viable candidates for class I mutations in both recessive NDI types. Possibly, searching in AVPR2 and AQP2 promoter regions might be helpful to reveal the causative mutations for the small percentage of yet genetically undefined NDI cases.

Development of therapeutic approaches

Conventional therapy

Conventional therapy for NDI consists of replacement of urinary water losses by adequate fluid supply, in combination with a limited electrolyte diet (low sodium intake) to decrease obligatory water excretion. This is combined with the administration of the current first-line drug combination consisting of hydrochlorothiazide (HTZ, 2–4 mg/kg/24 h) and amiloride (0.3 mg/kg/24 h) [148, 149]. This treatment replaced the earlier used combination of HTZ and indomethacin, since it has been reported to have the same efficiency, but without the strong gastrointestinal and hematopoietic side-effects the latter medication induces [150]. In young children below the age of 4 to 6 years, however, amiloride is less well tolerated because of persistent nausea. Therefore we advise the temporary use of the combination of indomethacin (2 mg/kg/24 h)-HTZ (2–4 mg/kg/24 h) in these young children. In patients who cannot tolerate indomethacin, selective inhibitors of cyclooxygenase-2 (COX-2) might be helpful. Caution in using indomethacin and selective COX-2 inhibitors in NDI is warranted, as their administration can potentially lead to the acute deterioration of renal function in dehydrated patients. Single-drug therapies show lower efficacy and are therefore not preferred.

The mechanism of action by which HTZ reduces NDI side-effects is somewhat counterintuitive. The drug is commonly known as a diuretic (increased urine excretion). This is achieved by the inhibition of sodium reabsorption in the distal tubule upon blocking the NaCl co-transporter (NCC). As water follows sodium, this leads to hypovolemia, resulting in a further activation of the renin-angiotensin II- aldosterone system. Increased angiotensin II-mediated sodium reabsorption in the proximal tubules is thought to be the main response. In this segment, sodium reabsorption is obligatorily coupled to increased water reabsorption via AQP1 [151]. Thereby, less pro-urine reaches the distal tubule and water homeostasis is less dependent on water reabsorption in the collecting duct.

Additionally, HTZ is believed to induce AQP2 upregulation, which might lead to increased water reabsorption even without AVP-dependent stimulation [152].

Amiloride acts comparable to HTZ by inhibiting epithelial sodium channels (ENaC), again resulting in lowered sodium reabsorption and increased hypovolemia [153]. Although these drugs reduce urine excretion in NDI patients, they are unable to achieve urine volumes produced in healthy individuals. Therefore, the general problem remains, although the symptoms are relieved.

Consequently, current research focuses on methods to treat NDI on a more causative level than solely trying to fight the symptoms. We next discuss the more recent developments in this field based on the hereditary type for which the approach is suited. In Fig. 2, an overview of all major mechanisms employed is depicted.

X-linked NDI-directed therapies

Based on the findings of molecular cell biological research regarding the underlying principles of X-linked NDI, several new approaches for treatment have evolved, all more based on a longer-lasting symptom reduction due to their more fundamental approach.

Generally, the principles currently investigated can be divided into three major groups: The first line tries to promote transport of misfolded, ER retained AVPR2s to the plasma membrane by assisting with protein folding [154]. Once rescued, receptors can then fulfill their natural function, since their missense mutations often do not lead to complete loss of function, but rather cause affinities for ligand and/or G-protein to be lowered. The second approach is to directly stimulate the retained receptors intracellularly, while they are still located in the ER, thereby circumventing the necessity for proper folding [155]. A third approach for NDI treatment has recently been provided by promising results that indicated the possibility to completely circumvent AVPR2 in stimulating AQP2 membrane translocation [156].

AVPR2 rescue

The extensive retention of misfolded AVPR2 in the ER led to the suggestion to develop pharmacological chaperones that can be administered to the patient and subsequently assist with proper folding of the mutant receptors. This could then lead to shielding of the mutants from ERAD and recognition by the ER transport system, eventually re-establishing expression of (at least partially) active AVPR2 on the plasma membrane.

