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Investigation of two clinically important G protein-coupled receptors: V2 vasopressin receptor and AT1 angiotensin receptor

Ph.D. Thesis

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List of abbreviations

7TM	seven-transmembrane
ACE	angiotensin-converting enzyme
ACE2	angiotensin-I converting enzyme 2
ACEI	angiotensin-converting enzyme inhibitor
ADAM	a disintegrin and metalloprotease
ADPKD	rapidly progressing autosomal dominant polycystic kidney disease
AGT	angiotensinogen
AQP2	aquaporin 2 water channel
ARB	angiotensin II type-1 receptor blocker
AngI	angiotensin I
AngII	angiotensin II
AngIII	angiotensin III
AngIV	angiotensin IV
AT1-R	angiotensin II type-1 receptor
AT2-R	angiotensin II type-2 receptor
AVP	arginine vasopressin
AVPR2	arginine vasopressin type 2 receptor gene
BRET	bioluminescence resonance energy transfer
CaMKII	calcium / calmodulin-dependent protein kinase II
cAMP	3',5'-cyclic adenosine monophosphate
CDI	central diabetes insipidus
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
dDAVP	1-deamino-8-D-arginine- vasopressin
DI	diabetes insipidus
DUSP	dual-specificity MAP kinase phosphatases
ECL	extracellular loop
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

Epac exchange factor directly activated by cAMP
ER endoplasmic reticulum
ERK extracellular signal-regulated kinase
FAK focal adhesion kinase
FGF fibroblast growth factor
FGFR fibroblast growth factor receptor
FNDI familial neurohypophyseal diabetes insipidus
GDP guanosine diphosphate
GEF guanine nucleotide exchange factor
GPCR G protein-coupled receptor
GRK G protein-coupled receptor kinases
GTP guanosine triphosphate
HA hemagglutinin
HB-EGF heparin-binding EGF-like growth factor
HD heptahelical domain
HEK-293 human embryonal kidney cell
ICL intracellular loop
IP_3 inositol 1,4,5-trisphosphate
JAK Janus kinases
JG juxtaglomerular
JNK c-Jun NH2-terminal kinase
MAPK mitogen-activated protein kinase
MEK mitogen-activated protein kinase kinase (MAPK/ERK kinase)
MKK MAPK kinase
MKP dual-specificity MAP kinase phosphatase (DUSP)
MKP-1 mitogen-activated protein kinase phosphatase-1
MMP matrix metalloproteinase
MrgD Mas-related GPCR member D
NDI nephrogenic diabetes insipidus
NO nitric oxide
NSAID nonsteroidal anti-inflammatory drugs
NSIAD nephrogenic syndrome of inappropriate antidiuresis disease

PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
Pyk2	proline-rich tyrosine kinase 2
RAAS	renin-angiotensin- aldosterone-system
SIADH	syndrome of inappropriate antidiuretic hormone
SnRK1	SNF1 (sucrose non-fermenting 1)-related protein kinase 1
TM	transmembrane
VSMC	vascular smooth muscle cell
V1R	type 1 vasopressin receptor
V2R	type 2 vasopressin receptor
V3R	type 3 vasopressin receptor
WT	wild type
X-NDI	X-linked nephrogenic diabetes insipidus

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1. INTRODUCTION

The focus of the thesis is the examination of signaling pathways initiated by two types of G protein-coupled receptors (GPCRs), namely the angiotensin II type 1 receptor (AT1-R) and type 2 vasopressin receptor (V2R).

Cardiovascular diseases are the leading cause of global morbidity and mortality [1]. The importance of the angiotensin II (AngII)/AT1-R arm of the renin-angiotensin-aldosterone system (RAAS) has been well described in cardiovascular physiology and pathophysiology, although the detailed molecular mechanisms are not completely uncovered, so they require further investigation. Of particular importance is the fact that the AngII activates numerous signaling pathways which cause vasoconstriction and aldosterone secretion, which, in turn, results, acutely, in increased peripheral vascular resistance and blood volume, and, chronically, long-term changes in blood pressure [2]. In addition, the AngII stimulus also induces gene expression changes and other long-term responses in cardiovascular tissues [2]. These responses cause proliferation, hypertrophy and vascular remodeling [2]. The cellular mechanisms by which these responses are generated have been the subject of myriad studies, and finding the novel aspect of these mechanisms was the motivation behind the experiments performed within this research.

Furthermore, besides receptor overactivation, mutations in GPCRs can also lead to pathological conditions such as mutation in V2R, which cause nephrogenic diabetes insipidus (NDI). Arginine-vasopressin system plays an essential role in the maintenance of the human body's water balance by regulating the concentrating function of the kidney via stimulation of V2R [3]. Loss-of-function mutations in V2R lead to NDI disease characterized by polyuria and polydipsia.

The first part of the thesis is focused on the exploration of the functional and biochemical properties of the NDI-causing mutation in the V2R. The second part of the thesis examines the AngII-induced gene expression changes of three dual-specificity MAP kinase phosphatase (DUSP) genes in vascular smooth muscle cells.

1.1. G protein-coupled receptors (GPCRs)

GPCRs, also known as seven-transmembrane (7TM) receptors, are key cell-surface proteins which constitute the largest class of receptor and membrane proteins in the

human proteome. They transduce environmental signals to various pathways across the membrane and mediate the majority of cellular responses to external stimuli, including taste, smell and light, and the effects of hormones, paracrine factors and neurotransmitters on cells, thus playing essential roles in physiological processes. Because of that and not surprisingly, GPCRs are commonly targeted by pharmacological agents. The common structural feature of these receptors is a seven transmembrane α helical arrangement (often called the heptahelical domain, or HD). However, the size and function of the extracellular N-terminus and loops, as well as the intracellular C-terminus and loops, vary greatly [4]. Although the mechanism of action of GPCRs was thought to be largely mapped out in the 80s and 90s, recent discoveries have revealed new and previously unappreciated mechanisms for GPCR activation and subsequent downstream signaling.

There are more than 800 individual GPCRs in humans, which can be divided into five main families, based on their sequence and structural characteristics: **G**lutamate family (family C), **R**hodopsin family (family A), **A**dhesion family (family B2), **F**rizzled/taste2 family (family F), **S**ecretin family (family B1) according to the "GRAFS" classification system [5]. More than 700 GPCRs belong to class A or rhodopsin-like receptors in humans [6], including the angiotensin type 1 receptor (AT1-R) and type 2 vasopressin receptor (V2R) examined in this dissertation.

The binding of ligands to GPCRs triggers ligand-specific conformational changes and by stabilizing the receptor conformation, the GPCRs can interact with intracellular "microswitches" through the α-helical transmembrane (TM) domains. Upon activation, GPCRs couple to one or more members of G protein families and/or β-arrestin proteins (Figure 1). Activating ligands or agonists stabilize the active receptor conformation, allowing binding to G proteins, whereas the inhibitory ligands or antagonists stabilize the inactive receptor conformation. The inverse agonists are ligands which are capable of inducing a pharmacological response opposite to that of the agonist. The cellular function of GPCR is based on the activation of the heterotrimeric G proteins which are composed of three subunits: G_α, G_β, and G_γ [7].

In contrast to the very high number of human GPCRs, only a relatively small number of different G proteins have been identified. Based on the sequence similarity between each G_α subunit, heterotrimeric G proteins can be classified into four main families (G_s,

$G_{i/o}$, $G_{q/11}$, and $G_{12/13}$) that regulate the activation of different effector proteins, thereby generating distinct downstream signaling responses [8].

GPCR-mediated G protein signaling is initiated by the binding of activating ligands, which leads to stabilization of the active conformation of GPCR, therefore the receptor can interact with a guanosine diphosphate (GDP)-bound inactive heterotrimeric G protein. This interaction allows the receptor to act as a guanine nucleotide exchange factor (GEF), promoting the exchange of GDP for guanosine triphosphate (GTP) in the G_{α} subunit. The GTP-bound G_{α} subunit dissociates from $G_{\beta\gamma}$, and, thus, both subunits can mediate downstream signaling by modulating the activity of cytosolic effector proteins and changing the intracellular concentrations of second messengers such as 3',5'-cyclic adenosine monophosphate (cAMP), calcium ion or diacylglycerol (DAG). Since the G_{α} subunit has internal GTPase activity, it is thus able to hydrolyze GTP. Afterwards, it is possible to reassemble the heterotrimeric G protein by the GDP-bound G_{α} subunit and $G_{\beta\gamma}$. This process allows the G protein to enter another G protein activation cycle.

Beside G proteins, important interaction partners of GPCRs are the G protein-coupled receptor kinases (GRKs), which can phosphorylate intracellular peptide motifs of active GPCRs. The GRK family consists of seven homologs, of which GRK1 and 7 phosphorylate the visual opsins, whereas the GRK2–6 regulate various members of GPCRs family, as well as other cell-surface receptors [9]. The consequential accumulation of negative charges enables the high affinity binding of β-arrestin proteins, initiating desensitization and internalization of receptors [10]. There are four subtypes of arrestin proteins, which are found in most vertebrates. They are divided into visual and non-visual subtypes. Arrestin-1 and -4 are the visual subtypes expressed in photoreceptor cells in the retina and there they regulate the phototransduction cascades, and, thus, quench photopigment signaling in rods and cones. The nonvisual arrestins, arrestin-2 and -3 (also known as β-arrestin 1 and 2), regulate hundreds of GPCR subtypes [11]. As β-arrestin and G proteins utilize overlapping binding interfaces, G protein binding is sterically blocked, so β-arrestin-bound receptors are no longer able to induce their primary signaling [12]. In addition, GPCR-mediated β-arrestin activation also promotes the stimulation of other cytoplasmic signaling pathways, such as mitogen-activated protein kinase (MAPK) and non-receptor tyrosine kinase c-Src [9].

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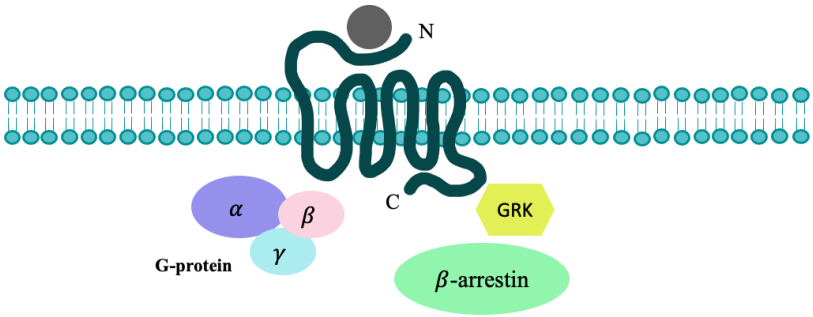
The GPCR receptor sensitivity can be changed due to various reasons. Receptor desensitization can occur after agonist stimulus, especially upon prolonged or repeated exposure to high agonist concentrations. An important mechanism in the regulation of the responsiveness of the GPCRs is the GRK- β-arrestin system, which is responsible for rapid signal termination [13]. Based on the different desensitization mechanisms of the GPCRs, homologous and heterologous desensitization can be distinguished.



- Trigger signaling cascades and cellular responses.

- GPCR internalization and desensitization.
- Blocking G-protein access.
- Activate additional signaling cascades.

Figure 1. **Schematic representation of the generic structure and signaling pathway of GPCRs.** GPCRs bound to G protein vs. β-arrestin show differential signaling behaviors (Based on [10]).

During homologous desensitization, the receptor is unable to initiate signal transduction by repeated agonist stimuli. The most important effectors of this mechanism are GRK kinases and β-arrestin proteins. Following receptor activation, similarly to G proteins, GRKs also can recognize the same activated GPCRs, leading to phosphorylation of the receptor at specific sites on the intracellular loops and carboxyl-terminal tail. After that, a β-arrestin is able to bind with high affinity to the phosphorylated region of the receptor. There is an overlap in the receptor region involved in the binding of G protein and β-arrestins, and, therefore, β-arrestin binding to the receptor inhibits further G protein binding and activation, resulting in desensitization of G protein-dependent signaling of the receptor [14, 15]. On the contrary, in case of heterologous desensitization, the agonist-induced signaling of a certain receptor causes desensitization of an agonist-unbound receptor, thus reducing the responsiveness of the other receptors. In contrast to the mechanism of homologous desensitization, heterologous desensitization involves the

activity of protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC), which can phosphorylate the intracellular parts of other receptors in the plasma membrane. The GRK-β-arrestin system performs other functions, including promoting the internalization of receptors from the cell surface through clathrin-coated pits. In addition, by binding to other signaling proteins, GRKs and β-arrestins are able to act as switches, thus activating G protein-independent pathways.

Advances in our understanding of the structure and functions of GPCRs have led to the development of new types of drugs. The biophysical studies of GPCR structures now allow for *in silico* docking of chemical compounds and structure-based drug design [16]. Furthermore, recent advances in identifying various formats of GPCR-targeted antibodies to lock receptors in specific conformational states or in complex with signaling partners, as well as advances in electron cryomicroscopy technology, now allow for atomic-resolution characterization of heterogeneous structural ensembles [16]. These technologies might continue to provide significant insights into the dynamic features of GPCR structure and function [16]. As new GPCR ligands and signaling mechanisms are discovered, many potential therapeutic targets may become evident to more precisely achieve the desired effect.

1.2. The arginine-vasopressin system

The diluting and concentrating function of the kidney plays a crucial role in regulating the body's normal water balance [3]. Water is lost during sweating, breathing, urination and defecation, and water is obtained through drinking, eating and metabolic processes. The maintenance of normal fluid balance and plasma osmolality (290-295 mOsm/kg H_2O) requires both tight regulation of water intake and urinary water excretion [3]. Maintaining water homeostasis is controlled by both the osmolality and intravascular blood volume. Decreased blood volume and/or increased plasma osmolality reflect a need for the body to conserve water. Changes in intravascular blood volume are sensed by vascular volume receptors and baroreceptors, which are activated by 5-10% changes in blood volume, while even smaller changes, less than 1%, in plasma osmolality stimulate osmoreceptors in the hippocampus [17, 18]. These processes regulate the release of the antidiuretic hormone, arginine vasopressin (AVP), from the posterior pituitary gland.

AVP is a 9 amino acid nonapeptide, which is biochemically cyclical with a critical disulfide bridge that determines biological activity. AVP is synthetized from the precursor protein, prepro-vasopressin, which is proteolysed to AVP, copeptin and neurophysin II. AVP is synthesized by neurons whose cell bodies are present within the magnocellular supraoptic and paraventricular nuclei in the hypothalamus and released in the posterior pituitary. AVP is accompanied by a carrier protein, neurophysin, traveling along the supraoptic pituitary tract to reach the axonal terminals of the posterior pituitary gland of magnocellular neurons, where AVP is released. The secreted AVP molecules then enter nearby capillaries, and, through them, the body's systemic circulation, from where they set out to the kidney and primarily affect its to reabsorb water. Presence of AVP induces expression of water transport proteins in the late distal tubule and collecting duct to increase water reabsorption [19]. Under physiological conditions, the glomerular filtration rate is 180 L/day, of which approximately 90% is constitutively reabsorbed in the proximal tubule and descending limb of Henle's loop. The remaining fluid can be reabsorbed in the renal collecting duct, depending on the needs of the body. This process is primarily regulated by the hormone AVP allowing the body to adapt to periods of water load or water restriction.

AVP is a key hormone for adequate tonicity homeostasis, and, thus the hormone release is most strongly regulated by hyperosmolarity or hypernatremia and hypovolemia [19]. The osmoreceptors in the hypothalamus, where AVP is stored, respond perfectly to very small changes in blood osmolarity [20]. Therefore, a slight increase in osmolarity results in the secretion of AVP, which acts primarily to increase water reabsorption in the kidneys, thereby restoring osmolarity to its baseline [21]. In hypovolemia or volume deprivation, baroreceptors sense decreased arterial blood pressure and volume and the information is transmitted to the central nervous system causing the release of AVP, which then promotes reabsorption of water in the kidneys and also causes vasoconstriction in high concentrations. Thus, these two mechanisms together increase the effective arterial blood volume and increase blood pressure to maintain tissue perfusion. In the hypovolemic state, AVP is excreted even in the hypoosmotic state, whereas, in contrast, hypervolemia inhibits AVP secretion [19]. Therefore, in hyperosmotic hypervolemic states, AVP secretion decreases [19].

In addition to osmolarity and blood volume status, several other factors affect AVP secretion. Nausea, hypoglycemia, nicotine, opiates, angiotensin II, pain, and certain medications promote AVP secretion [22]. On the contrary, AVP secretion is negatively affected by ethanol, atrial natriuretic peptides and alpha-adrenergic agonists [22].

