Altered agonist sensitivity of a mutant V2 receptor suggests a novel therapeutic strategy for nephrogenic diabetes insipidus

László Sándor Erdélyi[1,3], András Balla[1,3], Attila Patócs[2], Miklós Tóth[2], Péter Várnai[1,3], László Hunyady[1,3]

[1] MTA-SE Molecular Physiology Research Group, Budapest, Hungary

[2] 2nd Department of Internal Medicine, Semmelweis University, Budapest, Hungary

[3]Department of Physiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary

Correspondence author: László Hunyady

PO Box 259, H-1444 Budapest, Hungary, hunyady.laszlo@med.semmelweis-univ.hu

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Abstract

Loss of function mutations of the type 2 vasopressin receptor (V2R) in kidney can lead to nephrogenic diabetes insipidus (NDI). We studied a previously described, but uncharacterized mutation of V2R (N321K missense mutation) of an NDI patient. The properties of the mutant receptor were evaluated. We constructed a highly sensitive Epac based BRET (bioluminescence resonance energy transfer) biosensor to perform real-time cAMP measurements after agonist stimulation of transiently transfected HEK293 cells with V2Rs. β-arrestin binding of the activated receptors was examined with luciferase-tagged β-arrestin and mVenus-tagged V2Rs using BRET technique. Cell surface expressions of HA-tagged receptors were determined with flow cytometry using anti-HA-Alexa488 antibodies. Cellular localization examinations were implemented with fluorescent tagged receptors visualized with confocal laser-scanning microscopy. The effect of various vasopressin analogues on V1R was tested on mouse arteries by wire myography. N321K mutant V2R showed normal cell surface expression but the potency of AVP for cAMP generation was low, while the clinically used desmopressin (dDAVP) was not efficient. The β-arrestin binding and internalization properties of the mutant receptor were also different compared to the wild type. Function of the mutant receptor can be rescued with administration of V2R receptor agonist dVDAVP, which had no detectable side effects on V1R in the effective cAMP generating concentrations. Based on the findings we could propose a therapeutical strategy for NDI patients carrying the N321K mutation, since our in vivo experiments suggest that dVDAVP could rescue the function of the N321K-V2R without significant side effect on V1R.

Introduction

Members of the G protein-coupled receptor (GPCR) superfamily are a major group of cell surface receptors, which recognize hormones, neurotransmitters and sensory information, thus they play essential roles in physiological processes (1). In addition to their physiological importance, the pathological significance of GPCRs cannot be emphasized enough since at least 40% of the modern therapeutic drugs target directly or indirectly these receptors and their signaling (2). Mutations of GPCRs are responsible for numerous human diseases and more than 600 loss of function mutations of GPCR's have been identified (3). Investigation of these mutations helps to reveal the functions and structures of the different GPCRs, moreover it may provide clues to find drugs targeting receptors (4). One of the most extensively investigated receptor regarding inactivating mutations is the type 2 vasopressin receptor (V2R). Loss of function mutations of V2R can cause nephrogenic diabetes insipidus (NDI) with different mechanisms (5,6).

V2Rs are localized to the basolateral plasma membrane of the principal cells in the kidney collecting ducts and have essential roles in mediating the water-conserving effect of arginine-vasopressin (AVP). AVP is secreted from the neurohypohysis in response to increased plasma osmolality, and its effect on water reabsorption is mediated by V2R. Binding of AVP to V2R, which is a G_s-coupled receptor, leads to cAMP-mediated translocation of aquaporin-2 (AQP2) to the apical plasma membrane. This regulation of AQP2 water channel localization is crucial to increase urine osmolality and to reduce urine output in humans (7). Ligand binding of GPCRs also stimulates mechanisms that can lead to termination of signaling. Impairment of this process can cause diseases as well (8,9). Desensitization and internalization of GPCRs are regulated by GPCR kinases and β-arrestins (10,11). Binding of β-arrestin to the desensitized receptor is followed by the internalization, which decreases the amount of receptors in the plasma membrane. The balance between the

internalization, degradation, synthesis and recycling determinates essentially the hormone sensitivity of a tissue (12).

Diabetes insipidus is a syndrome characterized by polyuria, hyposthenuria and polydipsia. NDI is caused by the impaired effect of AVP in the kidney, although the hormone secretion is normal. Almost 90% of NDI cases are caused by loss of function mutations of V2R. More than 200 mutations have been identified worldwide, much of them are missense mutations, which act by different mechanisms (13). Thus, mutations can be classified into several groups based on their consequences (14). Class I mutations of the AVPR2 gene lead to impaired transcription, mRNA processing or translation of the receptor resulting in truncated and rapidly degraded proteins. Class II mutations lead to the formation of misfolded full length proteins, which are recognized by the quality control system of the endoplasmic reticulum (ER), which result in ER retention (15). Thus, class I and II mutations lead to hormone insensitivity due to decreased number of cell surface receptors. Class III mutants are another group of missense mutations, which interfere with either G protein coupling or AVP binding, leading to inappropriate signal transduction without affecting the cell surface expression of the receptors. Class IV mutants have normal ligand binding but their intracellular trafficking is altered causing impaired cAMP signal production mostly due to constitutive β-arrestin dependent internalization into endosomal vesicles (16).

Identification of the altered properties of mutated receptors could help to define therapeutic strategies for the treatment of NDI patients. Possible therapeutic mechanisms include direct stimulation of signal generation, bypassing the receptor (14), and different strategies to rescue the receptor function. The most extensively investigated V2R mutants belong to the class II mutations, which cause ER retention. Pharmacological chaperons are chemical ligands, which facilitate the folding of receptors in the ER and rescue them from ER retention. This mechanism leads to increased plasma membrane expression of otherwise

functional receptors. Pharmacological chaperones of the V2R can be antagonists (17-21) or agonists (22,23).

In this study we have identified the N321K mutation of V2R in an NDI patient by genomic DNA sequencing. Although this mutation was reported previously, the mechanism of its pathogenic effect has not been identified (6). Here we characterize the pharmacological and functional properties of this mutant receptor, and based on these findings we propose a new therapeutic strategy for patients carrying this mutation.

Materials and methods

Materials

Molecular biology enzymes were obtained from Fermentas (Burlington, Canada), Stratagene (La Jolla, CA, USA), and Invitrogen (Carlsbad, CA, USA). Cell culture dishes and plates for BRET measurements were purchased from Greiner (Kremsmunster, Austria). Lipofectamine 2000 and coelenterazine h were from Invitrogen (Carlsbad, CA, USA). The anti-HA-Alexa488 mouse monoclonal antibody was purchased from Life Technologies (Grand Island, NY, USA). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). The human embryonic kidney (HEK293) cells were from ATCC (American Type Culture Collection, Manassas, VA, USA).

Mutation analysis

Written informed consent was obtained from a male NDI patient. Genomic DNA was extracted from peripheral blood leucocytes using DNA isolation kit (Boehringer Mannheim Corporation, Indianapolis, IN, USA). The AVPR2 gene was amplified with PCR in fractions using forward primer (5'-ATCACCTCCAGGCCCTCAGA-3') and reverse primer (5'-ATGGGACGGCAGATGGCAC-3'), as well as forward primer (5'-

TGATCCTGGCCATGACGCTG-3') and reverse primer (5'-AGAGGCAAGACACCCAACAGC-3'). The sizes of the PCR products were determined in agarose gel and were purified for DNA sequencing. The PCR products were sequenced in both directions.

Molecular biology

The cDNA of the human arginine vasopressin receptor 2 (Clone ID: AVR0200000, GenBank Accession Number: ACC#AY242131) was purchased from S&T cDNA Resource Center (Rolla, MO, USA). The untagged and the HA-tagged V2Rs were subcloned into pcDNA3.1. For the construction of the super *Renilla* luciferase (Sluc) tagged V2R, the receptor sequence was amplified from the cDNA clone and subcloned into a pEYFP-N1 vector (Clontech, Mountain View, CA, USA) containing the sequence of super *Renilla* luciferase (24). In order to create the mVenus-tagged V2R, the amplified receptor was subcloned into a pEYFP-N1 vector containing the sequence of mVenus. Venus contained an A206K mutation holding the protein in monomeric form (25). β-arrestin2-Rluc was constructed as described previously (26). The β-arrestin2 was subcloned into a pEYFP vector with replacement of eYFP with humanized *Renilla*-luciferase (Promega, Madison,WI). The generation of the MP-YFP was described previously (27). Mutagenesis was performed using standard site-directed mutagenesis techniques in order to generate N321K receptor constructs. After verifying the mutations with dideoxy sequencing, the mutated fragment was exchanged between the wild type and mutated portion with suitable restriction sites to avoid the generation of unwanted mutations outside the sequenced regions. The Epac-BRET sensor was based on the $^{T}EPAC^{VV}$ construct developed and kindly provided by Dr. Kees Jalink (28). For the construction of the Epac-BRET sensor the mTurquoise part of the $^{T}EPAC^{VV}$ was replaced with Sluc. The Sluc sequence was amplified with PCR and was subcloned into $^{T}EPAC^{VV}$.