The physical principle of small pharmaceutical chaperones lies in the fact that aggregation of misfolded proteins may lead to a thermodynamically more stable state than folding into the dimeric native conformation. Yet, the energetic barrier, separating the native state from the misfolded state that can undergo oligomerization, usually is too high to be of any biological importance. This is why under normal conditions the kinetically favorable product (e.g., the native conformation) is preferentially formed. However, in an unnatural situation, such as a mutation that destabilizes the native conformation, as it is

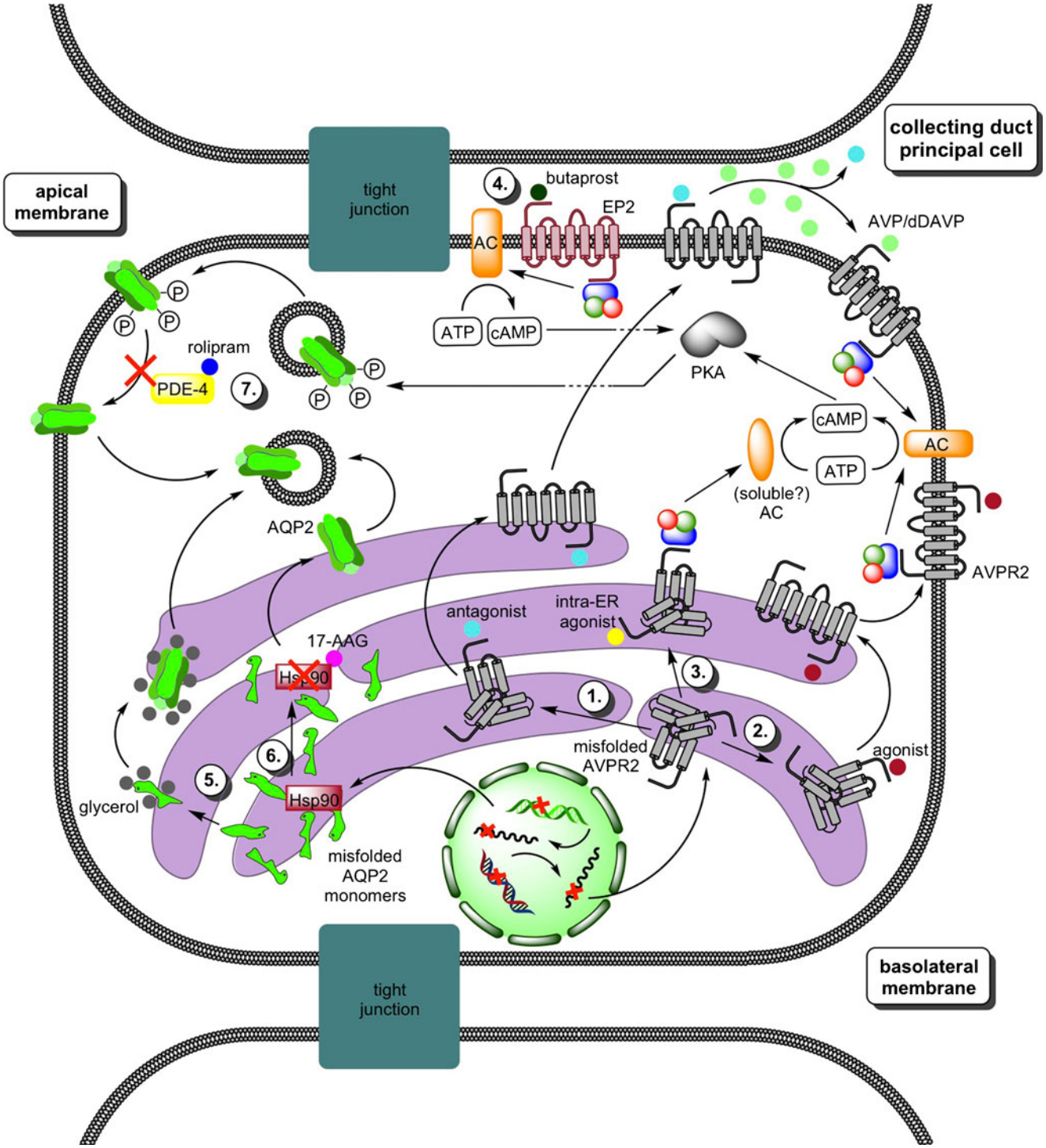

Fig. 2 Therapeutic approaches to treat nephrogenic diabetes insipidus (NDI). Approaches 1–4 focus on XNDI, 5–6 are suited for ARNDI, 7 applies to ADNDI. (1) Cell-penetrating AVPR2 antagonists induce native folding and rescue AVPR2 to the basolateral membrane. Displacement of the antagonist by high AVP/dDAVP concentrations is required to induce cAMP signaling. (2) Agonists function similarly, but do not need displacement to activate AVPR2. (3) ER-penetrating agonists can stimulate misfolded AVPR2 without inducing maturation and induce prolonged signaling. (4) Stimulation of EP2 by butaprost activates cAMP production as well as AQP2 phosphorylation and targets AQP2 to the apical membrane without involvement of AVPR2. (5) Glycerol acts as pharmacological chaperone in high concentrations, rescuing AQP2 mutants from the ER. (6) Hsp90-inhibitor 17-AAG enables AQP2 mutant escape from the ER. (7) Rolipram inhibits PDE-4 and increases AQP2 concentration in the apical membrane by slowing down dephosphorylation and downregulating re-internalization

seen in most missense mutations of AVPR2, this equilibrium might change, thereby promoting misfolding and aggregation.

Small-molecule ligands that bind to the native state can in turn either stabilize the native conformation or increase the energy barrier separating the native state from the aggregation-prone misfolded conformation, enhancing proper folding and promoting transport to the natural cellular location by circumventing ER quality control-associated retention [157].

Proof of principle has already been provided as early as in 1986 [158], but its applicability to X-linked NDI has not been tested until the year 2000 by Morello et al. [159]. They showed that, in cell cultures, the cell permeable, non-peptidic AVPR2 antagonists SR121463A and VPA-985 could rescue receptors by promoting proper folding, increasing cell membrane receptor load 15-fold and restoring AVP responsiveness of cells. These drugs are lipophilic small-molecule AVPR2 antagonists and were therefore anticipated to be cell-permeable and, as they were developed against the normal AVPR2 structure, able to specifically bind the "native" conformation of the mutants. Since then, in a variety of studies a large number of AVPR2 mutants have shown the ability of being rescued by molecular chaperones and generally being (at least partially) functional [160–162]. However, two major problems prevent progress in this field of research.