1.2.1. Vasopressin receptors

AVP hormone has multiple actions in the body. In addition to its antidiuretic effect, the AVP is a potent neurohormone involved in the regulation of arterial blood pressure, sympathetic activity, baroreflex sensitivity, glucose homeostasis, release of glucocorticoids and catecholamines, stress response, anxiety, memory, and behavior [23]. These multiple actions of AVP are mediated by at least three G protein-coupled receptors: type 1 vasopressin receptor (V1R, also known as V1aR), type 2 vasopressin receptor (V2R) and type 3 vasopressin receptor (V3R, also known as V1bR).

1.2.1.1. Type 1 vasopressin receptor (V1R) and type 3 vasopressin receptor (V3R)

The V1 receptors are found on vascular smooth muscle cells in arteries, arterioles, and veins, hepatocytes, the brain, and other tissues. These receptors mediate vasoconstriction, and they increase in vascular resistance in most of the vascular beds [24]. The V3 receptors are mainly found on cells within the central nervous system, especially in the anterior pituitary and are involved in adrenocorticotropin hormone release and also in cognitive functions in the brain [24, 25]. Activation of V1 and V3 receptors stimulates phospholipase C (PLC) via G_q protein, which mediates the hydrolysis of inositol 4,5-biphosphate to inositol 1,4,5-trisphosphate (IP_3) and DAG [18]. These second messengers activate enzymes, such as PKC, which leads to an increase in intracellular Ca^{2+} [18].

1.2.1.2. Type 2 vasopressin receptor (V2R)

AVP exerts its water-conserving effect through the V2R, which is a member of the GPCR superfamily, localized on the basolateral side of the principal cells of the kidney's collecting duct. When AVP reaches the kidney, it binds to the V2R receptor on the basolateral membrane of the collecting duct principal cells and initiates a signaling cascade. Binding of AVP activates and promotes receptor interaction with the G_s

heterotrimeric protein, which, in turn, activates adenylate cyclase. Adenylate cyclase generates a second messenger, intracellular cAMP, which leads to the activation of PKA. The PKA phosphorylates the aquaporin 2 (AQP2) water channels and causes translocation from intracellular storage vesicles to the apical plasma membrane, rendering this membrane permeable to water (Figure 2A) [19, 26, 27]. The AQP2 functionally exists as homotetramers and is the rate-limiting entry site for water reabsorption along an osmotic gradient [28, 29]. Water enters the principal cell through AQP2 along the osmotic concentration gradient and leaves the cell through AQP3 and AQP4 water channels, which are constitutively expressed on the basolateral side of these cells (Figure 2A). Upon recovery of the isotonic state, a decrease in AVP levels in the blood plasma induces AQP2 internalization, which, in turn causes lower rate of water reabsorption [30]. In addition, activation of V2R by AVP leads to C-terminal phosphorylation of V2R and consequent recruitment of β-arrestin and internalization of the receptor, which negatively regulates the effects of V2R activation thus preventing prolonged and excessive absorption of water [31].

1.2.2. Pathology of the arginine-vasopressin system

Diabetes insipidus

Diabetes insipidus (DI) is an inherited or acquired clinical syndrome, which is associated with inadequate AVP hormone secretion or renal response to AVP, characterized by increased urinary excretion (> 50 mL/kg), diluted urine (<300 mOsm/kg), and increased water intake of up to 20 L/day [32]. The kidneys excrete large amounts of water regardless of the hydration state of the body. Because of that, untreated DI patients or inadequate water intake, needed to compensate for loss of water, can cause a serious risk of dehydration [33, 34].

There are four fundamental forms of DI: central DI (CDI), nephrogenic DI (NDI), gestational DI, and primary polydipsia. There are two congenital forms of DI, familial neurohypophyseal DI (FNDI) and congenital NDI, which together account for <10% of all DI clinical cases [35]. While FNDI is the central form of DI due to deficient secretion of AVP from the pituitary gland, NDI occurs primarily due to a decreased or defective effect of AVP in the principal cell of the collecting duct. Unlike congenital forms, both CDI and NDI can also be acquired. These conditions, especially NDI, are generally more

common. Other forms of DI include gestational diabetes insipidus caused by a lack of AVP due to the increased metabolism of AVP in the placenta and due to primary polydipsia, resulting from AVP suppression due to excessive water intake [36]. AVP deficiency in CDI can be corrected by treatment with commercially available desmopressin, which is an AVP analog specific for the V2R, while defects in the action of AVP at the level of kidneys can rarely be corrected. In the dissertation, the congenital NDI form, which is related to the concept of the research to be presented is discussed.

Nephrogenic Diabetes Insipidus

The main characteristic of NDI is the impaired AVP-induced water reabsorption, occurring regardless of normal or elevated plasma concentrations of AVP. The incapacity of the late distal tubules and collecting ducts to respond to AVP results in defective urine concentration, which causes several clinical manifestations such as polyuria, polydipsia, and hyposthenuria. Most commonly, the acquired form of NDI manifests as a complication in numerous common clinical conditions, such as electrolyte abnormalities (hypercalcemia, hypokalemia), or in treatment with drugs such as lithium and cisplatin-therapy.

Congenital Nephrogenic Diabetes Insipidus

Congenital NDI is a result of a mutation in *AVPR2* or *AQP2* genes. The *AVPR2* gene encodes the vasopressin V2R receptor, while *AQP2* encodes the AQP2 water channel. Different forms of congenital NDI are distinguished based on different inheritance patterns. Mutations affecting the *AVPR2* gene are inherited in an X-linked, recessive manner. The *AQP2* mutation shows autosomal dominant or recessive inheritance. X-linked NDI (X-NDI) accounts for 90% of congenital cases, while in the remaining 10%, congenital NDI has an autosomal dominant or recessive inheritance pattern with mutations in the *AQP2* gene.

V2R is predominantly expressed in the distal convoluted tubules and renal collecting tubes. V2R responds to AVP, and, thus, urine concentration is possible, whereas in the case of X-NDI, the distal nephron is insensitive to AVP, resulting in blunted water reabsorption in the collecting ducts. Disorders of urinary concentration are present from birth and symptoms appear early, in the first weeks. Infants often suffer from

hypernatremic dehydration, with symptoms of irritability, poor feeding, and poor weight gain, and occasionally high fever due to dehydration and constipation, as well as occasional seizures [37]. Without treatment, most patients cannot grow normally, and mental retardation can occur as a serious complication, presumably as a result of repeated episodes of brain dehydration and brain edema (resulting from too rapid rehydration attempts) [38]. However, such complications may occur in the case of a delayed clinical diagnosis, in *de novo* mutations. In contrast, those at risk of inheriting the disease gene are diagnosed and treated much earlier [38].

Mutation in the *AVPR2 gene*

Various mutations in the *AVPR2* gene can be classified as loss-of-function mutations, which result in impaired receptor signaling transduction or reduce the number of cell surface receptors [30]. These mutations lead to X-NDI, which causes the characteristic symptoms of NDI [39]. Concentration of urine cannot be observed even after exogenous AVP administration in male X-NDI patients, whereas some heterozygous women have varying degrees of polyuria and polydipsia due to skewed X-chromosome inactivation [40, 41]. Gain-of-function mutations of the V2R are also described, which result in the nephrogenic syndrome of inappropriate antidiuresis disease (NSIAD), causing constitutive activation of the receptor [42]. The clinical appearance of NSIAD syndrome is completely asymptomatic, with defective urine dilution only manifesting upon water loading [43].

To date, more than 200 X-NDI-inducing mutations have been described, most commonly affecting the transmembrane domain of the receptor and various mechanisms are responsible for the impaired functions of the mutated receptors [44, 45]. The cellular manifestations of the *AVPR2* gene mutation can be classified into five classes (Figure 2B) [46]. The class I mutations of the *AVPR2* gene result in improperly processed or unstable mRNA, frameshift or nonsense mutations that lead to truncation of V2R receptors [46]. The class II mutations are the most common, caused by missense/nonsense mutations that result in misfolding of the receptor protein. They are recognized by the endoplasmic reticulum (ER) quality control system. These mutant receptors are usually trapped in the ER and their degradation occurs often. The class III mutations also cause misfolding of receptor proteins, although here the V2R receptor reaches the plasma

membrane and interacts with AVP, but it does not interact fully with G proteins, resulting in impaired cAMP production. The class IV mutations also result in V2R misfolding, but in this case the mutant V2R reach the plasma membrane, but do not interact properly with AVP, while class V mutations are missorted to an incorrect cellular compartment [46].

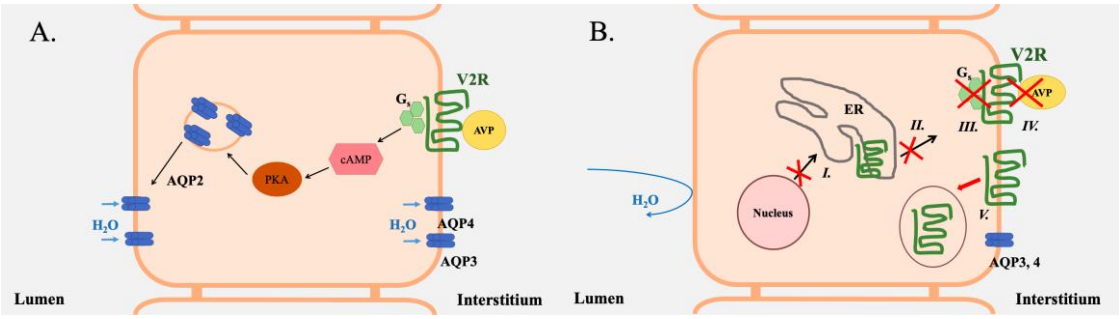


Figure 2. **(A) Schematic representation of the mechanisms of urine concentration by vasopressin (AVP).** Circulating AVP binds to the G protein-coupled vasopressin receptor (V2R) in the basolateral membrane of cells of the renal collecting ducts. G_s protein-mediated signaling leads to activation of adenylate cyclase. The adenylate cyclase is then activated and it increases the cAMP production and PKA activity leading to AQP2 phosphorylation and accumulation in the apical plasma membrane. Water is reabsorbed from urine through AQP2, AQP3 and AQP4, thereby concentrating the urine. **(B) Cell physiological consequences of the V2R mutation leading to NDI.** In the schematic figure of the collection duct cells, the manifestation of the different mutation classes is indicated by Roman numerals. V classes of V2R mutation in NDI: mutation can lead to incomplete proteins (I.); ER retention of full-length proteins (II.); impaired G_s coupling (III.); impaired AVP binding (IV.); and missorting in the cell (V.) (Based on [46]).

1.2.3. Therapy of congenital NDI

Currently, no specific treatment is available to restore the function of the mutant V2R in patients with X-linked NDI. The main strategy for treating cNDI patients is directed toward the specific symptoms. This strategy consists of the replacement of urinary water loss by adequate water intake in combination with a low-salt and low-protein diet to minimize water excretion. However, this can seriously impact the quality of life, due to excessive drinking and urine voiding. Conventional treatment strategies also include the use of thiazide diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs) and amiloride, used isolated or in combination, which have been proven effective in reducing urine output by up to 50% [47]. Treatment of a polyuric condition with diuretics blocks the Na-

Cl-cotransporter in the distal tubule and promotes the reabsorption of sodium and water in the proximal tubule, thus delivering less water to the collecting ducts [37]. However, diuretics may affect the sodium and potassium balance in patients. Therefore, in addition to diuretics, the administration of potassium sparing agents, such as amiloride, can have an additive effect with diuretics and compensate for potassium loss induced by thiazides. However, these treatments require tight monitoring of serum electrolytes and osmolality. Additionally, treatment with diuretics, sometimes in combination with NSAID, such as indomethacin can also efficiently decrease the degree of polyuria [36], but latter drugs main limiting factors, that they often cause gastrointestinal bleeding, and their administration can potentially lead to the acute deterioration of renal function in dehydrated patients [37].

Although conventional treatment can alleviate the symptoms of NDI, the urine concentrating defect is still considerable, representing a serious problem for the patient's quality of life. Alternative approaches must be considered for treatment of NDI patients who do not tolerate currently available drugs due to their side effects.

1.2.3.1. Pharmacological chaperones as a possible therapeutical strategy for the treatment of congenital NDI

Recent studies discuss new and alternative approaches for treating patients with X-linked NDI, of which the latest therapeutical strategies include pharmacological chaperones. Although the diversity of mutations in NDI may complicate finding a universal therapeutic strategy for these patients, approximately 50% of all NDI mutations are missense, in which a pharmacological chaperone-based therapy may represent a possible general treatment for this protein-misfolding disease [48].

Several studies have revealed that class II mutant, ER retained V2R receptor proteins are often functional and the cell surface expression of these proteins can be rescued by cell-permeable ligands of the V2R (pharmacological chaperones, pharmacochaperones, or pharmacoperones). The V2R was the first of such receptor proteins, for which the ability of pharmacochaperones is described as a way to promote cell surface trafficking of mutant receptors retained in the ER [49]. Pharmacochaperones can alter the configuration of a misfolded mutant receptor, and, thus, allow the transport of intracellularly trapped receptors to the plasma membrane, where rescued receptors can

bind AVP, the physiological ligand [48-50]. Both the cell-permeable nonpeptidic agonists and antagonists of V2R can act as pharmacological chaperones, although the use of both agonists and antagonists as pharmacological chaperones has limiting factors. One of the main limitations of pharmacochaperones is that their effects are often significantly dependent on the nature of the V2R mutation [27, 50]. Thus, different mutations may require different compounds to achieve the mutant V2R rescue [27, 50]. Furthermore, if the pharmacochaperone compound is not a completely selective agonist of V2R, side effects via other receptors may occur (e.g., undesirable effects via V1R), and the trade-off in affinity is required for these components, i.e., the need for sufficient receptor binding and easy release from the receptor [27]. In addition, stimulation of V2R with AVP helps to arrest the response by inducing internalization of the receptor as well as the delivery and degradation in lysosomes, which might be compensated for in the presence of a high level of AVP [27]. One promising non-peptide antagonists pharmacochaperone is the tolvaptan (OPC41061), which has been shown in several studies to be able to rescue some of the misfolded mutant V2Rs [51-53]. Tolvaptan is a selective V2R antagonist that can bind to V2R with 1.8-fold higher affinity than the native hormone vasopressin and it is also a partial antagonist of V1R, but it is 29-fold more selective for V2R than for the V1R receptor [54]. The tolvaptan (Samsca) has been approved in the USA and Europe for treatment of hyponatremia, for instance in the syndrome of inappropriate antidiuretic hormone secretion and congestive heart failure, and, more recently, for the treatment of polycystic kidney disease [55, 56]. In future, this ligand may also be of high therapeutic value in the treatment of cNDI.

Pharmacochaperone agonists possess advantages over antagonists since they are able to directly stimulate the V2R and induce receptor-associated signaling pathways. Nevertheless, agonists also promote V2R internalization, and a decrease in the cAMP signal, which can reduce the beneficial effects of these compounds. MCF14 compound is a cell-permeable, high-affinity non-peptide agonist of V2R, which has been previously reported to act as a pharmacochaperone [57]. Previous studies described that MCF14 can promote maturation and rescue of some V2R mutant receptors, and, in addition, these studies have shown that it can directly activate cAMP signaling of many V2R mutant receptors upon binding to the receptor [57]. Moreover, it was also noted that MCF14-induced receptor activation did not initiate β-arrestin binding, so it is considered to be a

biased agonist [57]. It does not induce receptor internalization and consequently receptor downregulation, which can be therapeutically beneficial [57]. These particular properties may lead to additional beneficial effects in developing a drug for patients with cNDI disease. Combining biased agonist properties with pharmacochaperone activity would be a great strategy in developing drugs for treating diseases related to protein misfolding, for which the drug-induced beneficial versus harmful effect ratio has to be improved.

1.3. Renin-angiotensin-aldosterone-system (RAAS)

In its classical view, the renin-angiotensin-aldosterone-system (RAAS) is involved in hormonal regulation of blood pressure and the salt/water homeostasis [58]. However, RAAS is now considered as an “ubiquitous” system that is expressed locally in various tissues and exerts a number of paracrine/autocrine effects that are involved in tissue physiology and homeostasis [59, 60]. It plays a key role in processes such as cell growth, proliferation, differentiation, migration, apoptosis, extracellular matrix (ECM) remodeling and inflammation [59, 60].