Cell culture and transfection

Cell culture and transfection protocols were described previoiusly (27). The DNA amounts were 0.25 μg receptor containing construct/well and 0.25 μg BRET partner containing construct/well; the amount of Lipofectamine 2000 was 0.5 μl/well.

BRET measurements

BRET measurements were performed as described previously (27). We used either a *Renilla* luciferase-fused receptor as the energy donor and an eYFP-tagged protein as the acceptor or we used intramolecular BRET probe (cAMP measuring Epac-BRET). Dose-response sigmoidal curves were generated using non-linear regression. The statistical analysis was carried out with two way ANOVA and one way ANOVA with Tukey's multiple comparison test.

Confocal microscopy

The cells plated on polylysine-pretreated glass coverslips (3×10^5 cells/35-mm dish) and were transiently transfected with the HA-tagged receptor constructs (2 μg receptor/well; the amount of Lipofectamine 2000 was 4 μl/well.). After 24 h, the cells were washed with PBS, and were fixed with 4% PFA solution for 10 minutes. Cells were washed three times with 10% FBS containing PBS solution. For the immunostaining the anti-HA-Alexa488 mouse monoclonal antibodies were diluted to 1:250 in the presence or absence of 1% saponin and the cells were incubated for 1 h. After washing with 10% FBS-PBS the coverslips were mounted on slides. The localization and distribution of the targeted probes were analyzed using a Zeiss LSM 510 confocal laser-scanning microscope.

Flow cytometry

The cells were plated on glass coverslips (3×10^5 cells/35-mm dish) were transiently transfected with the HA-tagged receptor constructs or pcDNA3.1 (2 μg DNA/well; the amount of Lipofectamine 2000 was 4 μl/well.). The cells were deattached by Versene reagent treatment and were centrifuged. The cells were suspended in ice cold PBS, and were

centrifuged on 4 ^{o}C. The cell pellets were suspended and incubated with diluted (1:100) anti-HA-Alexa488 mouse monoclonal antibodies for 40 minutes on 4 ^{o}C. After the labeling period the cells were washed in ice cold PBS. Flow cytometry measurements were performed with Beckman-Coulter SC. After measuring the fluorescent intensity of the cells, G_{mean} was calculated using WinMDI v2.9 (http://facs.scripps.edu). For the relative fluorescent intensity the background (pcDNA3.1) was subtracted and the data were normalized for the wild type receptor. Statistical analysis was carried out using two-way ANOVA.

Wire myography

Thoracic aortas from rats were removedand placed into cold Krebs solution containing (in millimolar) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.17 $MgSO_4 \cdot 7H_2O$, 20 $NaHCO_3$, 1.18 KH_2PO_4, 0.027 EDTA, 10.5 glucose. Aortic rings were mounted onto a multichannel isometric myograph system (Danish Myo Technology, Aarhus, Denmark). The thermostated (37 °C) organ chambers of the myographs were filled with Krebs solution, which was and bubbled with carbogen gas (5% CO_2 and 95% O_2). Resting tension of aortic rings was set to 10 mN and allowed to equilibrate for 30 min. The integrity and functionality of the aortic ringswere tested by124 mM K^+ containing KREBS solution (constriction) and after several washing cycles and waiting period by 10 μMachetylcholine (vasodilation). Recording was performed with the Powerlab data acquisition system and the LabChart evaluation program (ADInstruments, Oxford, UK). Vasoconstrictor responses were calculated as percent values of reference 1μM phenilephrine caused precontraction. Concentration-dependent vasoconstrictor response curves to agonists were obtained using parallel segments.

Results

The male patient was born in 1984 with polyuria and polydypsia, and NDI was diagnosed at the age of 18 month since desmopressine (dDAVP), a vasopressin analogue, was ineffective during the early water deprivation test. Currently his water consumption is approximately 12 liter/day. Thiazide and amiloride diuretics were ineffective and the water intake remained unchanged. Clinical laboratory tests of the patient revealed the following parameters (reference ranges shown in parenthesis): serum sodium 145 mmol/l (136-146), serum potassium 4.3 mmol/l (3.5-5.0), serum osmolality 282 mOsm/kg (without any medication, at his usual daily water intake, 275-295). The urine specific gravity was 1003 g/cm^3 (1002-1030) and the urine osmolality was 72 mOsm/kg (50-1200, depending on fluid intake).

The AVPR2 gene was amplified with PCR of the genomic DNA isolated from the peripheral blood of the patient (see details under "Materials and Methods") and the mutation was identified by DNA sequencing. A missense mutation was found in the patient (Fig. 1A) and this C→G substitution results an asparagine lysine change (N321K) in the 7^{th} transmembrane domain of the V2R (29). No other mutations in the AVPR2 gene were found. The family anamnesis of the patient suggests that the N321K substitution is not a de novo mutation, since symptoms of diabetes insipidus were presented in at least 3 generations of his family (Fig. 1B). However, it was not possible to achieve sequencing of the AVPR2 gene of other family members. According to the records, the grandmother's father had suffered from polydypsia and died at 82 years of age. The grandmother had also suffered from polydypsia and had a daily water consumption of 6-8 l. Her 11 months old child died because of exsiccosis. The mother and sister of the patient are healthy.

We expressed HA-tagged wild type or N321K mutant V2R transiently in HEK293 cells to examine the cellular localization of the receptors. Immunofluorescent staining of the receptor was performed using anti-HA antibodies tagged with Alexa488 both in

permeabilized and non-permeabilized cells. Confocal microscopy revealed that the mutant N321K-V2R is localized in the plasma membrane of the transfected cells very similarly to the wild-type receptor (Fig. 2A and 2B). Immunostaining of permeabilized cells expressing either wild type or mutant receptors showed marked intracellular fluorescence. The mock-transfected cells with empty-pcDNA3.1 did not show any fluorescent staining (data not shown). Taken together, the mutant receptors showed very similar cellular distribution compared to the wild type receptors. These data show that the N321K-V2R can reach the plasma membrane of the cells (Fig. 2A-D). Theoretically, the fusion of the mutant receptor with a tag (HA or fluorescent protein) could alter the trafficking, therefore we used another approach to determine the localization of the receptors. In order to confirm the plasma membrane localization of the mutant receptor, we have also used HEK293 cells transiently expressing fluorescently tagged receptors. The mVenus tagged WT-V2R and N321K-V2R had similar cellular distribution in living cells assessed by confocal microscopy (data not shown). We also compared the quantity of the expressed receptors on the surface of transfected cells performing flow cytometry measurements. HA-tagged WT-V2R and N321K-V2R were transiently transfected in HEK293 cells, and labeled with anti-Ha-Alexa488 antibodies as described in the Materials and Methods. We did not detect any significant difference in the relative fluorescence intensities (RFI) of the WT (RFI: 1.0) and N321K receptors (RFI: 0.954±0.05; n=3, $p>0.05$) on the cell surface indicating that the plasma membrane expression of the mutant receptor is similar to that of the wild type receptor (data not shown).