First, the ability of the AVPR2 to be stimulated by AVP after translocation to the plasma membrane has to be ensured, which requires dislocation of the pharmacological chaperone by AVP after rescuing. Therefore, low-affinity antagonists are believed to have the highest clinical value [163]. However, their efficiency in rescuing is lower than that of high-affinity ligands and the high concentrations required to be administered for sufficient activity by low-affinity antagonists might lead to severe complications in patients. Moreover, Robben et al. have shown that upon pre-treatment of AVPR2 mutant cells with clinically feasible blood concentrations of antagonists, subsequent treatment with dDAVP only led to an increased cAMP response in cells pre-treated with high-affinity AVPR2 antagonist [162]. These data indicated that at these antagonist concentrations, rescue of cell surface expression of the AVPR2 mutants becomes limiting over dislocation of the antagonists. These issues have to be addressed in mouse models or clinical studies to be resolved before usage of low-affinity ligands as drugs becomes viable.

Secondly, stimulation of AVPR2 on the plasma membrane by AVP leads to increased internalization and rapid degradation of the receptors by the beta-arrestin-MAPK-pathway [65, 164], thereby continuously lowering the AVPR2 concentration in the AVP-accessible membrane [155]. This requires high-frequency administration of pharmaceuticals to patients, probably also causing unwanted side-effects, again limiting the clinical value of these compounds. A small-scale clinical trial conducted in 2006 by Bernier et al. showed that the AVPR1 antagonist SR49059 could induce an increase in urine osmolality in all five patients [161], indicating a general agreement of in vitro results with a human setting. However, a rapid decline of the drug effect also was observed, probably associated with the AVP-induced receptor internalization. The clinical development of SR49059 was discontinued due to safety issues as the drug seemed to interfere with the cytochrome P450 metabolic pathway, showing the validity of the two major concerns mentioned above. Maybe because of the higher potential of other approaches to follow, we are not aware of further clinical studies in which cell-permeable AVPR2 antagonists are used to treat NDI patients.