The main effector peptide of this system is angiotensin II (AngII) which participates not only in physiological but also in pathological mechanisms leading to cardiovascular diseases. The excessive action of AngII leads to pathophysiological changes such as hyperplasia and hypertrophy in the blood vessels, vascular transformation, atherosclerosis and hypertension [2]. Given its diverse range of functions and its potency in affecting cardiovascular physiology, the extensive study of RAAS and the understanding of its physiological and pathophysiological function is essential because it is frequently pharmacologically targeted in clinical practice for the treatment of heart failure, hypertension, kidney complications of diabetes mellitus, acute myocardial infarction, atherosclerosis and stroke [2]. In particular, it is imperative to understand the characteristics of AngII receptors, and to investigate the mechanisms of AngII-induced signaling and the diverse roles of AngII, given the beneficial effects of angiotensin converting enzyme inhibitors (ACE-I) and angiotensin II type-1 receptor blockers (ARBs).

1.3.1. Regulation of classical RAAS pathway

The classical RAAS pathway involves successive enzymatic reactions, and regulation of the formation and degradation of AngII. Renin is synthesized as an inactive enzyme, as prorenin that is cleaved by microsomes to produce renin in juxtaglomerular (JG) cells within the afferent arterioles of the kidney. The prorenin is either constitutively secreted as an inactive precursor or converted to renin upon activation of JG cells, stored in granules, and released by regulated exocytosis [61]. Activation of these cells occurs in response to decreased blood pressure or hypovolemia, beta-activation, or activation by macula densa cells in response to a decreased sodium load in the distal convoluted tubule. Active renin from granules is released into the systemic circulation in a controlled manner by JG cells via an exocytic process [62]. Upon a stimulus by different mechanisms including AngII negative feedback, this process creates a rate-limiting step of the RAAS cascade in most species [62]. Once renin has been released into the blood, it can act on its target, angiotensinogen (AGT). AGT is constitutively released from the liver and is usually present in excess in the circulation. It is the only known substrate for renin. Although its main source is the liver, additional tissues of AGT synthesis have also been described, the most important of which are the brain, kidneys and adipose tissue [63, 64]. AGT production can be induced by a number of stimuli, including estrogen, insulin, glucocorticoids, thyroid hormone, inflammation and AngII [65].

In the plasma, renin acts to cleave AGT into angiotensin I (AngI), a decapeptide (Ang-(1-10)) which is then further processed by the angiotensin-converting enzyme (ACE), a membrane-bound exopeptidase on the endothelial cells, to release the biologically active octapeptide AngII (Ang-(1–8)). AngII is recognized by two different G protein-coupled receptors, the angiotensin II type I (AT1-R) and type 2 receptor (AT2-R). Type 1 and type 2 receptors have different vascular effects [66]. While AT1-R mediates hypertensive effects, the activation of AT2-R causes hypotension, and elicits an antihypertensive effect [66]. The effects of AngII on AT1-R include increasing of sodium retention, vasoconstriction, increased nervous system activity, and aldosterone release from the adrenal gland zona glomerulosa cells. The AT1-receptor is associated with most of the diseases in which chronic RAAS activation occurs [67]. In addition to AngII production, the ACE is able to degrade a number of vasodilatory peptides, including

angiotensin 1-7 (Ang-(1-7)), bradykinin, and kallikrein, thus playing a central role in vasoconstriction regulation [68].

The RAAS family was further supplemented with other peptides such as Ang-(1-7), angiotensin III (AngIII, Ang-(2-8)), angiotensin IV (AngIV, Ang-(3-8)), and angiotensin 1-12 (Ang-(1–12)). Ang-(1-7) can be formed by different enzymes and pathways, although the most well-known Ang-(1–7)-generating enzyme is angiotensin-I converting enzyme 2 (ACE2). ACE2 can generate Ang-(1–7) in two different ways, directly from AngII, or indirectly from AngI through Ang-(1–9) intermediate. Ang-(1–9) exerts its effect via AT2-R, thereby reducing AngII levels and increasing Ang-(1–7) metabolites with vasodilatory properties. Thus, the balance between ACE and ACE2 is an important factor in the regulation of AngII level [68]. Ang-(1-7) elicits its effect through the Mas receptor, whereby it mediates antagonistic effects of AngII such as vasodilation, antihypertensive and antifibrotic effects. Alamandine (Ala1-Ang-(1–7)) is endogenously synthesized from Ang-(1–7) and also promotes antihypertensive effects through the Mas-related GPCR member D (MrgD) to produce nitric oxide (NO) [69, 70].

1.3.2. Angiotensin receptors

As mentioned above, AngII is the major regulatory hormone in RAAS, which has pathological functions in addition to its physiological effects. RAAS hyperactivity also plays an important role in many cardiovascular diseases, such as the development of hypertension and atherosclerosis, and abnormal remodeling of the vascular wall and myocardium, mainly due to the excessive effect of AngII resulting in vascular hyperplasia and hypertrophy [2]. AngII exerts these pathological effects primarily through the AT1-receptor. As one of the main focuses of the dissertation are the AngII-induced cell signaling cascades and gene expression changes and their role in vascular smooth muscle cells (VSMCs), it is important to discuss in more detail the AngII-activated AT1-R signaling pathways and some of the proteins that were studied in the AngII-induced responses in this thesis.

The main target of AngII are the vascular smooth muscle cells, in which AngII stimulation activates several signaling pathways after its binding to AT1-R [2]. The cellular responses to hormone activation vary in time. The acute AngII stimulation regulates salt/water homeostasis, vasoconstriction, and arterial blood pressure, while the

long-term exposure to AngII causes changes in the expression of various genes, which contributes to the development of cardiovascular hyperplasia, hypertrophy and remodeling, in-stent restenosis, decreased fibrinolysis, and renal fibrosis [2]. Although there is a wealth of knowledge in understanding the mechanisms involved in the regulation of AT1-R activation-induced gene expression in various cells [71-76], there is less information about the affected signaling pathways of VSMCs.

While the effects of AngII on AT1-Rs have been studied well, it is now recognized that the AT2-R also plays an important role in cardiovascular physiology. Like AT1-R, AT2-R also belongs to the GPCR family, displaying a similar affinity to AngII as AT1-R, although they share only 34% amino acid sequence homology [77]. The AT2-R is widely expressed in embryonic tissues and its expression decreases dramatically shortly after birth in most organs, but lower expression of AT2-R is restricted in the brain, adrenals, kidney, ovaries, uterus and cardiovascular system [78, 79]. Although the AT1-R activities have been known for many years, the AT2-R activities are not yet completely elucidated. The AT2-R activation counteracts most of the AT1-R-mediated action by inhibiting cell proliferation and differentiation, promoting vasodilation, and reducing inflammation and oxidative stress [80]. However, the precise role and importance of AT2-R in the cardiovascular pathophysiology still remains to be determined.

1.3.2.1. AT1-R structural characteristics

Most of the physiological effects of AngII are mediated by AT1-R, which is found in a variety of tissues, including vascular smooth muscle, endothelium, heart, kidney, adrenal gland, brain, lung, and adipose tissue. The AT1-R receptor is composed of 359 amino acids (~40 kDa). It is a seven-transmembrane protein belonging to the GPCR receptor superfamily. The crystal structural study of AT1-R confirmed that the receptor has seven transmembrane α-helices with three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3), as well as with an extracellular N-terminal region and an intracellular C-terminal region. The AT1-R has several contact sites for docking AngII. The AngII binding site is composed by TM2-3-4-5-6-7 helices and the ECL2 loop. Ligand binding is facilitated by ionic bonds between the Asp281 side chain of AT1-R and the Arg2 side chain of AngII, and between the COOH-group of AngII and the Lys199 amino acid of AT1-R. Firstly, the C-terminal end (Tyr^4-Ile^5-His^6-Pro^7-Phe^8) of AngII binds to

the ECL2 region of the receptor, through ion-ion interactions between Phe8(AngII)/His256(AT1-R) and Tyr4(AngII)/Asn111(AT1-R) [81, 82]. This interaction is responsible for the partial activity of the receptor, which results in a rearrangement of ECLs and promotes the binding of N-terminal region of AngII to extracellular regions [81, 82].

The cytoplasmic tail of AT1-R contains many serine/threonine-rich residues that can be phosphorylated by various kinases such as GRK and PKC. Also, phosphorylation of these amino acid residues is required for receptor regulation by β-arrestin and for internalization. Recently, the crystal structure of AT1-R has been determined with an antagonist, the candesartan precursor ZD7155 [83]. The AT1-R-ZD7155 complex structure revealed three new AT1-R amino acids, Tyr35, Trp84, and Arg167, which formed a critical interaction with ZD7155 [83]. Among the three amino acids mentioned above, Arg167 may be essential in determining the binding and selectivity of the ligand.

1.3.2.2. AngII-mediated AT1-R receptor signaling mechanism

AngII exerts its short- and long-term effects by modifying cellular signaling processes, primarily through AT1-R [2, 84]. Upon agonist binding, the AT1-R receptor in VSMC acts largely through heterotrimeric $G_{q/11}$-protein activation, which generates the secondary messenger, Ca^{2+} signal via inositol IP_3, and DAG (Figure 3). Under the influence of the AngII stimulus, this classical signaling mechanism is responsible for the majority of cellular responses in its physiological target cells [85, 86]. However, several other signaling pathways are initiated from AT1-R. AT1-R can also activate other G proteins, such as $G_{i/o}$ or $G_{12/13}$ [85, 86].

However, AT1-R-mediated signaling mechanisms do not only occur through G protein activation [87]. AngII stimulation activates several non-receptor and receptor tyrosine kinases, of which epidermal growth factor receptor (EGFR) transactivation plays the most significant role leading to pathological remodeling of the heart, blood vessels and the kidney [2, 88]. Activation of matrix metalloproteinases (MMPs), such as A disintegrin and metalloprotease (ADAM), mediates EGFR transactivation by cleaving heparin-binding EGF-like growth factor (HB-EGF) to produce the ligand required for EGFR transactivation (Figure 3) [89, 90]. Although some studies show G_q independent EGFR transactivation, the ADAM17-mediated HB-EGF separation by AngII requires G_q

activation [2]. In the long-term effects of AngII, EGFR transactivation has been shown to be an important factor in VSMC, including cell proliferation, vascular remodeling, and the development of atherosclerosis [2].

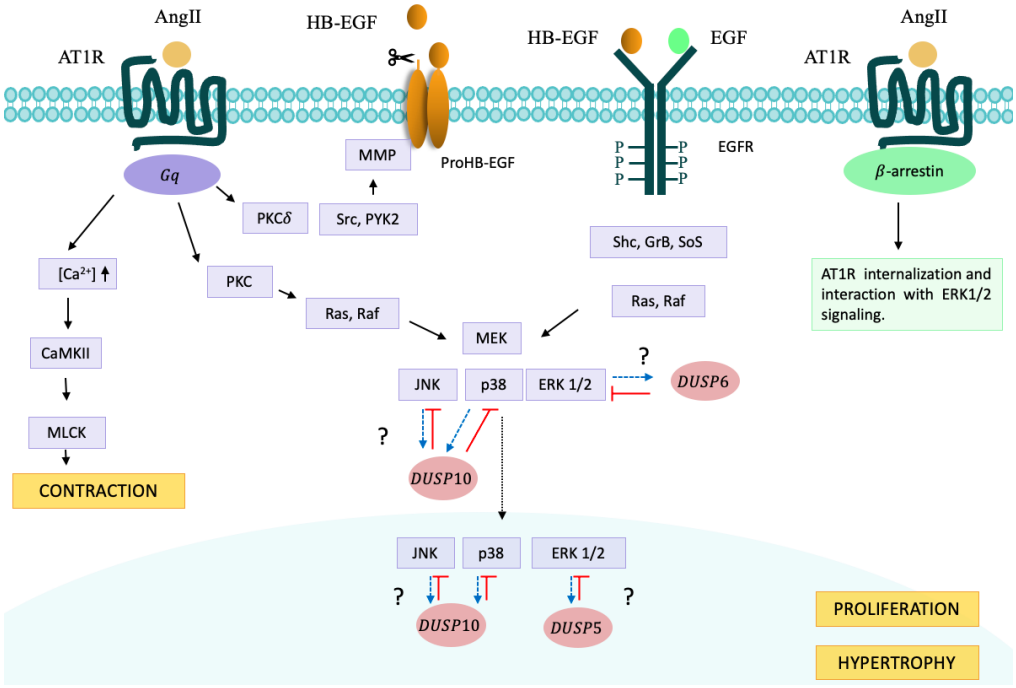


Figure 3. **AngII signaling via AT1-R mediate pathways in VSMCs. Abbreviations:** CaMKII: calcium / calmodulin-dependent protein kinase II, MLCK: myosin light chain kinase, PKC: protein kinase C, MMP: matrix metalloproteinase, HB-EGF: heparin-binding EGF-like growth factor, EGFR: epidermal growth factor receptor, PYK2: proline-rich tyrosine kinase 2, MEK: mitogen-activated protein kinase kinase, ERK1/2: extracellular signal-regulated kinase, DUSP: dual-specificity MAP kinase phosphatases (Based on [91]).

In addition to activating classical G protein-dependent pathways, AngII also cross-talks with non-receptor tyrosine kinases via AT1-R, such as c-Src family kinases, Ca^{2+}-dependent proline-rich tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK) and Janus kinases (JAK) [92]. AngII also activates serine/threonine kinases via AT1-R, such as MAPKs including ERK1/2, p38 MAPK, and c-Jun NH2-terminal kinase (JNK), which have been described to play important roles in the regulation of various functions of VSMCs such as cell growth and hypertrophy (Figure 3) [92]. MAPK regulates processes such as gene expression, protein stability, protein translation, protein localization, and enzyme activity, thereby affecting cell proliferation, differentiation, cell survival and cell

death. A specific property of MAPKs is that they require dual phosphorylation of both threonine (Thr) and tyrosine (Tyr) residues within the signature motif T-X-Y in the activation loop of the kinase for activity, and thus MAPK kinase (MKK or MEK) acts as a dual specificity (Thr/Tyr) protein kinase [93]. The duration and magnitude of MAPK activation play a major role in the biological outcome of signaling. These states of MAPK are primarily regulated by dual specificity (Thr/Tyr) MAP kinase phosphatases (MKP or DUSP) that can dephosphorylate both threonine and tyrosine residues [93]. Moreover, they also control the spatiotemporal properties of the MAPK pathways [93].

1.3.2.3. Dual-specificity MAP kinase phosphatases (DUSPs)

The family of DUSPs provide a mechanism of spatial and temporal feed-back control of key signaling pathways but can also mediate crosstalk between different MAPK cascades and facilitate interaction between MAPK pathways and other key signaling modules trough regulated dephosphorylation and inactivation of MAPK isoforms in mammalian cells and tissues [94]. Since the binding of DUSPs to MAPKs does not require the phosphorylated, active state of MAPKs, they can regulate the availability of different MAPKs. Recent studies have uncovered the key physiological functions of MAPK phosphatases (MKPs) and also revealed their potentially important roles in the pathophysiological outcome of signaling with relevance to human diseases, such as cancer, diabetes, inflammatory and neurodegenerative disorders. To date, 11 DUSPs have been described in the literature to regulate the activity pattern of MAPKs, which can be divided into three subgroups based on sequence homology, subcellular localization, and substrate specificity [93, 95, 96]. There are four nuclear DUSPs; comprising DUSP1, DUSP2, DUSP4 and DUSP5, which can dephosphorylate all three MAPKs in the nucleus, three DUSPs; DUSP6, DUSP7 and DUSP9, which are ERK1/2 MAPK selective in the cytoplasm, while a further four DUSPs; DUSP8, DUSP10, DUSP14 and DUSP16, are found in both the nucleus and cytoplasm, and they mainly deactivate the stress-activated c-Jun amino-terminal kinase (JNK) and p38 MAPKs, except the DUSP8 which is more specific for ERK1/2 [97].

During the analysis of the transcriptional effect of AngII in VSMC cells, our research group revealed the upregulation of several DUSPs, such as DUSP5, 6, 10, 4 and 14 [91]. In this thesis, one *DUSP* gene has been selected from each DUSP subfamily, which are

DUSP5, DUSP6 and *DUSP10*, for examination their gene-expression changes in response to AngII stimulation. More detailed information about these *DUSPs* will be provided below.