Since these data showed no evidence of the ER retention of transiently expressed N321K mutant receptors, the function of this mutant receptor was also evaluated. Since V2R is coupled to G_s, we used bioluminescence resonance energy transfer (BRET) technique to monitor cAMP generation in living HEK293 cells upon agonist stimulation. The HEK293

cells were transiently transfected with the Epac-BRET sensor and with either wild type or N321K-V2R constructs. The experiments were performed 24 hours after the transfection. The Epac-BRET probe reports when the Epac domain is loaded with cAMP, causing conformational changes that move away the energy acceptor from the donor, as was shown in a previous report that presented the corresponding FRET probes (30). Consequently, an increase in intracellular cAMP level results in a decreased BRET ratio in our measurements. Fig. 3A shows the real-time evaluation of cAMP levels in living cells expressing either WT-V2R or N321K-V2R. Cells were stimulated at the indicated time with 10 nM AVP (in case of the wild type receptor, square) or with 1 μM AVP (in case of the mutant receptor, triangle). It is noteworthy that the basal (before stimulus) BRET ratio is higher in the cells expressing the N321K-V2R than in the cells expressing the wild type receptor, indicating that the basal cAMP concentration in the N321K-V2R expressing cells is lower than that of the WT-V2R expressing cells. Basal cAMP production of wild type V2Rs was already reported in COS7 cells, and it could be blocked with antagonists (31). In agreement with these data, the WT-V2R also possesses basal activity in HEK293 cells, whereas the N321K-V2R lacks the constitutive activity in our expression system. Although the mutant receptor was able to stimulate cAMP production upon AVP stimulus with very similar amplitude than that of the wild type receptor, the kinetics of the activation was different. The cAMP production was sustained in the cells expressing the WT-V2R, where as it was apparently more transient in cells expressing the N321K-V2R mutant receptor (Fig. 3A). We also determined the dose-response curve of the mutant and the wild type receptor upon AVP stimulus (Fig. 3B). The effect of the hormone on the WT-V2R and N321K-V2R expressing HEK293 cells was calculated as the BRET ratio difference between the ligand and the vehicle treated cells at the first time points after the treatment. The maximal BRET changes were similar in case of both receptors, but the potency of the N321K-V2R is dramatically decreased compared to the wild

type. The pEC_{50} of AVP for the wild type receptor was 10.46 ± 0.04 M, while that of the mutant receptor was 6.49 ± 0.07 M.

The effect of the AVP analogue dDAVP is fundamental both in the diagnosis and the treatment of diabetes insipidus. Therefore we investigated the effect of dDAVP on cAMP production in HEK293 cells expressing WT-V2R and N321K-V2R (Fig. 3C). According to our data the dDAVP has pEC_{50} of 9.23 ± 0.07 M for WT-V2R, whereas in case of the N321K mutant receptor we could not measure detectable cAMP production upon dDAVP stimulation. The inefficiency of dDAVP on N321K-V2R cells is consistent with the clinical data of the patient who carries this mutation.

Next we examined the internalization properties of the mutant receptor and compared those with that of the wild type receptor. First, we investigated the β-arrestin2 binding of the receptors using BRET technique in living cells. Association of the receptor with β-arrestin2 was detected as BRET signal elevation after AVP stimulus indicating the interaction of mVenus-tagged WT-V2R with Rluc-β-arrestin2. Based on the kinetics of the binding, the WT-V2R is apparently a class B GPCR (Fig. 4A) (32) Fig. 4B shows the dose-response curves of β-arrestin2 binding to the receptors after 380 sec stimulation. The pEC50 of β-arrestin2 binding dose-response curve to the wild type receptor was 8.03 ± 0.005 M. Interestingly, we were not able to detect β-arrestin2 binding in case of the N321K-V2R even at high, supraphysiological levels of AVP (Fig. 4B, triangle). These data prompted us to examine the internalization kinetics of the receptors. In this set of experiments the receptors were tagged with a bioluminescence donor *Renilla* luciferase (V2R-Sluc), while the energy acceptor YFP was targeted to plasma membrane by fusing a small tag containing consensus sequences for myristoylation and palmitoylation (MP-YFP) (27,33). The BRET ratio monitored the non-specific resonance energy transfer, which is dependent on the distance between the donor and acceptor. As Fig 4C shows, stimulation of the wild type V2R with1

μM AVP (square) decreased the BRET ratio, which reflects the altered localization of the energy donor and the acceptor, indicating the internalization of the cell surface localized receptors into the endosomal compartments (33). The reduction in BRET ratio between MP-YFP and N321K-V2R-Sluc upon stimulation with 1 μM AVP (triangle) was smaller than that in case of WT-V2R-Sluc, suggesting that the internalization of N321K-V2R is reduced compared to the wild type receptor.

Theoretically the functional impairment of theN321K-V2R mutant receptor can be repaired with an agonist that activates the receptor and has a proper potency in cAMP production. We tested several commercially available peptides, which are known ligands of the V2R receptor. Our aim was to find a ligand that activates the mutant receptor initiated cAMP generation and has a high V2R selectivity over type-1 vasopressin receptor (V1R) in order to avoid the potential side effects in an *in vivo* system. Here, we present the results of cAMP measurements using a selective V2R agonist Val^4-dDAVP (dVDAVP) (34), a V2R agonist, but V1R antagonist deamino-Pen^1,Val^4-dDAVP (PVDAVP) (35), Lys^8-VP (LVP) and $Asu^{1,6}$-AVP (AsuAVP) (36). The dose-response curves were measured in transiently transfected HEK293 cells using our Epac-based cAMP sensitive BRET probe (Fig. 5). Table 1. shows the calculated EC_{50} values of the various peptides. The efficacy values of the dVDAVP, LVP, and AsuAVP peptides were similar to AVP (and dDAVP) after stimulation of the wild type receptor expressed in HEK293 cells (Fig. 5A, C and D, square). As it was expected, these peptides had dramatically decreased potency in N321K-V2R expressing cells (Fig. 5A, B and D, triangle). In case of the PVDAVP the potency is slightly lower than those of AVP, dVDAVP, LVP, and AsuAVP when the wild type receptor used (Fig. 5B, square), however PVDAVP did not cause detectable cAMP production of the mutant receptor (Fig. 5B, triangle). From the tested peptides, the agonist dVDAVP had the highest potency (pEC_{50}: 6.3 ± 0.19 M) to activate the mutant receptor, which was comparable to the potency of AVP

(6.492 ± 0.07 M) to stimulate the cAMP production of this receptor. This finding raised the possibility that dVDAVP can be used to rescue the function of N321K-V2R. Since the vasoconstrictor side effect of AVP analogues is a concern during the treatment of NDI, we have tested the effect of dVDAVP on V1R initiated vasoconstriction of isolated mouse arteries by wire myography vessels. Fig. 6 shows that increasing concentrations of AVP caused vasoconstriction through vascular smooth muscle cell, whereas even 10^{-5} M concentration of dVDAVP was not able to evoke this effect.

Discussion

In this study we have characterized an N321K missense V2R mutation, which was identified from a Caucasian male NDI patient. This mutation was already found previously in another patient, but cellular consequences of the mutation were not examined before (6). The water deprivation test was carried out in childhood, which clearly diagnosed diabetes insipidus, moreover the administration of dDAVP did not have any effect on urine concentration. The familiar anamnesis strongly suggested a genetically inherited mutation, since the symptoms of diabetes insipidus was presented for at least four generations, which raised the possibility of an X-linked NDI. Sequencing of the genomic DNA demonstrated a C→G substitution in the AVPR2 gene, which results in an asparagine-lysine change at position 321 of the V2R. This asparagine is in the NPXXY motif, which is a conserved sequence in G-protein coupled receptors and assumed to have a role in ligand binding, G-protein coupling and internalization of β-adrenergic receptor (37). Mutation of this asparagine residue in type I angiotensin receptor ($AT_{1a}R$) causes markedly reduced G-protein interaction and generation of second messengers, but has no effect on the internalization kinetics of the receptor (38). It was clearly shown that mutation of proline 322 in V2R leads to impaired coupling to G_s protein (4). According to our data, the plasma membrane expression of the

N321K-V2R was similar to that of the wild type receptor in transient expression systems (Fig. 2). This result suggested that the mutant receptor is delivered to the cell surface and a ER retention problem is not responsible for the phenotype of this patient. On the other hand, the stimulation of the mutant receptor with AVP revealed markedly decreased potency and unchanged efficacy in cAMP production compared to the wild type receptor (Fig. 3B). Interestingly, we have found that the basal activity of the N321K-V2R was also decreased compared to WT-V2R (Fig 2A). However, the basal BRET ratio values (before stimulus) of the unstimulated N321K-V2R in cAMP measurements are identical to the BRET ratios of the wild type receptor under maximal inhibition with high dose antagonists (data not shown). Taken together, the mutant receptor has reduced second messenger formation capability both in the absence and presence of agonists suggesting that the N321K mutation causes impaired G-protein coupling. However, it is also important that the classification of a receptor mutation is not always unambiguous. NDI causing R137H-V2R was shown to belong to class IV due to the constitutive β-arrestin dependent internalization of the receptors (16). Moreover, R137C-V2R and R137L-V2R mutations lead to nephrogenic syndrome of inappropriate dieresis due to the constitutive activity and β-arrestin dependent internalization. The R137H-V2R has also impaired G-protein coupling (39) and as it was more recently showed, this mutant has altered trafficking to the plasma membrane as well (40). Taken together, these raise the possibility that one mutation can cause multiple effects on receptor function.