The use of non-peptide agonists has somewhat circumvented the first problem, as it has been shown that the compounds MCF14, MCF18, and MCF57, all high-affinity agonists of AVPR2, could induce receptor maturation as well as translocation to the plasma membrane and elicited a cAMP response [165]. In contrast to AVP, however, they did not stimulate receptor internalization, but blocked the MAPK pathway responsible for arrestin-induced internalization.

Intracellular receptor activation

Recent studies by Robben et al. have indicated that it is possible to activate ER-retained, but intrinsically functional, AVPR2 by non-peptide agonists [155]. This activation surprisingly leads to sufficient cAMP increase to cause AQP2 to be transported to the apical membrane. They used the recently for oral administration developed OPC51 and the two novel VA88 and VA89 non-peptidic AVPR2 agonists to show that those are able to stimulate multiple mutants inside of the ER, that then activated their natural signal transduction pathway. In contrast to pharmaceutical chaperone-assisted folding and rescue of the receptors, the localization and maturation state of the AVPR2 did not change upon activation, indicating that these compounds do not act as molecular chaperones.

Additionally, activation of AVPR2 located in the plasma membrane by AVP leads to rapid degradation of the receptor as a mechanism of adaptation. This limits the efficacy of receptor rescue obtained with AVPR2 antagonists or the non-peptide agonists of the previous paragraph, since after normal stimulation, a large fraction of the rescued receptors is degraded and new assisting chaperones have to be administered. This is not the case for receptor activation within the ER; proteasomal degradation of the trapped receptors is not increased upon intracellular activation by the non-peptide agonists lacking pharmacochaperone function. Another advantage of the use of non-peptidic agonists – as well as antagonists – for intracellular AVPR2 stimulation is the high selectivity of non-peptide compounds for the AVPR2. This should prove to minimize the side-effects of administration

of those therapeutics, since no other cellular mechanisms are expected to be activated. Moreover, once a high-affinity antagonist is bound to the receptor in order to restore native folding, it tends to remain bound. Thereby, it competes with the natural agonist AVP, resulting in less activation of AVPR2 than predicted from the amount administered. Therefore, the more strictly dose-dependent effect of cell-penetrating agonists is expected to offer better predictability. Here, the new promising non-peptidic agonists seem to have a better ability to penetrate cell membranes and show higher specificity for AVPR2 than the peptide variants in vitro. Future in vivo and clinical testing has to confirm whether this type of drugs has the proposed positive effects in patients and meets the safety requirements.

The mode of action by which receptors trapped in the ER can still activate their coupled G-protein and how this stimulates adenylate cyclase is not yet understood. However, most recently, a new model for signaling by internalized GPCRs has been proposed by Calebiro et al. [166, 167], in which GPCRs localized in endosomal membranes can activate their natural pathways (e.g., activation of adenylate cyclase and production of cAMP), if stimulated by lipophilic agonists that are able to penetrate the endosomal membranes. They also showed earlier that internalized GPCRs can continue signaling from endosomes [168], suggesting that the classical model, in which internalization solely is a desensitization process, has to be reconsidered. In how far this model also holds for GPCRs completely trapped in the ER/endosomal compartments, remains to be elucidated. Clearly, both AVPR2 rescue and intracellular receptor activation will only work for mutations that affect folding or proper intracellular transport and not for truncated receptors, those that lost their affinity for AVP or their coupled Gs-protein or mutations that interfere with transcription and translation. Other approaches that can deal with these types of mutants remain to be found.