The DUSP5 is unique among the four nuclear DUSPs as it is absolutely specific for ERK1/2 and binds tightly to its substrate, and is able to anchor an inactive ERK in the nucleus [98]. On the other hand, growth factor inducible expression of *DUSP5* is mediated by ERK activity, i.e. the transcription of *DUSP5* mRNA is dependent on the phosphorylation of Elk-1 by ERK1/2, making it a classical negative feedback regulator of this signaling pathway [98]. The cytoplasmic DUSP6, like DUSP5, shows preferred substrate specificity for ERK1/2, having no significant activity toward JNK, p38, or ERK5. This selectivity is mediated by the high affinity binding of ERK and promotes the catalytic activation of DUSP6, involving a conformational change, thus greatly increasing enzyme activity. DUSP6 is primarily localized in the cytoplasm and binds to activated ERK1/2, which also indicates the role of this DUSP as a cytoplasmic anchor of inactive ERK, thus regulating the spatial and temporal activity of MAPK [99]. It was demonstrated that the *DUSP6* gene expression and *DUSP6* mRNA stability are controlled by the MEK-ERK1/2 pathway [100]. Both nuclear localized DUSP5 and cytoplasmic DUSP6 are induced by ERK1/2 activity and they play an important role in the negative feed-back loop to limit ERK1/2 activation. In zebrafish experiments, it has been described that DUSP5 is implicated in cardiovascular development, where it is expressed in angioblasts and mature vasculature and that *DUSP5* knockdown increased the angioblast population during early embryonic development [94]. Furthermore, *DUSP5* overexpression also antagonized the function of a serine threonine kinase, SnRK-1, which promotes angioblast development [101]. DUSP5 has also been shown to act as a regulator of cardiac fibroblast proliferation and cardiac hypertrophy [94]. Ferguson et al. demonstrated that the overexpression of DUSP5 in cardiomyocytes resulted in ERK1/2 inactivation and reduction of agonist-dependent hypertrophy [102]. However, it was later shown that albeit DUSP5 terminates nuclear ERK signaling and anchors ERK in the nucleus, the DUSP5 increases the ERK activation in MEF cells [103]. Several studies have described that *DUSP6* expression is regulated by active fibroblast growth factor (FGF) signaling and that it functions as a feedback inhibitor, attenuating FGF-stimulated MAPK signaling during embryonic development in mice [104]. *DUSP6* mutant mice

showed increased levels of phospho-ERK1/2 in several tissues and phenotypes reminiscent of hyperactive fibroblast growth factor receptor (FGFR) signaling. By contrast, Maillet et al. noted that *DUSP6* mutant mice were phenotypically normal in appearance but had an enlarged heart owing to increased cardiomyocyte proliferation during development [105]. However, that, did not result in increased or prolonged ERK1/2 activation in response to stimulation [105]. Thus, they concluded that the DUSP6 is responsible for fine-tuning of basal ERK1/2 activity [105]. DUSP5 and DUSP6 together can regulate ERK1/2 by anchoring dephosphorylated ERK1/2 in either the nucleus or cytoplasm [93].

DUSP10 can be found in both the nucleus and cytoplasm, and various studies have described a role of DUSP10 as a negative regulator of p38 and JNK through their dephosphorylation [106]. However, recent studies dispute the ability of DUSP10 to regulate ERK1/2 activity [106]. It has been shown that the DUSP10 dephosphorylates the p38 and JNK selectively and more effectively than ERK [107]. Another study has described that DUSP10 may act as a scaffold protein of ERK, thus negatively regulating ERK by retaining it in the cytoplasm, avoiding ERK enzymatic activity and downregulating the ERK-dependent transcription [108]. However, current studies have also shown that DUSP10 can dephosphorylate phosphoserine (pSer) residues of other substrates in addition to MAPK substrates [109]. In a *DUSP10* knockdown study, it was shown that the knockdown of *DUSP10* inhibited acute EGF-stimulated ERK activation, which can be reversed by pharmacological inhibition of p38 MAPK. Thus, these results suggest that DUSP10 acts as a positive regulator of MAPK activation by modulating crosstalk between the p38 MAPK and ERK pathways [110].

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2. OBJECTIVES

During this Ph.D. study, the functions and signaling mechanisms of two clinically important GPCRs, the V2R and the AT1-R were investigated.

The aims in the characterization of the disease-causing S127F mutant V2 receptors studies were:

- Investigation of the cellular consequences of the missense mutation of the V2R (S127F-V2R), such as examination of the localization, cAMP signal generation capability of the mutant receptor, and classification of the type of the disease-causing mutation based on the obtained data.
- Investigation of the possible functional rescue of the mutant receptor by pharmacochaperones.
- Examination of the effect of pharmacochaperone treatments on the cell surface expression of the S127F-V2R.

The aims in the investigation of long-term effects of AngII in VSMCs were:

- Investigation of the AngII-induced upregulation of *DUSP* genes in the VSMCs.
- Examination of the time kinetics of *DUSP* expression changes in response to AngII stimulation of VSMCs.
- Examination of the signaling pathways affecting the expression of the different *DUSPs*, including the identification of the involved receptor and G protein type.
- Investigation of the role of β-arrestin and calcium signal in the AngII-induced upregulation of *DUSP* genes.

3. RESULTS

3.1. Identification of the S127F mutation in the *AVPR2* gene

Our collaborating researchers have identified a nephrogenic diabetes insipidus patient in a Hungarian family [30]. The male patient was born in 2012 and has manifested the classic symptoms of NDI such as polyuria and polydipsia since birth. The 5000 g infant's daily urine production was around 1 L at the time of the study. The clinical diagnosis of the NDI is based on results of the water deprivation test and on the ineffective response to arginine vasopressin analogue, 1-deamino-8-D-arginine-vasopressin (dDAVP, desmopressin) therapy. While the mother had no severe symptoms of the disease, she was diagnosed with subclinical NDI only after investigation of her infant. She had an average daily fluid intake of 3-4 L and was in good general health. A molecular mutation analysis of the *AVPR2 gene* was performed after the PCR amplification of genomic DNA samples isolated from the peripheral blood of the proband and his mother. The DNA sequencing of the PCR products revealed a point mutation in the *AVPR2* gene of the proband (Figure 4A). It also showed that the mother also carries the same mutation, which confirmed that this is not a *de novo* mutation and confirmed that the mother is a heterozygous carrier because both wild-type (WT) and mutant alleles were present in the mother's DNA sample (Figure 4A and B, middle panel). The *AVPR2* gene is located in the chromosome region Xq28. Hence, *AVPR2* mutations cause recessive X-linked NDI, which explains why the proband shows severe clinical symptoms, while his heterozygous mother is less affected. The patient's younger brother had no symptoms and mutation in the *AVPR2* gene (Figure 4B).

This missense mutation caused a cytosine to thymine single-base substitution (g.741C→T) in the exon 2, resulting in an amino acid substitution from serine-127 to phenylalanine (S127F), which is located in the third transmembrane domain of the V2 vasopressin receptor (Figure 4C). No further mutations were identified in the *AVPR2* gene.

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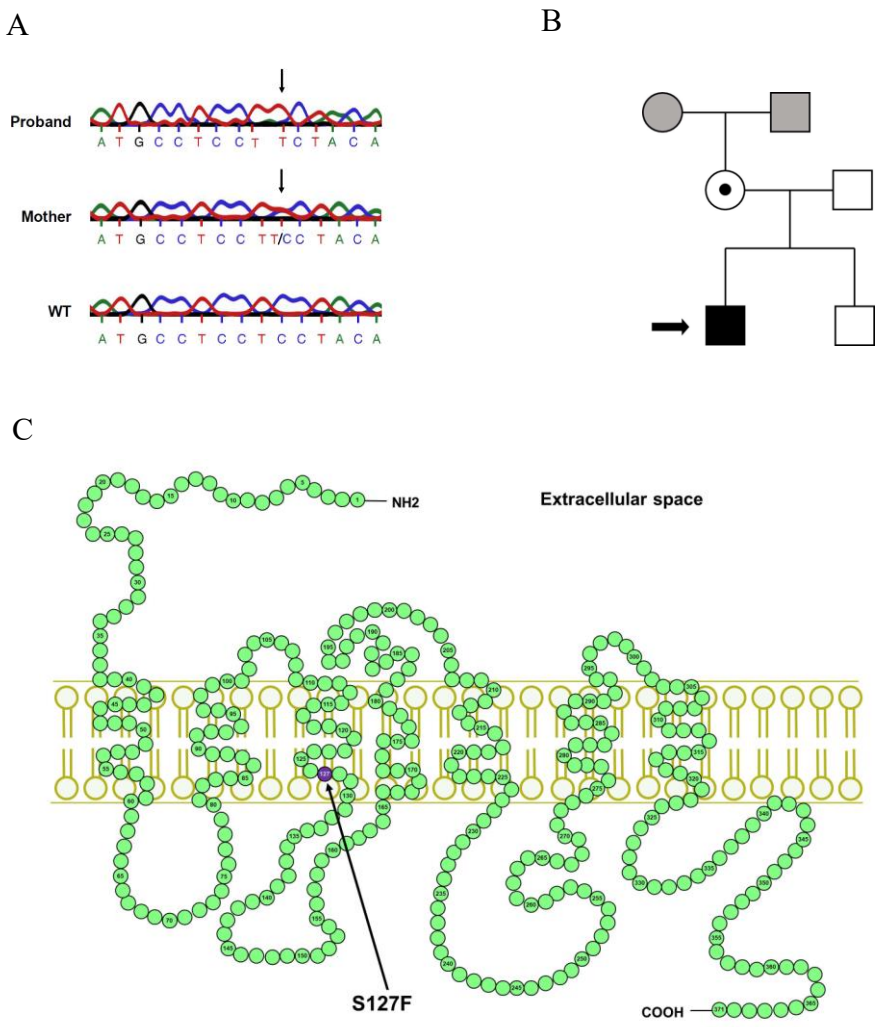

Figure 4. **The result of the genetic examination and the patient's family pedigree.** (A) Following the isolation of the proband's and his mother's genomic DNA from peripheral blood, the *AVPR2* gene was amplified by PCR and then the samples were sequenced. The chromatogram shows the Sanger sequencing of *AVPR2* gene from the proband (upper panel), the mother of the proband (middle panel), and a healthy control individual (lower panel). The arrows indicate the mutation in the DNA sequences. (B) Family pedigree of the proband. The arrow indicates the proband. Next to it, the solid black square represents the male individual with classic NDI symptoms, and the heterozygous mother with subclinical NDI is represented by an open circle with a central dot. In the sibling of the proband, the mutation was not present, which is indicated by an open square, while the genotype of the maternal parents is unknown, indicated by the grey square and circle. (C) The schematic representation of the human V2 vasopressin receptor. The arrow indicates the affected amino acid (purple circle) in the third transmembrane helix of the V2R [30].

3.2. Characterization of the mutant receptor

The S127F mutation has been previously mentioned in the literature, and identified in a Japanese family, but the functional consequences of the mutation have not been characterized [41]. We decided to characterize the impaired receptor functions caused by

the missense mutation. The first goal was to determine the type of the mutation that leads to disease. Despite the majority of missense *AVPR2* mutations being mainly class II, ER retention mutant receptors, it is important to investigate the cellular consequences of each new mutation [37, 45].

The first step in understanding the cellular effect of the S127F mutation is determining the intracellular location of the mutant receptor. Therefore, HEK-293 cells were transiently transfected with the plasmid of HA-tagged S127F-V2R mutant or WT human V2Rs. 24 hours after transfection, the expressed receptors were immunolabeled with anti-HA-Alexa488 fluorescence monoclonal antibodies on non-permeabilized cells, and fluorescence was detected by confocal microscopy. The obtained results showed that the WT V2Rs were located on the plasma membrane (Figure 5A), whereas the S127F mutant V2Rs were not detectable on the cell surface (Figure 5B), which excludes the possibility of the mutation belonging to class III or class IV mutation category. Subsequently, the goal was to determine the cause of the lack of mutant receptors on cell surfaces. It is presumably caused by inefficient synthesis of V2R (class I mutation) or misfolding of full-length receptor formation (class II mutation). For this assay, permeabilized cells expressing either HA-labeled WT-V2R or S127F-V2R mutant receptors were used to demonstrate intracellular receptors with immunofluorescent anti-HA antibodies. In permeabilized cells, WT-V2R was mainly present on the cell surface and showed only mild intracellular staining (Figure 5C). The small intracellular fraction of the transiently expressed WT receptors is probably in the secretory transport route to mature and reach the plasma membrane (Figure 5C). In contrast to WT receptors, the S127F-V2R mutant receptor showed marked intracellular staining, confirming receptor synthesis in cells, but apparently the synthesized receptors were not able to reach the cell surface due to intracellular trapping (Figure 5D). The possibility of a class V mutation could also be ruled out because the intracellular localization pattern of the mutant receptors is a characteristic of the ER, but not of the intracellular vesicles (Figure 5D). In summary, synthesis of the S127F-V2R mutant receptor was observed in the cells, but their localization was mainly present in the ER and in negligible amounts on the cell surface, indicating that S127F-V2R is a class II, ER retention mutant.

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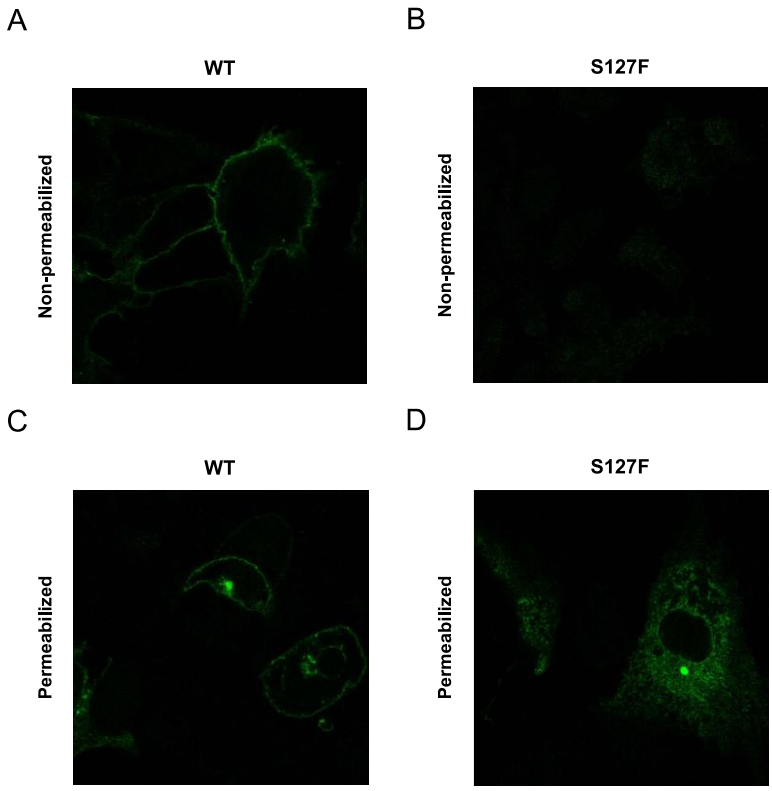

***Figure 5*. Investigation of the cell surface and intracellular localization of wild-type and S127F-V2R mutant receptors with a confocal microscope.** (A-D) HEK293 cells were transiently transfected with the plasmid of WT-V2R-HA or S127F-V2R-HA receptors. The HA-tagged wild-type and S127F mutant receptors were directly immunostained with anti-HA-A488 mouse monoclonal antibodies. Non-permeabilized fixed cells were used to confirm the plasma membrane expression of WT-V2R (A) and S127F-V2R (B), whereas the total cellular distribution of WT-V2R (C) and S127F-V2R (D) was assessed after staining permeabilized cells [30].

Next, a functional assay of the S127F-V2R mutant receptor was performed. Although the cell surface expression of the mutant receptor was extremely low, in some cases, amplification in the G_s protein-coupled receptor signaling cascades potentially allows for the measurement of the secondary messenger, cytoplasmic cAMP, if the receptor can be activated. The bioluminescence resonance energy transfer (BRET) technique was used for comparing cytoplasmic cAMP production of the WT-V2R and S127F-V2R in response to AVP stimulus. The BRET technique is suitable for measuring the change in cAMP concentration in living cells in real time [111]. As a result of cAMP binding to the Epac-BRET, the sensor undergoes a conformational change, shifting the relative position of the energy acceptor and donor within the intramolecular probe, and causing a change in the BRET ratio. Thus, an increase in cAMP level results in a decrease in the BRET

ratio. In this experiment, HEK293 cells transiently coexpressed the Epac-BRET probe with either the WT-V2R or S127F-V2R construct, and cells were stimulated with vehicle, 0.1 μM or 1 μM AVP to measure cAMP production.

The AVP stimulation of S127F-V2R expressing cells caused only minimal cAMP production compared to the cAMP signal generation of the WT receptor-expressing cells (Figure 6). Though, the 1 μM AVP stimulation caused a very low cAMP generation of the mutant receptor expressing cells, it was a statistically significant increase compared to the cAMP generation of the vehicle stimulated mutant receptor expressing cells, which demonstrated the partial functionality of the mutant receptor (Figure 6). This result raised the possibility of functional restoration or improvement of the mutant receptor.