The hormone sensitivity of a tissue is also dependent on the internalization processes of receptors, which affects the receptor amount in the plasma membrane of the cells. We characterized and evaluated the internalization properties of the WT-V2R and N321K-V2R in HEK293 cells using BRET technique to measure the β-arrestin2 binding of the stimulated receptors. The β-arrestin2 binding dose-response curve of the WT-V2R was right shifted compared to the cAMP dose-response curve, which reflects the presence of spare receptors in

the plasma membrane and the enhancement of the generation of second messengers (41,42). In contrary to the wild type receptor, we could not detect β-arrestin2 binding of the N321K-V2R, therefore we also examined the kinetics of the internalization properties of the receptors. We used BRET-based approach, where plasma membrane targeted YFP served as indicator of plasma membrane localization, and we measured the internalization of luciferase tagged receptors from the cell surface upon stimulation (27). Although this method can also detect intramembrane movements of the receptor, we have used this method to monitor internalization of activated receptors, since internalization of the receptor leads to its divergence form the plasma membrane marker (33). The results showed that the N321K-V2R had markedly reduced internalization compared to the wild type receptor. It is possible that the remaining internalization is the consequence of β-arrestin independent processes. It is also interesting that, in contrast to the angiotensin receptor (37), a mutation in the NPXXY motif in the V2R leads to impaired β-arrestin binding and internalization. It is also notable that the reduced internalization does not necessarily mean continuous signaling of the receptor from the plasma membrane: the transient kinetic of the cAMP signal of the N321K-V2R suggests that this mutation has no major effects on the desensitization processes (Fig. 2A). Since β-arrestin mediated uncoupling of the GPCR from G-proteins is not the only possible mechanism of desensitization, i.e. phosphorylation of the receptor by messenger dependent kinases also can lead to termination of the receptor activation (43).

The clinical diagnosis of the NDI is also based on functional tests. The widely used tests are the water deprivation test and administration of the dDAVP (44). Since the dDAVP is an essential compound not only in the diagnostic procedures of NDI, but also in the therapy of DI, we examined the effect of dDAVP on the mutant N321K receptor. As we mentioned before, we could not detect any cAMP signal upon dDAVP stimulus (Fig. 3). These results were consistent with the clinical findings: administration of dDAVP in childhood did not

exert any improvement in the observed parameters. The dose-response curve of the wild type receptor showed that the potency of the dDAVP was decreased compared to the AVP.It was already known that although the dDAVP is a V2R specific agonist it has a lower affinity to receptor than the AVP (45). Apparently the N321K mutation induces a conformational change of the V2R resulting in a decreased affinity and/or G-protein coupling, which led to lack of dDAVP effects on cAMP generation capability in the tested concentrations.

Theoretically, an altered receptor conformation can result decreased potency for a certain agonist but potency of other agonists can be affected differently. An agonist, which could activate the mutant receptor despite of the conformational change, can be the causal therapy in case of a mutation, such as N321K. In order to find such a compound, we tested several agonists with high affinity to the V2R (Fig. 5 and Table 1). Statistical analysis showed that the effects of the agonists were significant ($p<0,0001$) on the pEC50 values using two way ANOVA. The wild type receptor had similar potency for LVP, dVDAVP and AsuAVP. The PVDAVP had the lowest EC_{50} in case of wild type V2R, and we could not detect any cAMP signal with the N321K-V2R, although this peptide was thought to be beneficial for a potential therapy because of the V1R antagonistic effect (35). The dVDAVP had the highest potency on the N321K-V2R, moreover the N321K mutation had the reduced effect in case of this compound: the difference between the mutant and the wild type receptor in the potency was one order of magnitude less than with other agonist (ΔpEC_{50} (M) of dVDAVP is 3.71, for AsuAVP and LVP are 4.62 and 4.56 respectively). Using one way ANOVA statistical analysis with Tukey' multiple comparison test, the differences between the ΔpEC50 values of the dVDAVP and the other agonists (AsuAVP and LVP) were significant ($p<0,005$). The consequence of the N321K missense mutation is misfolding, resulting in a conformation with altered agonist sensitivity, which is more suitable for stimulation by dVDAVP than for other agonists. Fortunately, as was demonstrated earlier, the most effective AVP analogue

dVDAVP among the tested compounds is a selective V2R agonist (34). A potential high dose agonist treatment could be limited because of the cross reaction of the compound on the vascular V1Rs. The $V1_aR$ is expressed in various tissues such as in the walls of vascular vessels (46). Although the physiological concentration of AVP is lower than the concentration that exerts vasoconstriction, a high dose of vasopressin receptor agonist in the treatment of DI could provoke side effects (vasoconstriction, blood pressure elevation) through this system. The consequence of the agonist activity on V1R could be hypertension and according to studies in septic shocka decreased perfusion of the heart, kidney and intestines (47). In this present study we examined whether the high dose of dVDAVP, which is able to generate cAMP signal through the activation of N321K-V2R could promote vasoconstriction in peripheral arterioles. As Fig. 6 shows, we could not detect any constriction even at 10 μM final dVDAVP concentration. This result suggests that an appropriate (moderately high) dose treatment could have beneficial effects on the NDI symptoms including polyuria and polydipsia without the unsafe side effect vasoconstriction.

Although dVDAVP did not become an alternative drug of dDAVP in the clinical therapy of NDI over the years, but several studies are in the literature about the clinical use of dVDAVP. Czakó et al showed in a clinical trial that dVDAVP was not only more effective than dDAVP in patients with central DI, but that it also had a short term, moderate antidiuretic effect in patients with ‘ADH resistant diabetes insipidus’(48). The dVDAVP had three times longer antidiuretic effect in central DI than dDAVP after intravenous injection. It has also been shown that an intranasal use of dVDAVP was also similarly effective as dDAVP in these patients.

In this study we demonstrated that the disease causing N321K mutation of the V2R does not lead to ER retention and the N321K-V2R is present in the plasma membrane of HEK293 cells. The mutant receptor has an unchanged efficacy but dramatically decreased

potency for AVP. The N321K-V2R mutation leads to impaired internalization, most likely due the lack of β-arrestin binding upon stimulation with agonist concentrations which generate maximal cAMP signal. The N321K-V2R is biased among different ligands, as the misfolding and the conformational change due to the mutation causes different sensitivities of agonists. According to our data, the function of the mutant receptor can be rescued with administration of V2R receptor agonist dVDAVP, which had no detectable side effects on V1R in the effective cAMP signal causing concentration. Our in vivo experiments propose the possibility that appropriate dosage of dVDAVP can rescue the function of the N321K-V2Rin a NDI patient with no significant side effect on V1R. Based on these findings a therapeutic strategy can be formed for patients with N321K mutation in the V2R.

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References

1. Lagerstrom MC, Schioth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature reviews Drug discovery.* 2008;7(4):339-357.

2. Ma P, Zemmel R. Value of novelty? *Nature reviews Drug discovery.* 2002;1(8):571-572.

3. Schoneberg T, Schulz A, Biebermann H, Hermsdorf T, Rompler H, Sangkuhl K. Mutant G-protein-coupled receptors as a cause of human diseases. *Pharmacology & therapeutics.* 2004;104(3):173-206.

4. Ala Y, Morin D, Mouillac B, Sabatier N, Vargas R, Cotte N, Dechaux M, Antignac C, Arthus MF, Lonergan M, Turner MS, Balestre MN, Alonso G, Hibert M, Barberis C, Hendy GN, Bichet DG, Jard S. Functional studies of twelve mutant V2 vasopressin

receptors related to nephrogenic diabetes insipidus: molecular basis of a mild clinical phenotype. *Journal of the American Society of Nephrology : JASN.* 1998;9(10):1861-1872.

5. Tao YX. Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications. *Pharmacology & therapeutics.* 2006;111(3):949-973.

6. Arthus MF, Lonergan M, Crumley MJ, Naumova AK, Morin D, De Marco LA, Kaplan BS, Robertson GL, Sasaki S, Morgan K, Bichet DG, Fujiwara TM. Report of 33 novel AVPR2 mutations and analysis of 117 families with X-linked nephrogenic diabetes insipidus. *Journal of the American Society of Nephrology : JASN.* 2000;11(6):1044-1054.