Besides the various in vitro expression systems mentioned, a mouse model has recently been constructed, in which the endogenous AVPR2 gene is conditionally knocked out [156]. The mice developed extensively increased water intake and urine excretion, and showed dramatically reduced urine osmolality. Additionally, no functional AVPR2 could be detected in their kidneys. Earlier knockout mice did not survive for more than 1 week postnatal, because pups totally lacking renal water reabsorption are not viable [169]. In contrast, the new mouse model survived normally, since the AVPR2 knockout could be induced after reaching adulthood [170]. Therefore, this new model might provide a reliable in vivo system for studying the effects of drugs on XNDI. We recently developed a mouse model, in which a mutated AVPR2 can be expressed in an inducible manner. These mice will be used to test the viability and safety of rescuing antagonists or intracellularly activating agonists. As the agonists are still in clinical trials for their commercially more interesting indications, we have to wait for the generosity of the pharmaceutical companies to share their agonists or for the availability of the drugs on the market before we can test whether these compounds can relieve NDI in patients.

Circumvention of AVPR2

Yet another mechanism that was exploited to alleviate XNDI provides hope for patients with AVPR2 mutants that cannot be rescued or activated intracellular due to type 1 mutations. By stimulation of the E-prostanoid receptor EP4, NDI symptoms were greatly reduced in the conditional AVPR2-deletion mouse model [156]. This was due to raised AQP2 levels, most probably a consequence of cAMP production caused by EP4 stimulation.

Interestingly, in a very recent study on the effects of EP2 and EP4 stimulation on NDI symptoms, Olesen et al. further elaborated the underlying mechanisms and showed AVPR2-independent induction of AQP2 phosphorylation and its translocation to the apical membrane in MDCK cells [171]. Additionally, they reported relieved NDI symptoms in a rat model following the administration of the EP2 agonist butaprost. Due to its resistance to stimulation-induced internalization [172] and its more selective expression [173], the EP2 receptor is the more interesting candidate for NDI treatment in comparison to the EP4 receptor, also because EP2 agonists have already been tested in clinical studies for other diseases and have shown promising results concerning safety issues [174]. However, long-term safety studies, which are needed when being used in the treatment of NDI, will need to be performed.

Autosomal-recessive NDI

In general, AQP2 mutants causing autosomal-recessive NDI have features comparable to X-linked AVPR2 mutants. For class I mutants that are strongly truncated due to early stop codons, early frame shifts, or splice site mutations, it remains extremely difficult to think of therapeutic options other than gene therapy. Renal gene therapy has been hampered by a low level of accessibility and the absence of selectivity of gene transfer techniques to and expression in particular renal cells [95]. Yet, missense mutations are the largest group of causative alterations in AQP2 for NDI, and generally lead to aberrant folding of AQP2 inside the ER. As it is the case for AVPR2, those mutants often retain partial functionality whenever folding into native conformations is restored. Hence, finding substances that are able to re-establish natural AQP2 folding holds comparable promises for ARNDI treatment as it has already been shown for XNDI. The challenge is thus to find pharmacological chaperones aimed at

rescuing ER-retained AQP2 mutants from ERAD and thereby restoring the transport of partially functional AQP2 to the plasma membrane. In CHO and MDCK cells, glycerol has proven the applicability of chemical chaperones to AQP2 by restoring ER export in high concentrations [117]. Recently, Yang et al. described partial restoration of cellular AQP2 processing upon treatment of conditional AQP2-T126M knock-in mice with an Hsp90 inhibitor, 17-allylamino-demethoxygeldanamycin (17-AAG), eventually resulting in improved urinary concentrating ability [175]. The authors proposed several explanations for a possible mechanism. Hsp90 interacts with and promotes ERAD of several aberrantly folded proteins, including the mutant cystic fibrosis protein ΔF508-CFTR [176]. By disrupting the interaction of Hsp90 with AQP2-T126M, 17-AAG might enable AQP2-T126M to escape from the ER and to be processed further by the Golgi complex. Another possibility is that 17-AAG directly interacts with heterotetramers containing AQP2-T126M and residual wild-type AQP2 and inhibits their degradation, consequently inducing accumulation. There is also some evidence for the involvement of Hsp70 in the constitutive recycling pathway of AQP2 and inhibition of Hsp90 has occasionally been reported to be associated with induction of Hsp70. Further studies are necessary to elucidate the mechanism in more detail.