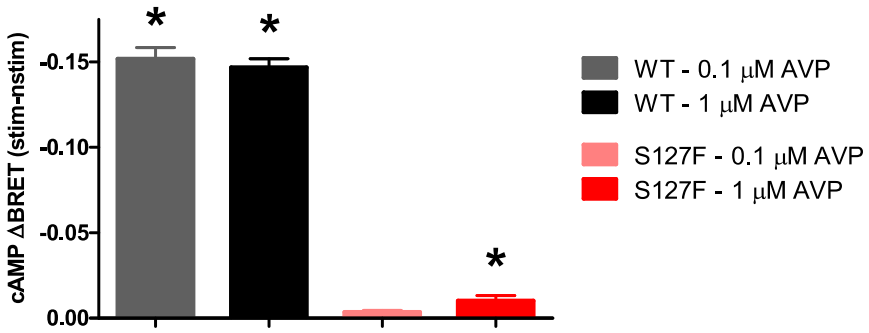

Figure 6. **cAMP signaling of the wild-type and S127F mutant V2 receptors.** To measure cAMP production, HEK293 cells were transiently transfected with the plasmids of the Epac-BRET sensor and either WT-V2R or S127F-V2R. BRET measurements were taken 24 hours later. The cells were stimulated with vehicle (nstim), 0.1 μM or 1 μM AVP (stim) and BRET ratios were monitored. The effect of stimulation was calculated as the BRET ratio difference between the AVP stimulated (stim) and non-stimulated (nstim) exposed cells after 500 sec of treatment. The values are from three independent experiments, each performed in triplicates. Mean values ± S.E. are shown (n = 3). Significance was calculated with one-way ANOVA test (*$p < 0.05$), *: statistically significant from vehicle stimulation (nstim) [30].

3.3. Functional rescue of the S127F-V2R

In the following set of experiments, we wanted to investigate the possibility of restoring the mutant receptor function. Firstly, the pharmacochaperone effect of the V2R inverse agonist, tolvaptan in rescuing the mutant receptor was investigated. In the experimental setup, WT-V2R or S127F-V2R expressing HEK293 cells were pretreated with tolvaptan or vehicle for 18 hours. It should be noted that different basal BRET ratio

values were observed between vehicle-treated WT-V2R (Figure 7A, black trace) and S127F-V2R (Figure 7A, red trace) expressing HEK293 cells.

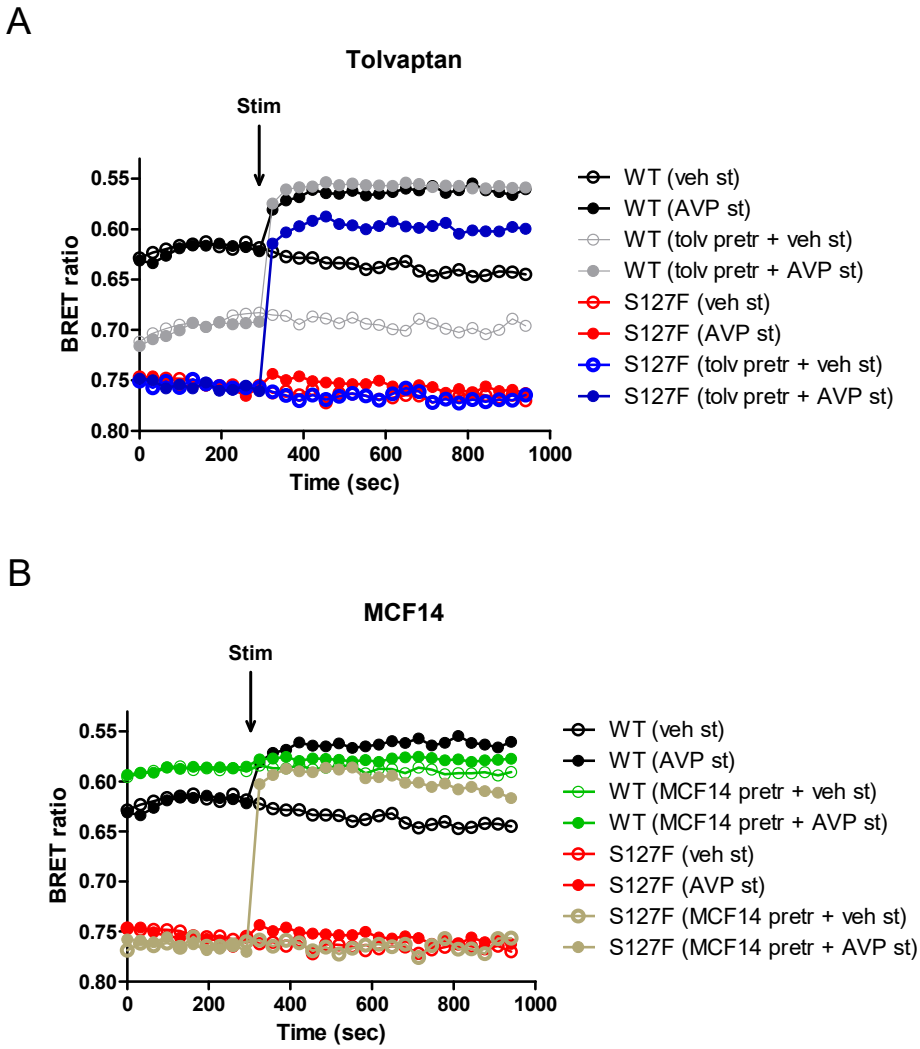

Figure 7. **Investigation of the pharmacochaperone effect of V2R ligands on the cAMP generation of the WT-V2R and S127F-V2R.** Effect of tolvaptan (A) and MCF14 (B) pretreatment on the intracellular cAMP production in response to the vehicle or AVP stimulation. HEK293 cells were transiently transfected with the plasmids of the Epac-BRET sensor and either WT-V2R or S127F-V2R. 24 hours after the transfection, the cells were pretreated with either 100 nM tolvaptan or 10 µM MCF14 or vehicle (DMSO) for 18 hours. Before the BRET measurements of the pharmacochaperone treated cells, the medium of the cells was replaced every 15 minutes for one hour, to wash out the remnants of tolvaptan or MCF14. In the BRET assay, cells were exposed to either vehicle (open circles) or 1 µM AVP (filled circles) at the time point indicated by the arrow. The BRET curves are an average of three independent experiments, each performed in triplicates. Mean values are shown, but error bars were omitted for better clarity. Since increasing cAMP decreases the BRET ratio, the y axis was inverted to visually represent cAMP level changes [30].

The BRET ratio was higher (more positive, closer to the background level) in S127F-V2R expressing cells than in WT-V2R expressing cells because the cAMP concentration is lower for the mutant receptor at the beginning of the measurement. This shows that the overexpressed WT-V2R receptor has constitutive activity (fact known from relevant literature) and confirms the minimal expression of the mutant receptor on the cell surface.

Despite the one-hour washing process before the BRET measurements, tolvaptan pretreatment reduced the basal activity of WT-V2R expressing cells, confirming its inverse agonist effect on cAMP production (Figure 7A, grey trace). The tolvaptan did not alter the basal cAMP level in S127F-V2R expressing cells, confirming that the expression of the mutant receptor did not cause detectable constitutive activity (Figure 7A, blue and red traces). Cells expressing WT-V2R or S127F-V2R were stimulated with 1 μM AVP at the indicated time point. The increase in the intracellular cAMP levels upon AVP stimulation (Figure 7A, filled circles) in WT-V2R expressing cells showed a rapid and robust change in both vehicle or tolvaptan pretreated cells (Figure 7A, black and grey traces). However, when the mutant receptor expressing cells were pretreated with the vehicle, only a minimal cAMP response was generated upon AVP stimulus (Figure 7A, red trace), but pretreatment with 100 nM tolvaptan significantly increased the cAMP response (Figure 7A, blue trace). This demonstrates the pharmacochaperone effect of tolvaptan and the successful partial functional rescue of the mutant receptor.

In the following stage, the role of the pharmacochaperone biased agonist of V2R in functional rescue of S127F-V2R was examined. Pretreatment of wild-type receptor-expressing cells with 10 μM MCF14 caused an increase in basal cAMP levels (Figure 7B, green open circles), and this cAMP level could not be further increased by AVP stimulation (Figure 7B, green filled circles). Despite the washing steps performed prior to measurement, the 10 μM MCF14 concentration used was probably difficult to eliminate, which was also previously reported [57]. As in the previous measurement, S127F-V2R expressing cells did not show basal activity upon MCF14 pretreatment (Figure 7B, brown traces). The MCF14 pretreated S127F-V2R expressing cells showed a rapid and robust increase in intracellular cAMP level upon AVP stimulation (Figure 7B, brown filled circles). This result indicates that the V2R agonist MCF14 acts as a pharmacochaperone, which demonstrates the ability of the compound to

reach and bind to ER-trapped receptor molecules, but that is not an activator of the S127F-V2R receptor.

In the next experiment, we examined whether an acute stimulus with MCF14 could directly activate misfolded receptors in S127F-V2R expressing cells (Figure 8). Based on our measurements, MCF14 stimulation was able to induce a cAMP signal similar to ones induced in AVP stimulation in WT-V2R cells (Figure 8, red and grey traces), but the MCF14 stimulation did not result in significant cAMP signal generation in S127F-V2R cells (Figure 8, green trace).

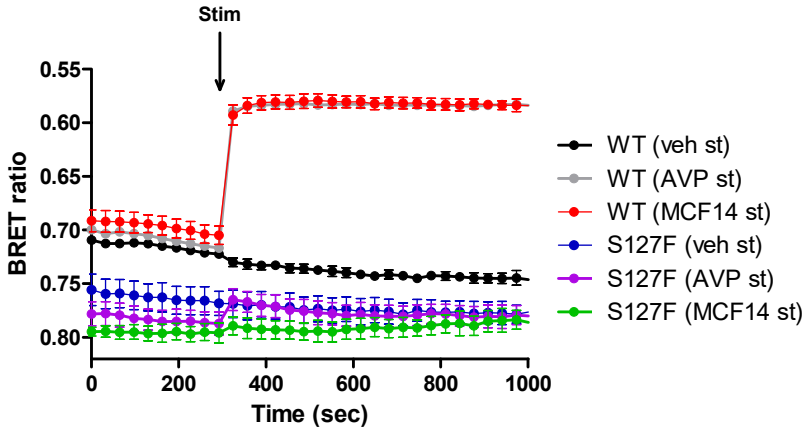

***Figure 8*. Effect of acute MCF14 stimulation on the cAMP level of WT-V2R and S127F-V2R mutant receptor expressing cells.** HEK293 cells were transiently transfected with the plasmids of the Epac-BRET sensor and either WT-V2R or S127F-V2R. BRET measurements were performed 24 hours after the transfection. The cells were stimulated with vehicle or 1 μM AVP or 10 μM MCF14 at the indicated time point. The BRET curves are an average of three independent experiments, each performed in triplicates. Mean values ± S.E. are shown (n = 3). Since increasing cAMP decreases the BRET ratio, the y axis was inverted to visually represent cAMP level changes [30].

3.4. Determination of the useful concentrations of pharmacochaperones to initiate cAMP generation in response to AVP stimulation

We investigated the possibility of functional rescue of the mutant receptor by using lower concentrations of tolvaptan and MCF14 than, previously used [57, 112]. From the obtained results, the dose-response curves of the functional rescue effects of tolvaptan and MCF14 in S127F-V2R expressing cells were determined. Based on the results, it appears that the previously used concentrations of tolvaptan (100 nM) and MCF14 (10 μM) were adequate to elicit a possible maximal cAMP response of the S127F-V2R

receptor, and that the used concentrations could be potentially decreased by half based on the dose-response curve (Figures 9A-D).

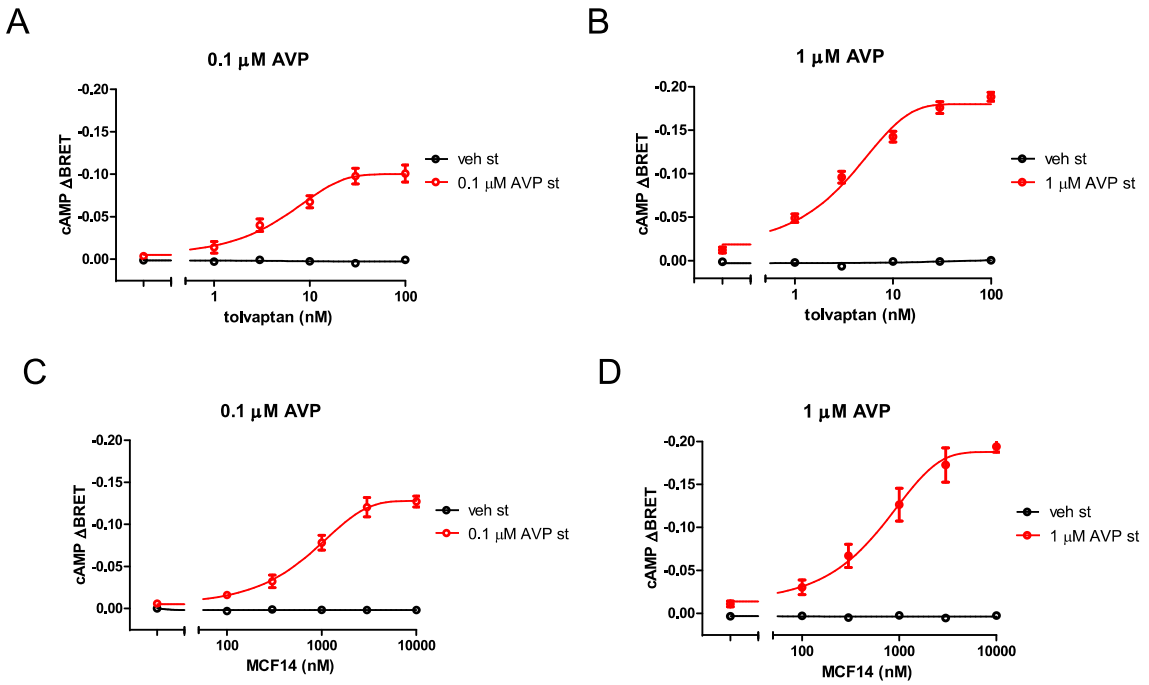

Figure 9. **BRET-based cAMP monitoring in S127F-V2R expressing cells following various concentrations of pharmacochaperone pretreatment.** Effect of different concentrations of tolvaptan (A-B) and MCF14 (C-D) pretreatment on the cAMP generation in response to 0.1 µM (A and C, red traces) or 1 µM AVP (B and D, red traces) stimulation. HEK293 cells were transiently transfected for 24 hours with the plasmids of the Epac-BRET sensor and S127F-V2R. After the transfection, the cells were incubated with different concentrations of tolvaptan or MCF14 for 18 hours. The effect of stimulation was calculated as the BRET ratio difference (ΔBRET) between the BRET ratio values taken before and 5 min after the stimulation. The concentration-response sigmoidal curve was generated using non-linear regression with the GraphPad Prism software. The BRET curves are an average of three independent experiments, each performed in triplicates. Mean values ± S.E. are shown (n = 3). Since increasing cAMP decreases the BRET ratio, the y axis was inverted to visually represent cAMP level changes [30].

3.5. Effect of V2R pharmacochaperone ligands on the cell surface expression of the S127F-V2R

Following the successful functional rescue of the S127F-V2R mutant receptor, the cell surface expression of the rescued mutant receptor by flow cytometry was examined. Therefore, to perform the assay, HEK293 cells were transiently transfected with HA-WT-V2R or HA-S127F-V2R, and after 24 h of transfection, the cells were

pretreated with vehicle or 100 nM tolvaptan or 10 μM MCF14 for 18 hours. Based on our measurements, both tolvaptan and MCF14 slightly increased the cell surface expression of the WT-V2R (Figure 10), while no significant change in the cell surface expression level of S127-V2R could be detected (Figure 10). That may reflect the fact that only a very small proportion of intracellular retained receptors were rescued by treatment with tolvaptan or MCF14.