7. Moeller HB, Rittig S, Fenton RA. Nephrogenic diabetes insipidus: essential insights into the molecular background and potential therapies for treatment. *Endocrine reviews.* 2013;34(2):278-301.

8. Ligeti E, Csepanyi-Komi R, Hunyady L. Physiological mechanisms of signal termination in biological systems. *Acta physiologica (Oxford, England).* 2012;204(4):469-478.

9. Rochdi MD, Vargas GA, Carpentier E, Oligny-Longpre G, Chen S, Kovoor A, Gitelman SE, Rosenthal SM, von Zastrow M, Bouvier M. Functional characterization of vasopressin type 2 receptor substitutions (R137H/C/L) leading to nephrogenic diabetes insipidus and nephrogenic syndrome of inappropriate antidiuresis: implications for treatments. *Molecular pharmacology.* 2010;77(5):836-845.

10. Bohm SK, Grady EF, Bunnett NW. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *The Biochemical journal.* 1997;322 (Pt 1):1-18.

11. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of cell science.* 2002;115(Pt 3):455-465.

12. Gaborik Z, Hunyady L. Intracellular trafficking of hormone receptors. *Trends in endocrinology and metabolism: TEM.* 2004;15(6):286-293.

13. Spanakis E, Milord E, Gragnoli C. AVPR2 variants and mutations in nephrogenic diabetes insipidus: review and missense mutation significance. *Journal of cellular physiology.* 2008;217(3):605-617.

14. Wesche D, Deen PM, Knoers NV. Congenital nephrogenic diabetes insipidus: the current state of affairs. *Pediatric nephrology.* 2012;27(12):2183-2204.

15. Robben JH, Knoers NV, Deen PM. Cell biological aspects of the vasopressin type-2 receptor and aquaporin 2 water channel in nephrogenic diabetes insipidus. *American journal of physiology Renal physiology.* 2006;291(2):F257-270.

16. Barak LS, Oakley RH, Laporte SA, Caron MG. Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(1):93-98.

17. Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, Bouvier M. Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *The Journal of clinical investigation.* 2000;105(7):887-895.

18. Robben JH, Sze M, Knoers NV, Deen PM. Functional rescue of vasopressin V2 receptor mutants in MDCK cells by pharmacochaperones: relevance to therapy of nephrogenic diabetes insipidus. *American journal of physiology Renal physiology.* 2007;292(1):F253-260.

19. Wuller S, Wiesner B, Loffler A, Furkert J, Krause G, Hermosilla R, Schaefer M, Schulein R, Rosenthal W, Oksche A. Pharmacochaperones post-translationally enhance cell surface expression by increasing conformational stability of wild-type and mutant vasopressin V2 receptors. *The Journal of biological chemistry.* 2004;279(45):47254-47263.

20. Bernier V, Lagace M, Lonergan M, Arthus MF, Bichet DG, Bouvier M. Functional rescue of the constitutively internalized V2 vasopressin receptor mutant R137H by the pharmacological chaperone action of SR49059. *Molecular endocrinology.* 2004;18(8):2074-2084.

21. Ranadive SA, Ersoy B, Favre H, Cheung CC, Rosenthal SM, Miller WL, Vaisse C. Identification, characterization and rescue of a novel vasopressin-2 receptor mutation causing nephrogenic diabetes insipidus. *Clinical endocrinology.* 2009;71(3):388-393.

22. Robben JH, Kortenoeven ML, Sze M, Yae C, Milligan G, Oorschot VM, Klumperman J, Knoers NV, Deen PM. Intracellular activation of vasopressin V2 receptor mutants in nephrogenic diabetes insipidus by nonpeptide agonists. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(29):12195-12200.

23. Jean-Alphonse F, Perkovska S, Frantz MC, Durroux T, Mejean C, Morin D, Loison S, Bonnet D, Hibert M, Mouillac B, Mendre C. Biased agonist pharmacochaperones of the AVP V2 receptor may treat congenital nephrogenic diabetes insipidus. *Journal of the American Society of Nephrology : JASN.* 2009;20(10):2190-2203.

24. Woo J, von Arnim AG. Mutational optimization of the coelenterazine-dependent luciferase from Renilla. *Plant methods.* 2008;4:23.

25. Zacharias DA, Violin JD, Newton AC, Tsien RY. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science.* 2002;296(5569):913-916.

26. Turu G, Szidonya L, Gaborik Z, Buday L, Spat A, Clark AJ, Hunyady L. Differential beta-arrestin binding of AT1 and AT2 angiotensin receptors. *FEBS letters.* 2006;580(1):41-45.

27. Balla A, Toth DJ, Soltesz-Katona E, Szakadati G, Erdelyi LS, Varnai P, Hunyady L. Mapping of the localization of type 1 angiotensin receptor in membrane microdomains using bioluminescence resonance energy transfer-based sensors. *The Journal of biological chemistry.* 2012;287(12):9090-9099.

28. Klarenbeek JB, Goedhart J, Hink MA, Gadella TW, Jalink K. A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PloS one.* 2011;6(4):e19170.

29. Moeller HB, Rittig S, Fenton RA. Nephrogenic Diabetes Insipidus: Essential Insights into the Molecular Background and Potential Therapies for Treatment. *Endocr Rev.*

30. Klarenbeek JB, Goedhart J, Hink MA, Gadella TW, Jalink K. A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PloS one.*6(4):e19170.

31. Takahashi K, Makita N, Manaka K, Hisano M, Akioka Y, Miura K, Takubo N, Iida A, Ueda N, Hashimoto M, Fujita T, Igarashi T, Sekine T, Iiri T. V2 vasopressin receptor (V2R) mutations in partial nephrogenic diabetes insipidus highlight protean agonism of V2R antagonists. *The Journal of biological chemistry.*287(3):2099-2106.

32. Shenoy SK, Lefkowitz RJ. Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *The Journal of biological chemistry.* 2003;278(16):14498-14506.

33. Toth DJ, Toth JT, Gulyas G, Balla A, Balla T, Hunyady L, Varnai P. Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in

endocytosis of the G-protein-coupled receptor. *Journal of cell science.* 2012;125(Pt 9):2185-2197.

34. Sawyer WH, Acosta M, Balaspiri L, Judd J, Manning M. Structural changes in the arginine vasopressin molecule that enhance antidiuretic activity and specificity. *Endocrinology.* 1974;94(4):1106-1115.

35. Manning M, Lowbridge J, Stier CT, Jr., Haldar J, Sawyer WH. [1-deaminopenicillamine,4-valine]-8-D-arginine-vasopressin, a highly potent inhibitor of the vasopressor response to arginine-vasopressin. *J Med Chem.* 1977;20(9):1228-1230.

36. Hase S, Sakakibara S, Wahrenburg M, Kirchberger M, Schwartz IL, Walter R. 1,6-Aminosuberic acid analogs of lysine- and arginine-vasopressin and -vasotocin. Synthesis and biological properties. *J Am Chem Soc.* 1972;94(10):3590-3600.

37. Barak LS, Menard L, Ferguson SS, Colapietro AM, Caron MG. The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor. *Biochemistry.* 1995;34(47):15407-15414.

38. Hunyady L, Bor M, Baukal AJ, Balla T, Catt KJ. A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor. *The Journal of biological chemistry.* 1995;270(28):16602-16609.

39. Rosenthal W, Antaramian A, Gilbert S, Birnbaumer M. Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. *The Journal of biological chemistry.* 1993;268(18):13030-13033.

40. Kocan M, See HB, Sampaio NG, Eidne KA, Feldman BJ, Pfleger KD. Agonist-independent interactions between beta-arrestins and mutant vasopressin type II receptors associated with nephrogenic syndrome of inappropriate antidiuresis. *Molecular endocrinology.* 2009;23(4):559-571.

41. Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Molecular endocrinology.* 2006;20(5):953-970.

42. Ehnis T, Hocher B, Abou-Rebyeh H, Oelkers W, Hensen J. Expression of vasopressin receptors (V2-subtype) on LLC-PK1 cells during cell culture. *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies.* 1993;31(5):273-276.

43. Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacological reviews.* 2001;53(1):1-24.

44. Nephrogenic Diabetes Insipidus. 2007. Accessed 2010.

45. Manning M, Misicka A, Olma A, Bankowski K, Stoev S, Chini B, Durroux T, Mouillac B, Corbani M, Guillon G. Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics. *J Neuroendocrinol.*24(4):609-628.