However, inhibition of ER chaperones as common as Hsp90 might also have severe side-effects. Hsp90 is an abundant cytosolic chaperone and contributes to various cellular processes like protein degradation and signal transduction. For example, it had been shown that Hsp90 inhibition with 17-AAG induced ER stress. This caused increased expression of ER chaperones, for example Grp94, and an increase in cytoplasmic calcium levels, eventually leading to the disruption of mitochondrial homeostasis and promoting mitochondria-induced apoptosis [177]. So it is likely that there is a complex mechanistic interplay between various factors, that in summary resulted in a partial restoration of urinary concentration ability in AQP2-T126M knock-in mice after treatment with 17-AAG. Lengthened studies addressing safety issues of Hsp90 or other chaperone inhibitors have to be conducted in order to elucidate the applicability of these compounds in NDI therapy.

Autosomal-dominant NDI

For dominant NDI patients, the approaches based on molecular chaperones that were already suggested for other forms of NDI, are not applicable, since native folding occurs. Instead, a search for therapeutics is on-going that interfere with misrouting and restore normal apical membrane targeting and transport. Since the number of patients is extremely low, and the conventional therapy with diuretics can relieve ADNDI to a certain extent, the amount of research performed considering this type of NDI is substantially lower than for the other subtypes, and in vitro data about possible therapies for dominant NDI is lacking [25].

The AQP2-Δ763–772 knock-in mouse model generated by Sohara et al. [140] is the first model for autosomal-dominant NDI, which can be used to test therapeutic approaches in vivo. After administration of dDAVP to the mice, the urine osmolality was increased and administration of dDAVP was proposed as the therapy of choice. However, high doses of dDAVP and long-term administration are required to induce lasting increased AQP2 synthesis, which can be beneficial to patients based on the residual wild-type tetramers discussed earlier.

Based on the improvement of AVP-dependent cAMP signaling of collecting duct cells in a hypercalcemia-induced NDI mouse model [178], Sohara et al. also tested the phosphodiesterase 4 inhibitor rolipram in the knock-in dominant NDI mice [140]. Their data indicated that rolipram is able to increase cAMP levels in the papillae and AQP2 phosphorylation as well as translocation to the apical membrane. If these results are reproducible in humans, rolipram might become a first therapeutic agent able to improve life quality of autosomal-dominant NDI patients.

However, as for the Hsp90-inhibition approach, phosphodiesterase 4 is a common protein that also is involved in immunosuppressive and anti-inflammatory pathways and its inhibition may have unwanted effects that outweigh the advantages. Side-effects already discovered in clinical trials, that tested rolipram as antidepressant, were emesis and nausea [179].

Summary and concluding remarks

Besides acquired forms of NDI, three congenital types of this disorder exist. X-linked recessive NDI accounts for the vast majority of patients. They show loss-of-function mutations in the AVPR2 gene, disrupting the cellular response to AVP. Most mutations lead to aberrant folding and retention of usually partially functional receptors in the ER. For autosomal-recessive NDI, mutations are mainly present in the transmembrane regions of the AQP2 protein. The major group of the mutations consists of missense mutations inducing improper folding and ER retention, as it is the case in XNDI. Therefore, further research in X-linked as well as autosomal-recessive NDI focuses on the identification of pharmacological chaperones to restore routing of intrinsically (partially) functional proteins to the plasma membrane. In X-linked NDI, another line of research utilizes membrane-permeable agonists to intracellularly stimulate AVPR2 and thus initiate responses comparable to normal stimulation by AVP.

For autosomal-dominant NDI, the failure of water transport can be explained by the importance of the AQP2 C-terminal tail in trafficking of tetrameric protein to the apical membrane.

Phosphorylation of S_{256} in the C-terminus is necessary for AVP-induced accumulation of AQP2 in the apical membrane. In addition to the disruption of this phosphorylation site, AQP2 gene mutations can introduce basolateral or remove apical sorting signals of wt-AQP2, also leading to misrouting. Other approaches than pharmacological chaperones have to be invented in order to treat ADNDI patients.

Insights into the pathogenesis of the disease have for all NDI subtypes led to the development of in vivo mouse models, which provide opportunities for clinical safety assessment of possible therapies. Future research into the promising approaches mentioned above, combined with clinical trials, will show which of those strategies can improve or replace the current standard therapies for patients suffering from different NDI types.

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