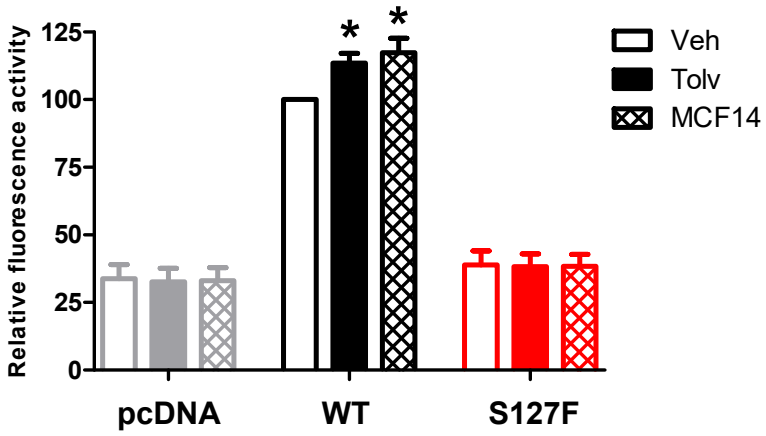

Figure 10. **Determination of cell surface expression of WT-V2R and S127F-V2R receptor by flow cytometry.** HEK293 cells were transiently transfected with HA-tagged wild-type or S127F mutant V2R or pcDNA3.1 construct and after 24 hours of transfection, the transfected cells were treated with 100 nM tolvaptan or 10 μM MCF14 or vehicle (DMSO) for 18 hours. Thereafter, the cells were immunostained with anti-HA-Alexa488 monoclonal antibodies, and the cell surface expression values of the immunofluorescent labeled receptors were analyzed and quantified by flow cytometry. Non-specific fluorescence was determined by measuring empty pcDNA3.1 transfected HEK293 cells incubated with anti-HA-Alexa 488 antibodies. The cell surface expression values are expressed as a percentage of the vehicle treated V2R median fluorescence values detected in the same experiment. The values are from three independent experiments, each performed in triplicates. Mean values ± S.E. are shown (n = 3). Significance was calculated with one-way ANOVA test (*$p < 0.05$), *: statistically significant when compared to vehicle stimulation [30].

Furthermore, the cell surface location of the receptors was also examined by confocal microscopy. We found that tolvaptan pretreatment significantly, induced S127F-V2R mutant receptor expression on the cell surface in microscopic determination, while MCF14 induced it weakly (Figure 11).

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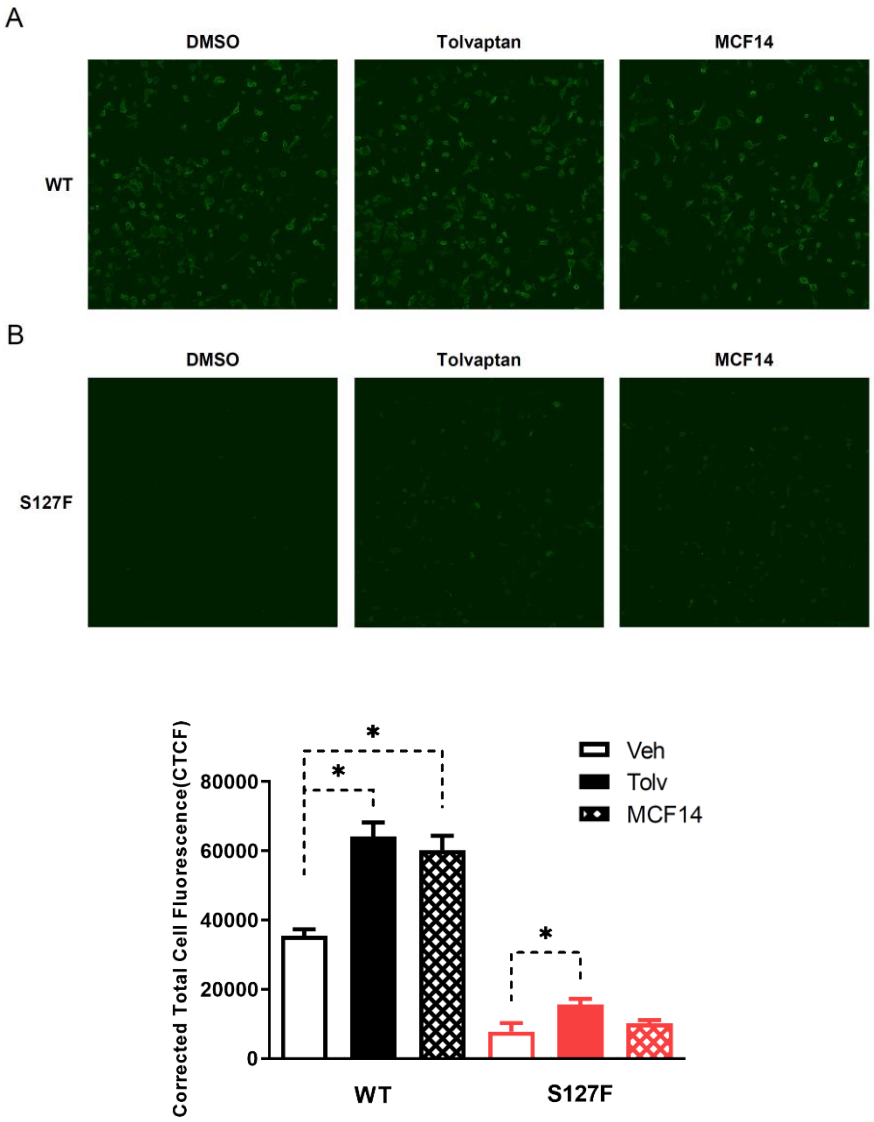

***Figure 11*. Visualization of the cell surface expression of the WT-V2R or S127F-V2R by confocal microscopy.** HEK293 cells were transfected with WT-V2R-HA (A) or S127F-V2R-HA (B) and 24 hours after the transfection, the cells were treated with 100 nM tolvaptan or 10 µM MCF14 or vehicle (DMSO) for 18 hours. The HA-tagged receptors expressing cells were directly immunostained with anti-HA-Alexa488 mouse monoclonal antibodies without using permeabilized staining conditions. Imaging of cells was performed with the ImageXpress Micro Confocal microscope system keeping the same exposure parameters (acquisition time and gain) between wells. The microscopic images were obtained by using MetaXpress software. (C) The cell fluorescence was determined by the CTCF method, using ImageJ software. The values are from three independent experiments (n=3). Mean values ± S.E. are shown. Significance was calculated with one-way ANOVA test (*$p < 0.05$), *: statistically significant when compared to vehicle stimulation [30].

In the second part of the thesis, the other, medically important GPCR, the AT1-R, was examined. This receptor is implicated in the development of many cardiovascular diseases, and in order to obtain new data on the long-term effect of AT1-R stimulation, the AngII-induced gene expression in vascular smooth muscle cells was analyzed.

3.6. Affymetrix GeneChip analysis and NGS measurements of the AngII stimulation upregulated genes in VSMCs

In this experiment, we compared the gene expression profile of vehicle and 100 nM AngII-treated VSMC cells by using the Affymetrix GeneChip Rat Gene 1.0 ST array. For our experiments, primary rat vascular smooth muscle cells were used until the third passage, in order to obtain physiologically relevant data. However, the primary VSMC of the rat can be maintained for up to 20–30 passages, and it has been shown that cultured VSMCs undergo phenotypic modulation [113]. These changes can appear in cell culture as early as in 7-9 days, so up to 3 passages were used in our experiments in order to keep the cells' molecular machinery as similar as possible to their *in vivo* conditions [114]. In order to confirm that these isolated cells have VSMC-specific properties, smooth muscle α-actin immunostaining and agonist stimulation were performed on these early passages. Our results confirmed that these cells have their characteristic properties of VSMCs, inasmuch as they showed smooth muscle α-actin expression and the typical ERK1/2 pattern upon 50 ng/ml EGF or 100 nM AngII stimulation (Figure 12).

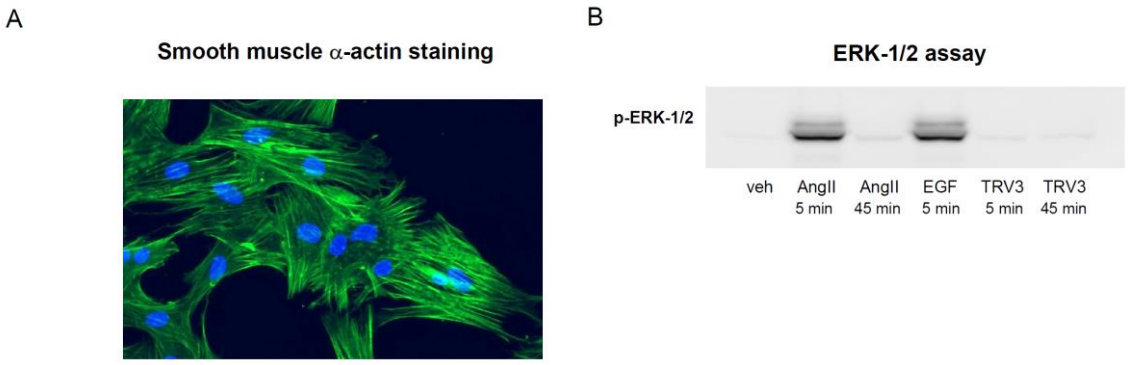

Figure 12. **Verification of the basic hallmarks of the isolated VSMCs.** (A) Immunostaining of rat primary VSMC culture with smooth muscle α-actin (green), the nuclei were stained by DAPI (blue). Bar: 50 μm. (B) ERK-1/2 MAPK activation. VSMCs were exposed to vehicle, 100 nM AngII for 5 and 45 min or 50 ng/ml EGF for 5 min. The western blot is a representative of three independent experiments [91].

Affymetrix GeneChip experiments were performed to analyze the effects of AngII stimulation on gene expression in vascular smooth muscle cells. In our experimental setup, more than 100 gene expressions were altered by AngII stimulation in smooth muscle cells (data not shown). Furthermore, three genes whose transcription is significantly enhanced by AngII in the primary smooth muscle cells (*DUSP5, DUSP6*, and *DUSP10*) were investigated.

3.7. qRT-PCR measurements validate the upregulation of *DUSP5*, *DUSP6* and *DUSP10* gene expression in response to AngII stimulation. Time kinetics of gene expression changes in response to AngII stimulation of VSMCs.

Among the upregulated genes, several *DUSP* genes, such as the *DUSP 5, 6, 10, 4*, and *14* genes were significantly upregulated according to Affymetrix GeneChip analysis. However, one *DUSP* was selected from each *DUSP* subfamily, namely *DUSP5, DUSP6*, and *DUSP10*, for further study in this dissertation. We wanted to confirm the effect of AngII stimulus on the expression of these *DUSP* isoforms by qRT-PCR determination. The primary aim was to confirm the Affymetrix GeneChip results and to determine the time course of the AngII-induced upregulation of *DUSP5* (Figure 13A), *DUSP6* (Figure 13B), and *DUSP10* (Figure 13C) expression levels. For qRT-PCR measurement, VSMCs were stimulated with 100 nM AngII for different time durations (1 to 6 hours), and then mRNA levels were determined.

It was clearly observed in case of *DUSP5* and *DUSP10* that mRNA levels were highest 2 hours after AngII stimulation, while the *DUSP6* mRNA levels showed an intense increase as early as after the first hour, peaking at 2 hours, but remaining continuously elevated, although showing a slightly reduced tendency at later time points. Based on this determination, 2 hours AngII stimulation was chosen for further experiments to analyze gene expression changes.

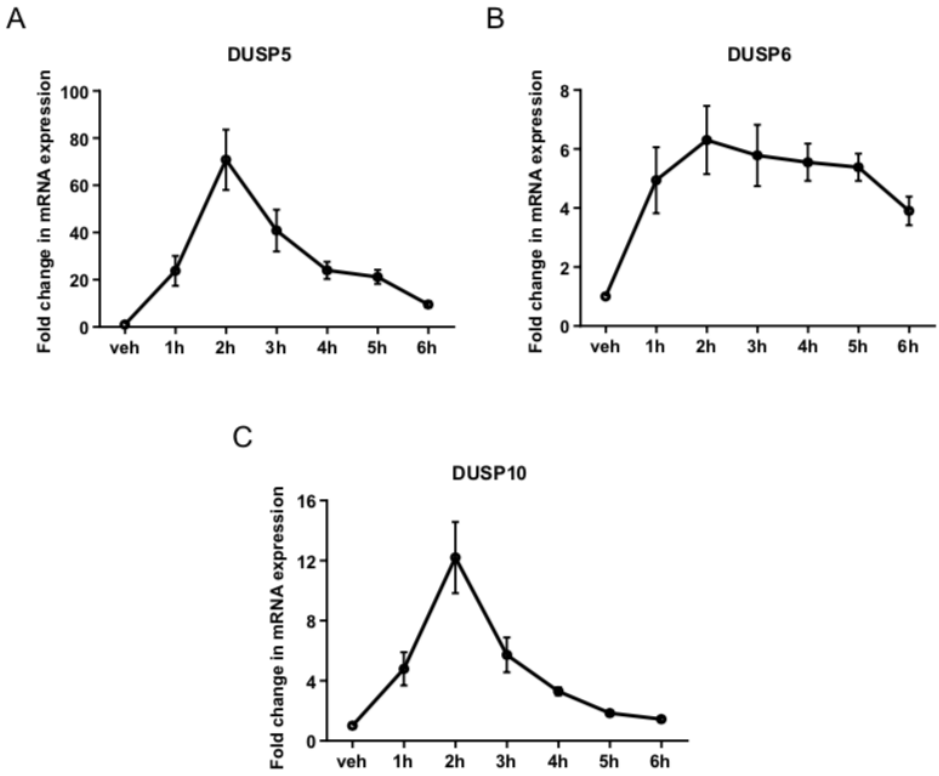

Figure 13. **Investigation of the time-dependent effect of AngII stimulation on gene expression in VSMCs by RT-PCR.** VSMCs were serum-depleted for 24 hours, then cells were treated for various lengths of time with 100 nM AngII, and the control group was treated with vehicle. Time kinetics of *DUSP5* (A), *DUSP6* (B), and *DUSP10* (C) expression levels upon different durations of AngII treatment. Standardization was made against the GAPDH housekeeping gene. The mRNA levels were normalized to values of vehicle samples and expressed as fold change. The values are from five or six independent experiments. Mean values ± S.E. are shown (n = 5-6) [91].

3.8. Investigation of signaling pathways involved in the AngII-mediated responses

Thereafter, we wanted to determine which angiotensin receptor type and associated G protein play a role in the expression regulation of the studied *DUSP* genes. The AngII can mediate its effect in VSMCs through stimulation of the AT1 and AT2 angiotensin receptors. The AT1-R is more important and expressed in higher amounts in blood vessels [97]. However, in the interest of excluding, AT2-R's potential role in the *DUSP* gene expression changes, candesartan, a selective AT1-R antagonist with insurmountable binding properties, was used. The pretreatment with 10 μM candesartan completely inhibited the AngII-induced upregulation of *DUSP* mRNA levels (Figure 14). This result indicated that the AT1-R mediates the observed AngII-induced gene expression changes that predominantly bind to the $G_{q/11}$ heterotrimeric protein in VSMCs [115]. EGF

stimulation was also used to demonstrate the amplitude of the direct EGFR activation induced expression changes (Figure 14, blue columns).

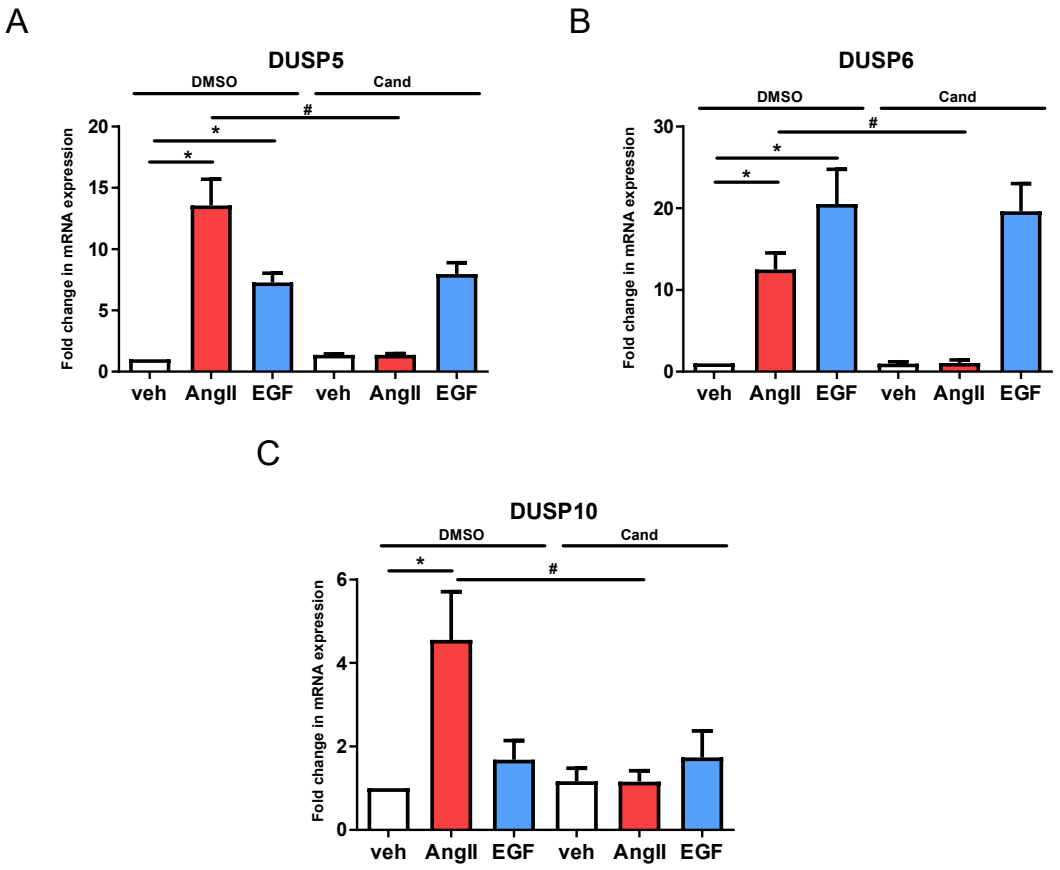

Figure 14. **Examination of the effect of AT1-R antagonist treatment on the agonist induced gene expression changes of *DUSP* isoform in vascular smooth muscle cells by qRT-PCR.** Serum-starved cells were pretreated with 10 μM candesartan (Cand) or vehicle (DMSO) for 30 minutes, and then exposed to either 100 nM AngII (red columns) or 50 ng/ml EGF (blue columns) or vehicle (white columns) for 2 hours. Standardization was made against the *GAPDH* housekeeping gene. The mRNA levels were normalized to values of DMSO vehicle samples and expressed as fold change. The values are from four independent experiments. Mean values ± S.E. are shown (n = 4). Significance was determined with multiple linear regressions. $p < 0.05$ was considered as statistically significant. *: Statistically significant from vehicle stimulation. #: Statistically significant when compared to DMSO pretreated agonist induced response [91].