46. Koshimizu TA, Nakamura K, Egashira N, Hiroyama M, Nonoguchi H, Tanoue A. Vasopressin V1a and V1b receptors: from molecules to physiological systems. *Physiological reviews.* 2012;92(4):1813-1864.

47. Russell JA, Walley KR, Singer J, Gordon AC, Hebert PC, Cooper DJ, Holmes CL, Mehta S, Granton JT, Storms MM, Cook DJ, Presneill JJ, Ayers D, Investigators V. Vasopressin versus norepinephrine infusion in patients with septic shock. *The New England journal of medicine.* 2008;358(9):877-887.

48. Czako L, Laszlo F, Manning M. [Treatment of diabetes insipidus with synthetic vasopressin derivatives (effect of 1-deamino-8-D-arginine vasopressin and 1-deamino-valine-8-D-arginine vasopressin)]. *Orvosi hetilap.* 1980;121(13):773-778.

Figure legends

Figure 1: The results of DNA sequencing and the familiar anamnesis. (A) The genomic DNA was isolated from peripheral blood. After PCR amplification the AVPR2 gene was sequenced. The chromatogram shows the results of the sequencing in the patient and in a healthy control. (B) Familiar anamnesis of the patient. Filled mark indicates polydipsia-polyuria syndrome in the male (squares) and female (circles) members of the family. The scored marks indicate deceased family members.

Figure 2: Examination of the cell surface expression of the N321K-V2R. Immunofluorescence microscopy analysis of HEK293 cells transiently expressing wild type (A and C) or N321K (B and D) HA-tagged V2R. The samples were stained with anti-HA-Alexa488 mouse monoclonal antibodies under permeabilized (C and D) and non-permeabilized (A and B) conditions. Scale bars represent 10 μm.

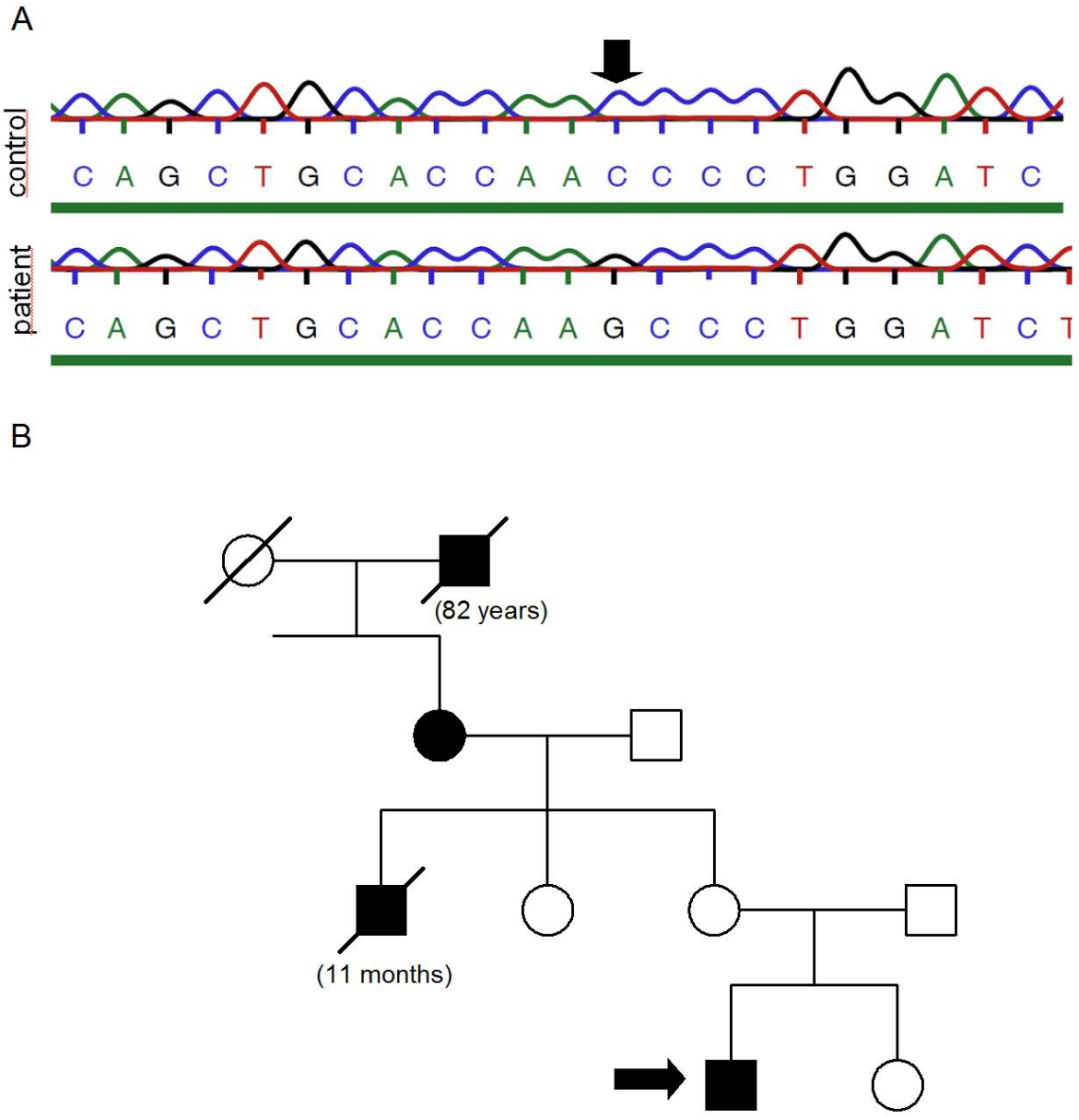
Figure 3: Measurement of the cAMP signal upon AVP and dDAVP stimuli. HEK293 cells were transiently transfected with the wild type or the N321K-V2R and the Epac-BRET sensor. After 24 h, the BRET measurements were implemented. (A) The cells were stimulated with 10 nM AVP in case of the wild type (square) and with 1 μM AVP in case of the mutant receptor (triangle) at the indicated time. Dose response curve of AVP (B) and dDAVP (C). The effect of the hormone on the WT-V2R and N321K-V2R expressing HEK293 cells was calculated as the BRET ratio difference between the ligand (stim) and the vehicle (nstim) treated cells at the first time points after the treatment. Mean values ± S.E. are shown (n = 3).

Figure 4: Examination of the internalization and β-arrestin binding properties of N321K-V2R. HEK293 cells were transiently transfected with the plasmids of the indicated BRET partners and after 24h, the cells were exposed to AVP or vehicle. (A-B) β-arrestin binding was measured with the transfection of wild type- or N321K-V2R-mVenus and β-arrestin-Rluc plasmids. (A) Cells were exposed either to 1μM AVP (square) or vehicle (dashed line) at the indicated time points. (B) Dose response curve of β-arrestin binding of AVP. The effect of the hormone on cells was calculated as the BRET ratio difference between the ligand (stim) and the vehicle (nstim) treated cells. (C) Internalization kinetics was measured with the transfection of wild type- or N321K-V2R-Sluc and MP-YFP plasmids. Cells were exposed either to 1μM AVP or vehicle at the indicated time points. Mean values ± S.E. are shown (n = 3).

Figure 5: Measurement of the cAMP signal upon different agonist stimuli. HEK293 cells were transiently transfected with the wild type or the N321K-V2R and the Epac-BRET sensor. After 24 h, the BRET measurements were implemented. Dose response curves of dVDAVP (A), PVDAVP (B), LVP (C) and AsuAVP (D). The effect of the hormones on the WT-V2R and N321K-V2R expressing HEK293 cells were calculated as the BRET ratio difference between the ligand (stim) and the vehicle (nstim) treated cells at the first time points after the treatment. Mean values ± S.E. are shown (n = 3).