Furthermore, in addition to activation of the $G_{q/11}$ protein, AT1-R is also able to bind to $G_{i/o}$ and $G_{12/13}$ heterotrimeric proteins, leading to inhibition of adenylyl cyclase or activation of Rho-kinase and phospholipase D, and to regulation of Ca^{2+} channels [105]. The YM-254890, a selective $G_{q/11}$ inhibitor was used to investigate the role of the

$G_{q/11}$ pathways. The application of YM-254890 at a concentration of 1 μM completely abolished the AngII-mediated gene expression upregulation in all examined *DUSPs* (Figures 15A-C).

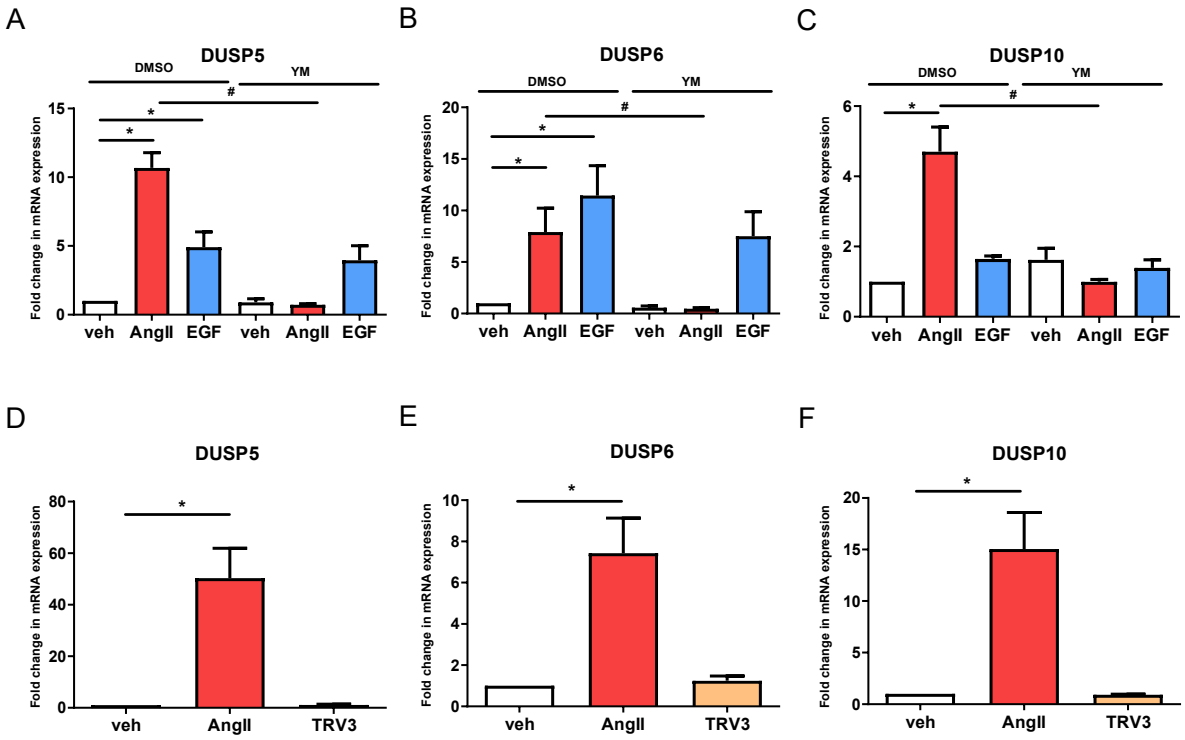

Figure 15. **Estimation of the contribution of G protein-dependent and independent mechanisms in the AT1-receptor simulation induced changes in *DUSP* levels in vascular smooth muscle cells.** Serum-depleted VSMCs were pretreated with 1 μM YM-254890 (YM) or vehicle (DMSO) for 30 minutes, and then exposed to either 100 nM AngII (red columns) or 50 ng/ml EGF (blue columns) or vehicle (white columns) for 2 hours (A-C). In the next experiment, serum-starved VSMCs were exposed to either vehicle (white columns) or 100 nM AngII (red columns) or 3 μM TRV3 (beige columns) for 2 hours (D-E). In both series of experiments, RNA was isolated from VSMCs, which was converted to cDNA and the cDNA levels of *DUSP5* (A and D), *DUSP6* (B and E) and *DUSP10* (C and F) were measured by qRT-PCR. Standardization was made against the *GAPDH* housekeeping gene. The values are from four or five independent experiments. Mean values ± S.E. are shown (n = 4-5). Significance was determined with multiple linear regression. $p < 0.05$ was considered as statistically significant. *: Statistically significant when compared to vehicle stimulation. #: Statistically significant when compared to DMSO pretreated agonist induced response. (A-C). In case of D-F significance was determined with one-way ANOVA-test (*p < 0,05) [91].

Next, we wanted to investigate the role of the β-arrestin mediated signaling pathway in the regulation of AngII-induced gene expression changes. For this study, a biased

agonist of the AT1-R receptor, the TRV3 peptide, was used, which does not induce or only partially induces G protein activity, but induces β-arrestin-mediated signaling through β-arrestin binding [76, 89, 92]. In contrast to the AngII stimulus, the use of 3 μM TRV3 did not induce significant changes in *DUSP* gene expression, suggesting that *DUSP* upregulation is controlled exclusively in a G protein-dependent manner (Figures 15D-F).

3.9. The role of calcium signaling and calcium dependent kinases in the upregulation of DUSP levels

The agonist activation of AT1-R and EGFR induces a cytosolic calcium level increase by phospholipase Cβ and γ (PLCβ and γ) [108, 115, 116], which in turn, activates several important regulatory mechanisms, such as PKC, Pyk2 and activation of calcium/calmodulin-dependent protein kinase in VSMC [115, 117-119]. Prior to the agonist stimulation, the VSMCs were pretreated with 50 μM BAPTA-AM, a calcium chelator, to investigate the role of intracellular calcium in the regulation of *DUSP* mRNA levels. The results obtained with 50 μM BAPTA-AM pretreatment show that the induced intracellular calcium increase is essential for the upregulation of the *DUSP* genes in response to both AngII and EGF stimulations in VSMCs (Figures 16A-C).

In the next experiment, a specific calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor, 50 μM CK59, was used to explore additional mechanisms involved in calcium signaling. The CaMKII plays a significant role in AngII-induced vascular reactivity, hypertrophy of VSMCs, and vascular remodeling [86, 90]. The pretreatment with 50 μM CK59 significantly inhibited the increase in AngII-induced *DUSP5* mRNA levels, but not the EGF caused response (Figure 16D). The AngII-induced regulation of *DUSP6* and *DUSP10* gene expression was also significantly reduced by CaMKII inhibition, but the effect was much lower in EGF-stimulated cells (Figures 16E and D).

In the next step, the role of calcium-dependent PKC was determined in the presence of a highly selective inhibitor of PKC, RO31-8425 (1 μM for 10 min) [120]. The pretreatment decreased the AngII-induced *DUSP5* mRNA level increase, but not the EGF-induced response (Figures 17A-C). In the case of *DUSP6* and *DUSP10*, the pretreatment significantly reduced AngII-induced upregulation, but had only a moderate effect on EGF-stimulated cells (Figures 17A-C).

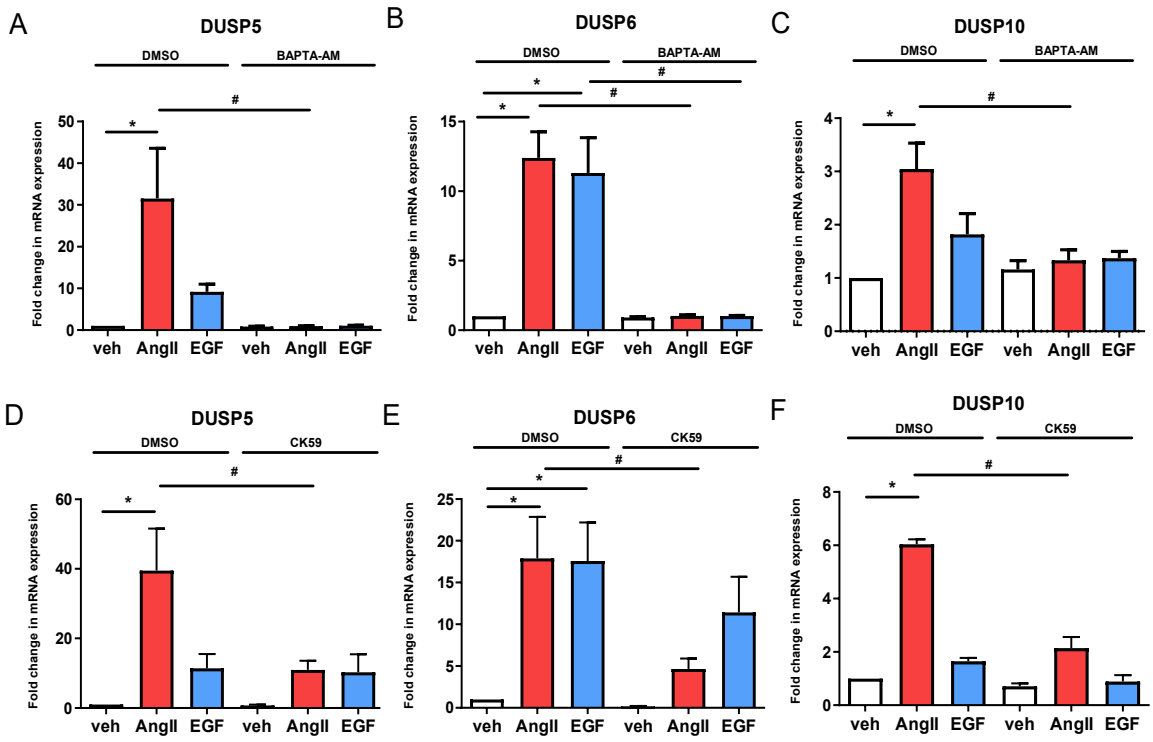

Figure 16. **Investigation of the role of calcium signaling in gene expression changes of *DUSP* isoforms in VSMCs.** Serum depleted VSMCs were pretreated with 50 μM BAPTA-AM or vehicle (DMSO) for 10 minutes (A-C), while in the other experiment series, the cells were pretreated with 50 μM CamKII inhibitor, CK59 or vehicle (DMSO) for 30 minutes (D-F). After the pretreatment, the cells were exposed to either 100 nM AngII (red columns) or 50 ng/ml EGF (blue columns) or vehicle (white columns) for 2 hours. RNA was isolated from VSMCs, which was converted to cDNA, and the cDNA levels of *DUSP5* (A and D), *DUSP6* (B and E) and *DUSP10* (C and F) were measured by qRT-PCR. Standardization was made against the *GAPDH* housekeeping gene. The mRNA levels were normalized to values of DMSO vehicle samples and expressed as fold change. The values are from three to six independent experiments. Mean values ± S.E. are shown (n = 3-6). Significance was determined with multiple linear regression. $p < 0.05$ was considered as statistically significant. *: Statistically significant when compared to vehicle stimulation. #: Statistically significant when compared to DMSO pretreated agonist induced response [91].

Furthermore, the role of Pyk2 (a regulator of EGFR transactivation in VSMCs) in the AngII-induced upregulation of *DUSPs* was investigated, using 1 μM PF-562271, a potent ATP-competitive FAK and Pyk2 kinase inhibitor. Pretreatment with 1 μM PF-562271 reduced the AngII-induced upregulation of *DUSP5* and *DUSP6* slightly, but not significantly (Figures 17D and E). Based on our data on the Pyk inhibitor, AngII-induced *DUSP5* upregulation is partially, but not completely Pyk2 dependent. It also reduced not

just the AngII-induced *DUSP6* upregulation, but also the *DUSP6* mRNA baseline. However, based on the results of multiple linear regression, the effect of Pyk2 inhibition remained significant. As for *DUSP10*, the 1 μM PF-562271 pretreatment did not affect the increase in AngII-mediated gene expression (Figure 17F).

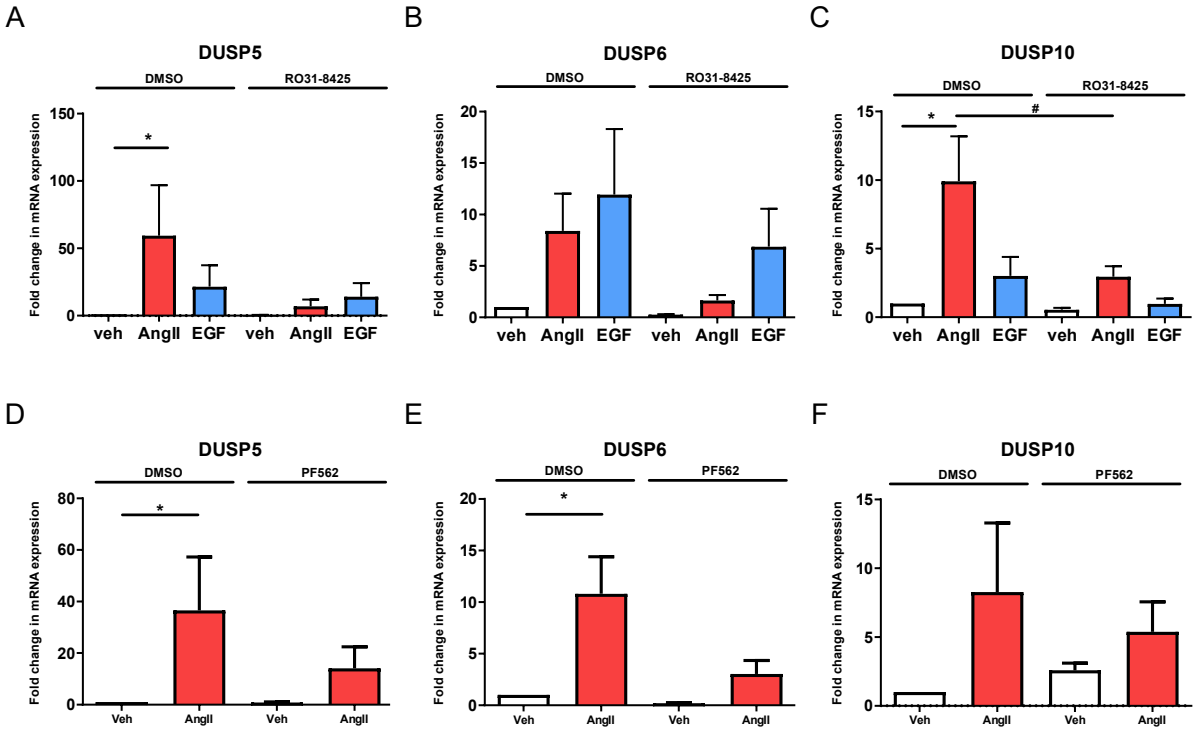

Figure 17. **Examination of the contribution of the PKC signal transduction in the AngII and EGF-induced gene expression changes of the *DUSP* isoform in VSMCs.** Cells were serum starved and then incubated with 1 μM of PKC inhibitor RO31-8425 or vehicle (DMSO) for 10 minutes (A-C). In the other experiment, the cells were incubated with 1 μM PF-562271, Pyk2 inhibitor or vehicle (D-F), and then exposed to either 100 nM AngII (red columns) or 50 ng/ml EGF (blue columns, A-C) or vehicle (white columns) for 2 hours. RNA was isolated from VSMCs, which was converted to cDNA, and the cDNA levels of *DUSP5* (A and D), *DUSP6* (B and E) and *DUSP10* (C and F) were measured by qRT-PCR. Standardization was made against the *GAPDH* housekeeping gene. The mRNA levels were normalized to values of DMSO vehicle samples and expressed as fold change. The values are from three independent experiments. Mean values ± S.E. are shown (n = 3). Significance was determined with multiple linear regression. $p < 0.05$ was considered as statistically significant. *: Statistically significant when compared to vehicle stimulation. #: Statistically significant when compared to DMSO pretreated agonist induced response [91].

4. DISCUSSION

In the first part of the dissertation, we identified and characterized a S127F (p.Ser127Phe) missense mutation of *AVPR2* in a Hungarian family (Figure 4). The patient's water deprivation test and the administration of dDAVP were ineffective. The administration of the compound was not followed by urine concentration increase, which confirmed the presence of the NDI disease. The molecular mutation analysis of the *AVPR2* gene revealed a point mutation in the *AVPR2* gene in the proband (Figure 4), and this missense mutation caused a cytosine to thymine single-base substitution (g.741C→T), resulting in an amino acid substitution from serine-127 to phenylalanine (S127F). Furthermore, the molecular examination of the *AVPR2* gene revealed that the proband was hemizygous for the mutation, while his mother was heterozygous for the S127F mutation, confirming her carrier status (Figure 4). The male proband shows the classic clinical symptoms of NDI, while his heterozygous mother has mild, subclinical symptoms. Since, *AVPR2* mutations cause recessive X-linked NDI, male patients show severe clinical symptoms, while heterozygous females are less affected. The mild symptoms of the proband's mother can be explained by the skewed X-chromosome inactivation. In previous studies, the female members of few congenital NDI families showed variable, but usually mild symptoms of diabetes insipidus, and these heterozygous female individuals possessed both normal and mutated *AVPR2* alleles [121-123]. It is assumed that the severity of symptoms depended on the rate of skewed methylation on the X chromosome, and that the NDI phenotype is caused by dominant methylation of the normal allele of the *AVPR2* gene [41, 121].

In characterization of properties of the loss-of-function S127F-V2R mutant receptor, our microscopic analysis showed that the expressed S127F-V2R is primarily located in the intracellular compartments, presumably due to ER retention caused by a misfolded mutant receptor structure (Figure 5D). The cell surface expression of the S127F-V2R was not significantly detectable (Figure 5B and 11B), and, in line with this, the mutant receptor has negligible signal transduction capacity compared to the wild-type receptor upon vasopressin stimulation. Even so, our high-sensitivity, real-time BRET measurements revealed that the AVP stimulation of S127F-V2R was able to generate a meagre, but statistically significant cAMP signal (Figure 6).

Mutations in GPCRs cause misfolding of the receptor proteins and, consequently, intracellular retention very frequently, leading to decreased cell surface presence of the receptors and reduced sensitivity to agonists [30]. These cellular changes may cause conformational or protein-misfolding disease [124]. The majority of congenital NDI mutations belong to this category. Several missenses, in-frame deletions, and insertions, and last exon mutations have been identified in the *AVPR2* gene, which cause abnormal folding of the receptor protein, resulting in the misfolded mutant receptors remaining in the ER [46]. The pharmacological chaperones can offer a new therapeutic approach for the functional restoration of damaged and ER retained receptors [125, 126]. Since the pharmacological chaperones have been proven to be a promising therapeutic candidate in rescuing several V2R mutant variants [48-50, 57, 127, 128], we investigated the therapeutic effect of the tolvaptan and MCF14 compounds on intracellularly trapped S127F-V2R. These compounds can serve as pharmacochaperones for certain V2R mutants since, in addition to being specific, non-peptide V2R ligands, they are also able to cross the plasma membrane, and, thus, potentially bind to intracellularly retained V2Rs.

In our studies we showed that tolvaptan pretreatment was able to inhibit the constitutive activity of V2R (Figure 7A) and increase the cell surface expression of the transiently expressed WT-V2R (Figures 10 and 11), which is consistent with other published data [53]. In our microscopic images, a significantly increased cell surface expression was observed in S127F-V2R mutant receptor expressing cells after tolvaptan pretreatment (Figure 11), but it was not observed in flow cytometry measurements (Figure 10). Although the tolvaptan pretreatment had no robust effect on receptor translocation from intracellular compartments to the plasma membrane, the tolvaptan pretreatment effectively rescued the cAMP-generating ability of the S127F-V2R mutant in response to AVP stimulation (Figure 7A). The third transmembrane helix of V2R (including residue 127) is involved in the AVP binding [129, 130], but S127 has no direct contact with AVP based on the 3D structure of the AVP-V2R complex [131]. Given these results, the impaired response to AVP stimulation is mostly due to intracellular-endoplasmic reticulum retention, but it cannot be excluded that the S127F mutation may alter both the plasma membrane expression of the receptor and the AVP affinity of the receptor. Functional rescue of S127F-V2R by tolvaptan pretreatment has demonstrated that pharmacochaperones can be used in clinical practice to treat NDI cases caused by the

S127F mutation. However, the potential clinical use of tolvaptan in NDI may be limited due to both its hepatotoxic [56] and its antagonistic effect.

Due to the limited clinical application of tolvaptan and in order to explore further possibilities we investigated the potential pharmacochaperone effect of the MCF14 compound to rescue the S127F-V2R mutant receptor. In our experiments, MCF14 pretreatment alone was unable to induce any increase in the basal cAMP level, suggesting that it is unable to acutely activate intracellularly trapped S127F-V2R mutant receptors (Figures 7 and 8), but that it acts as an effective pharmacochaperone for the S127F-V2R. The pretreatment with 10 μM MCF14 for 18 hours was able to functionally rescue the S127F-V2R mutant receptor since a significant increase in intracellular cAMP level was detectable after AVP stimulation (Figure 7B). Interestingly, the pretreatment with MCF14 pharmacochaperone, in contrast to the effective functional rescue effect, was unable to induce a significant increase in cell surface expression of S127F-V2R (Figures 10 and 11). This apparent discrepancy can be explained with the robust amplification of the signaling cascades [132, 133], which is an advantage of GPCR, including the G_s protein-coupled V2R induced signaling cascades [134].

We assume that the pharmacochaperone treatments, either with tolvaptan or by MCF14 administration caused the translocation of only a negligible proportion of the intracellular trapped receptors to the plasma membrane. This small number of translocated receptors did not yield a significant detectable increase in the cell surface receptor level in our experiments. Despite this, it was sufficient to functionally rescue the cAMP generation ability of the S127-V2R mutant receptor. A V2R receptor can simultaneously activate multiple G_s proteins, and each G_s protein activates several downstream adenylyl cyclases, which can lead to the production of numerous cAMP molecules. All results considered, no difference was detected between the signaling capability of tolvaptan and MCF14 rescued receptors, which suggests that both tolvaptan and MCF14 are able to bind the misfolded conformation of the S127F-V2R mutant receptor. However, MCF14 appears to be a more advantageous starting compound than tolvaptan for the development of a therapeutic agent in the treatment of NDI caused by S127F-V2R mutation, since it is a functional-selective agonist which does not promote hormone stimulation-induced β-arrestin binding and receptor-mediated endocytosis of the ligand-bound V2R [57]. The affinity of β-arrestin for the AVP-stimulated receptor

determines the fate of the internalized receptor, such as dephosphorylation and recycling to the cell surface or the downregulation of V2R by degradation**.** We attempted to investigate the ability of the rescued S127F-V2R mutant receptor to bind β-arrestin upon AVP stimulation, but due to the very low level of plasma membrane S127F-V2R, no β-arrestin interaction was detected even after pharmacochaperone treatments (data not shown).

In the second part of the dissertation, we examined AngII-induced gene expression changes in primary rat VSMCs to explore the sustained, long-term effects of AngII and to provide a novel insight into the transcriptomic effects of AngII in primary rat VSMCs. Furthermore, we wanted to explore previously unidentified mechanisms and new potential therapeutic targets for the alleviation of AngII-induced cardiovascular symptoms.

Firstly, we have examined the transcriptomic effect of AngII by stimulating serum-deprived early passage VSMCs with vehicle or 100 nM AngII for two hours, which was examined by Affymetrix GeneChip analysis. The Affymetrix GeneChip analysis revealed a number of genes that were significantly up- or downregulated after AngII stimulation, and which are consistent with the previously described genes in the VSMC [72, 73]. In our measurement data, the upregulated genes also included *DUSPs,* which can play an important role in the regulation of long-term MAPK signaling mechanisms of AT1-R. Since the effects of AngII on *DUSP* gene expression have not been described in previous articles, we wanted to investigate their role in AngII signaling and identify which branch of the AT1-R signaling pathway is responsible for these effects. From the upregulated genes, one member of each subfamily of *DUSP* isoforms, namely, *DUSP5, DUSP6*, and *DUSP10* was selected for analysis. Our measurements by Affymetrix GeneChip analysis were confirmed by real-time PCR measurements, which showed that the expression levels of the tested *DUSP* genes increased 1 hour after AngII stimulation, reached the peak at 2 h, and persisted until 6 h (Figure 13). The kinetics of *DUSP6* are slightly different from those of *DUSP5* and *DUSP10*, indicating that the involved signaling pathways may be different in terms of AngII-evoked responses. The results obtained in the study of the pathways responsible for the upregulation of *DUSP* genes showed that AngII effects are due to AT1-R activation caused by $G_{q/11}$-protein binding (Figures 14A-C and 15A-

C). Since the activated AT1-Rs interact with β-arrestins, which are scaffold platforms of signaling complexes, such as in the activation of the MAP kinase cascade [135], we have examined the possible role of β-arrestin-mediated signaling in the regulation of expression changes. The results obtained with the TRV3, a β-arrestin-biased AT1-R agonist, showed that the β-arrestin did not play a significant role in the regulation of *DUSP* expression levels (Figures 15D-F).

Subsequently, our results on the effects of the calcium chelator, BAPTA-AM, and specific inhibitors of CaMKII, PKC, and Pyk2 confirmed that the calcium signal and CaMKII play an important role in all the investigated *DUSP* expression changes (Figures 16 and 17), whereas PKC and Pyk2 calcium-dependent kinases do not play a significant role in the AngII-induced *DUSP5* and *DUSP6* mRNA level changes (Figure 17), while the *DUSP10* levels appear to be regulated only by PKC, and not by Pyk2 activity (Figures 17C and F).

The AngII-induced upregulation of *DUSPs* may regulate MAPK signaling in the VSMC in the long term. Both nuclear-localized *DUSP5* and cytoplasmic *DUSP6* are induced by ERK1/2 activity, and they have an important role in the negative feedback loop to limit ERK1/2 activation. DUSP10 is able to retain ERK MAPKs in the cytoplasm and downregulate ERK-dependent transcription [108]. DUSP10 has a complex role in the regulation of MAPK signaling. In a recent study it has been reported that the knockdown of *DUSP10* inhibits acute EGF-stimulated ERK activation, but that this inhibition can be reversed by the pharmacological inhibition of p38 MAPK, suggesting that DUSP10 may alter the crosstalk between the ERK1/2 and p38 MAPK pathways [110].

It is also conceivable that the DUSPs induced by AngII stimulation may suppress or modify certain types of MAPKs due to the non-exclusive substrate preference of certain DUSPs and the crosstalk mechanisms in parallel MAPK pathways, thus modifying the interactions of the MAPK network, after repeated hormone stimuli. However, further studies are needed to confirm this possibility. Furthermore, the upregulated DUSP enzymes may regulate other proteins in addition to MAPK, either by dephosphorylation and/or by binding to them. As the AngII-activated MAPK signaling has an implication in the phenotypic switching of VSMCs [136], we consider it worthwhile to later investigate the role of DUSPs in the physiological and pathological processes of the vascular system.

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5. CONCLUSION

In our experiments, we examined the cell physiological consequences of the S127F-V2R mutation and the possibility of restoring the mutant receptor function by pharmacochaperones. In the second part of the dissertation, the AngII-induced upregulation of *DUSPs* in the VSMCs and the signaling pathways affecting the expression of the different *DUSPs* were examined. Based on our results, the following conclusions can be drawn:

The S127F mutation belongs to class II mutation category of V2R, which leads to the production of misfolded and intracellularly, ER retained receptors. They can initiate cAMP signaling after their pharmacochaperone-induced translocation to the plasma membrane. In our experiments, S127F-V2R was able to generate a very low cAMP signal by the AVP stimulus, indicating partial functionality of the mutant receptor. This partial functionality of the S127F-V2R mutant receptor was successfully enhanced by pretreatment of V2R ligands with either tolvaptan and MCF14, which demonstrated their ability to reach and bind to ER-trapped receptor molecules, and, thus, to act as a pharmacochaperone. Our data shows that the pharmacochaperone treatments with administration of either tolvaptan or MCF14, caused the translocation of only a negligible proportion of the intracellular trapped receptors to the plasma membrane and that this small number of translocated receptors did not yield a significant detectable increase in the cell surface receptor level.

The results obtained in the study of the pathways responsible for the upregulation of *DUSP* genes showed that AngII effects are due to AT1-R activation caused by $G_{q/11}$-protein binding. Subsequently, our results confirmed that the Ca^{2+} signal plays an important role in all the investigated *DUSP* expression changes. Our data demonstrated that the regulation of expression changes of *DUSP* genes is probably determined by the interaction of the signaling cascades involved.

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6. SUMMARY

Dysfunction of G protein-coupled receptors can lead to pathophysiological conditions and a huge percentage of currently marketed drugs target these receptors. The water homeostasis of the body is regulated by the arginine-vasopressin system through the type 2 vasopressin receptor (V2R). Loss-of-function mutations of the V2R in the kidney can lead to nephrogenic diabetes insipidus (NDI), which results in several symptoms, such as polyuria and polydipsia.

In summary, our data show that the S127F mutation in the V2R leads to the production of misfolded and intracellularly retained receptors. Our results demonstrated that non-peptide V2R ligands, such as tolvaptan and MCF14, can serve as pharmacological chaperone molecules to functionally rescue the S127F-V2R mutant receptor. Our results indicate that pharmacological chaperones could be the ideal therapeutics to treat patients suffering from NDI caused by class II mutations, including the S127F mutation of the V2 vasopressin receptor.

AngII hormone and its main receptor, the AT1-receptor, are important regulators of the cardiovascular system. The VSMCs are the main targets of AngII and their stimulation does not just induce contraction but, it also results in gene expression changes. The long-term effects of AngII account for the development of several pathophysiological conditions, such as vascular and cardiac hypertrophy, hypertension, vascular remodeling and atherosclerosis. In conclusion, our data demonstrated that the regulation of AngII-induced *DUSP* gene expression changes in VSMCs is probably determined by the interaction of the signaling cascades involved. Based on our data, the expression changes of the studied genes can occur through the following mechanism: AngII binds to AT1-receptor, causing classical $G_{q/11}$ activation that triggers Ca^{2+}-dependent mechanisms. However, further studies are needed if we would like to determine how the ancillary signaling pathways can be successfully targeted in the treatment of diseases caused by AT1-R overactivation. Our data presented in this dissertation can provide new insight into the physiology of VSMCs in response to AngII stimulation, and a better understanding of the mechanism of AT1-R-mediated gene expression changes in primary VSMCs may lead to the development of novel types of drugs for treatment of cardiovascular and other diseases.

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8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Publications related to the present thesis:

1. Gem JB, Kovacs KB, **Szalai L**, Szakadati G, Porkoláb E, Szalai B, Turu G, Toth AD, Szekeres M, Hunyady L, Balla A. (2021) Characterization of Type 1 Angiotensin II Receptor Activation Induced Dual-Specificity MAPK Phosphatase Gene Expression Changes in Rat Vascular Smooth Muscle Cells. Cells, 10(12):3538. doi: 10.3390/cells10123538. IF: 7,666

2. **Szalai L**, Sziraki A, Erdelyi LS, Kovacs KB, Toth M, Toh AD, Turu G, Bonnet D, Mouillac B, Hunyady L and Balla A. (2022) Functional rescue of a nephrogenic diabetes insipidus causing mutation in the V2 vasopressin receptor by specific antagonist and agonist pharmacochaperones. Front Pharmacol, 13: 811836. doi: 10.3389/fphar.2022.811836. IF: 5,811

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