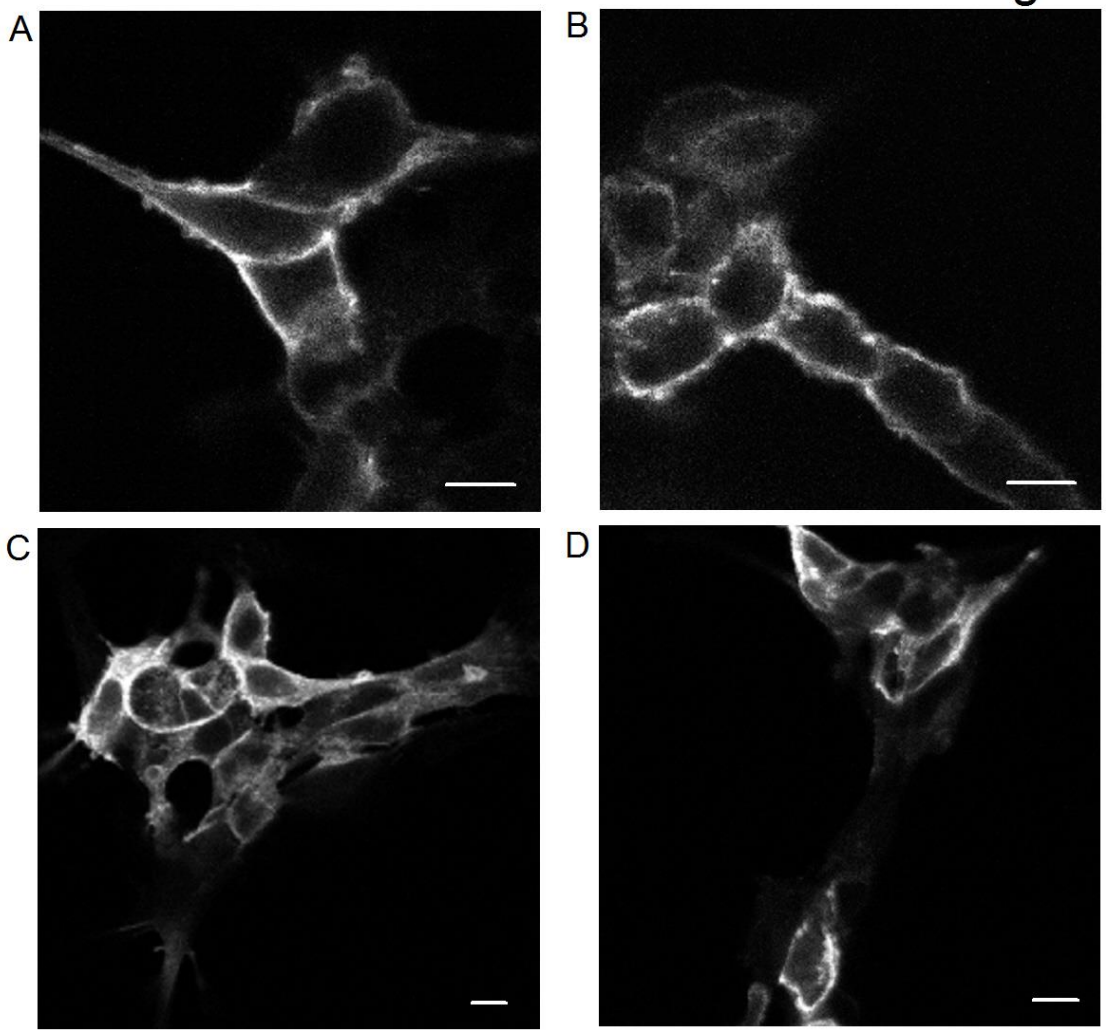
Figure 6: Effects of AVP and dVDAVP on vasoconstriction of mouse arterioles. Isolated mouse arterioles were exposed to increasing concentrations of AVP (square) or dVDAVP (triangle). The values of the vasoconstrictor responses were calculated as percent values of reference 1μM phenilephrin caused precontraction. The values are average of 3 independent experiments. Mean values ± SEM are shown (n = 3).

Figure 1


A
control
C A G C T G C A C C A A C C C C T G G A T C
patient
C A G C T G C A C C A A G C C C T G G A T C T
B
(82 years)
(11 months)

680

Figure 2



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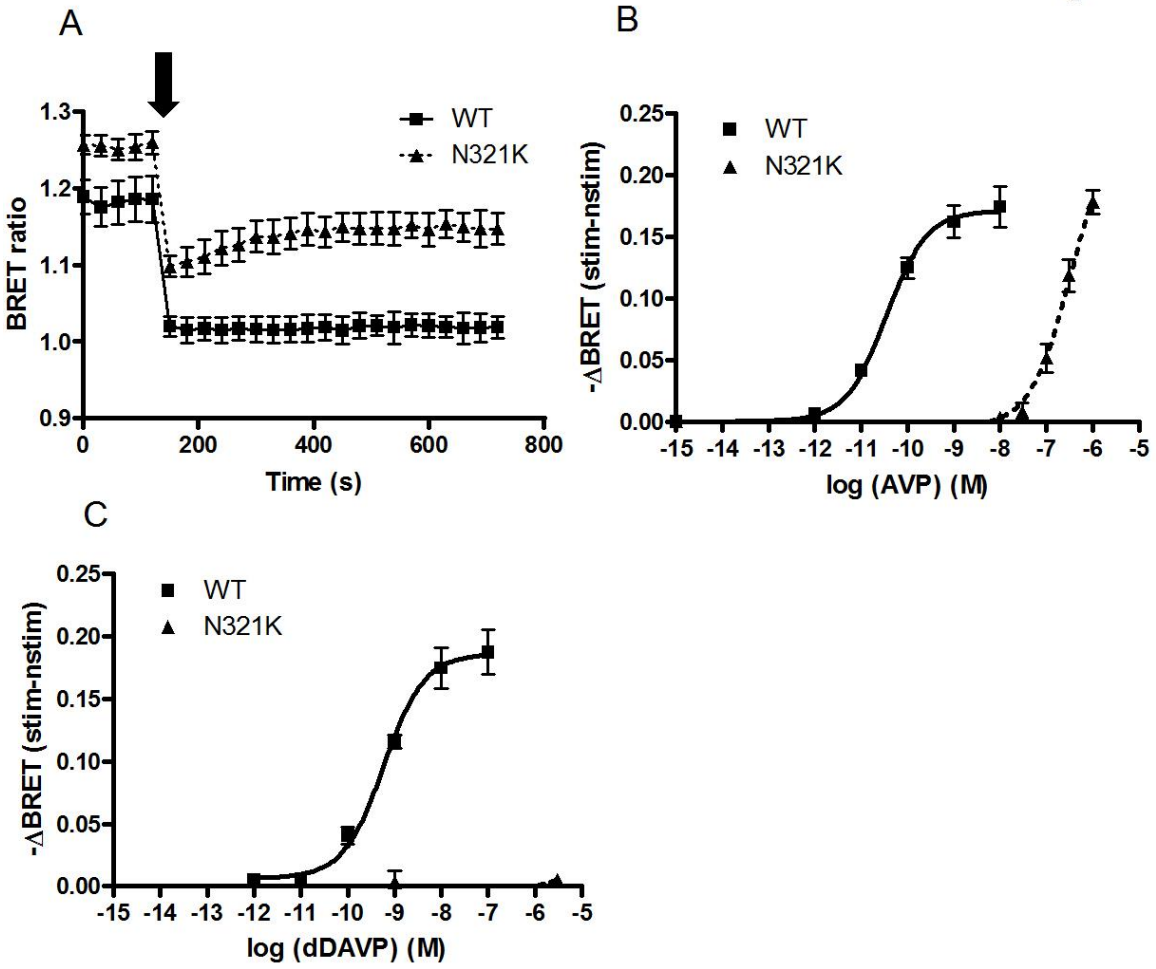
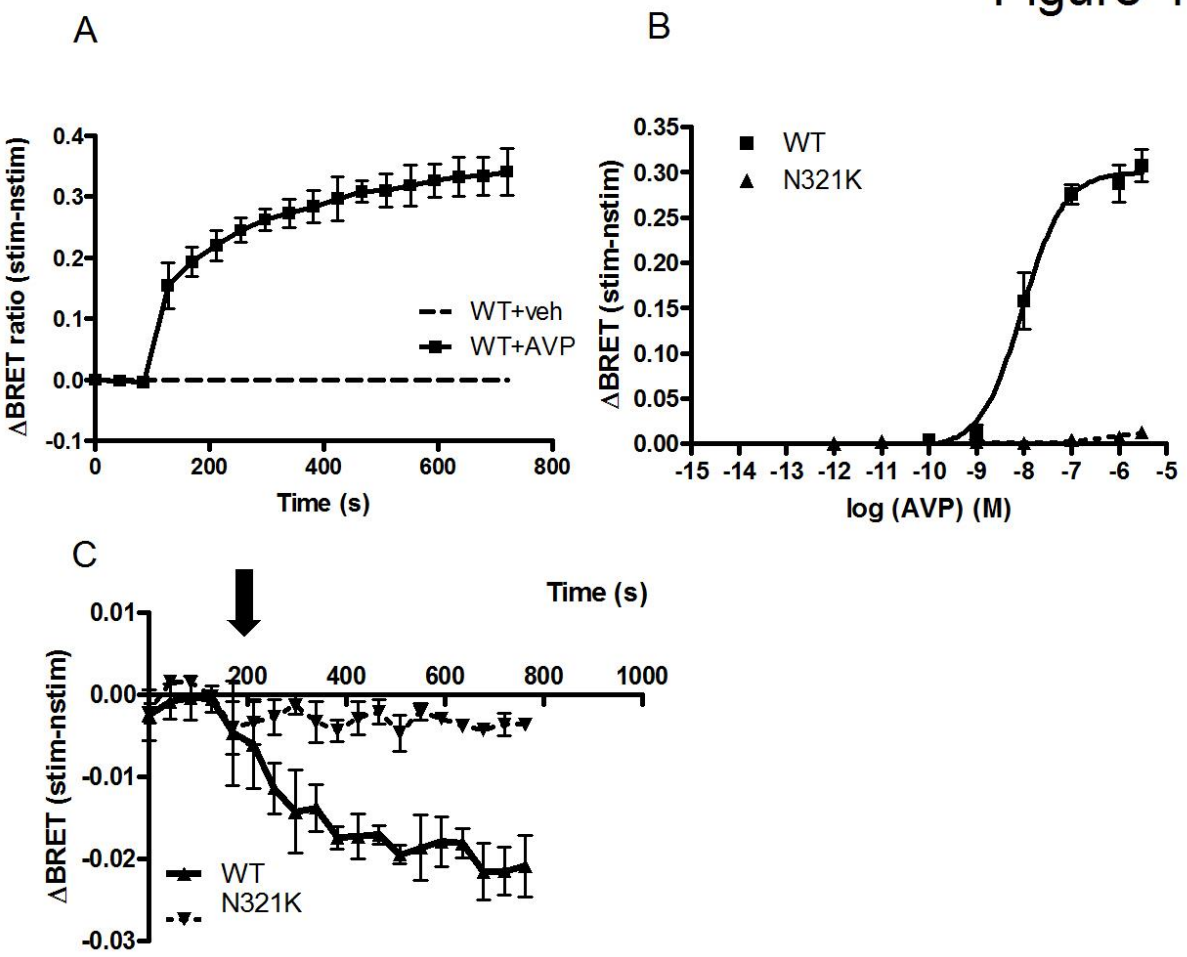
682

Figure 3


A
BRET ratio
Time (s)
WT
N321K
B
-ΔBRET (stim-nstim)
log (AVP) (M)
WT
N321K
C
-ΔBRET (stim-nstim)
log (dDAVP) (M)
WT
N321K

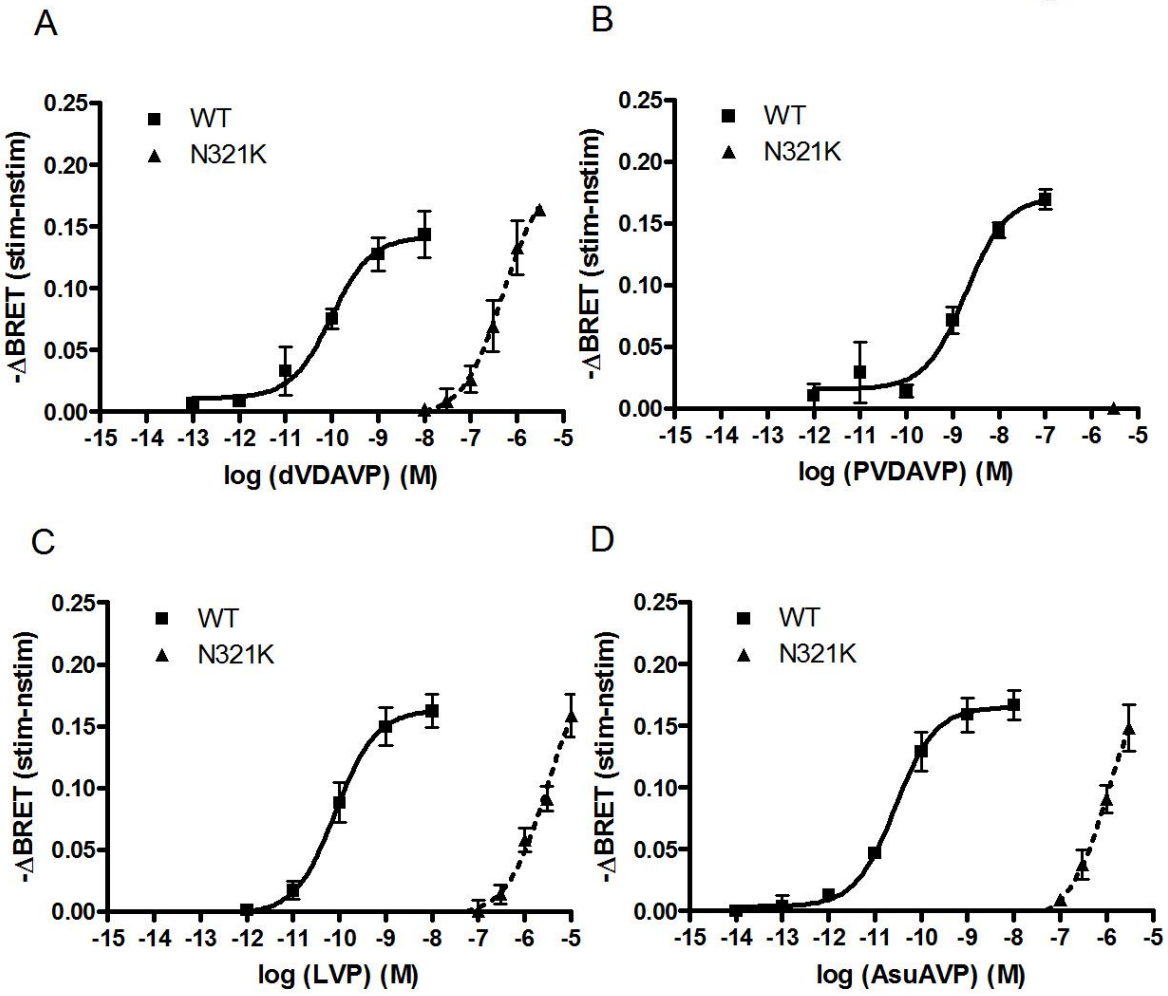
683

Figure 4


A
ΔBRET ratio (stim-nstim)
0.4
0.3
0.2
0.1
0.0
-0.1
0
200
400
600
800
Time (s)
WT+veh
WT+AVP
B
ΔBRET (stim-nstim)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log (AVP) (M)
WT
N321K
C
Time (s)
ΔBRET (stim-nstim)
0.01
0.00
-0.01
-0.02
-0.03
200
400
600
800
1000
WT
N321K

684

Figure 5


A
B
C
D
WT
N321K
-ΔBRET (stim-nstim)
0.25
0.20
0.15
0.10
0.05
0.00
-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log (dVDAVP) (M)
log (PVDAVP) (M)
log (LVP) (M)
log (AsuAVP) (M)

685

Figure 6

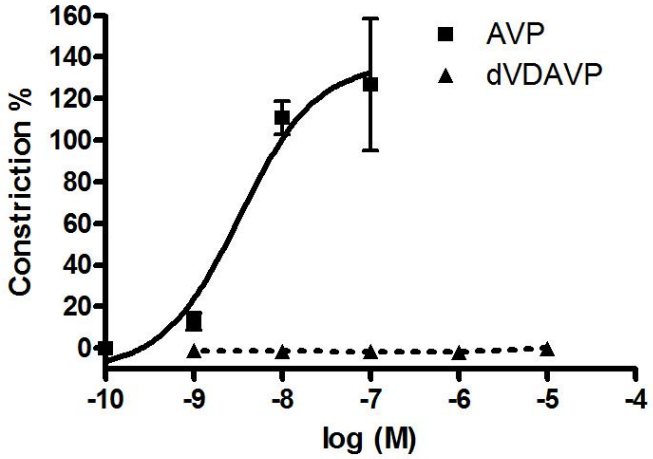


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Table 1.

pEC50 (M)	WT	N321K
AVP	10.46±0.04	6.492±0.07
dDAVP	9.229±0.07	
dVDAVP	10.02±0.11	6.30±0.05
AsuAVP	10.56±0.04	5.94±0.02
LVP	10.08±0.02	5.52±0.13
PVDAVP	8.75±0.01	