# *On the enigmatic mechanism of signal transduction of "arrestin-biased" angiotensin peptides: spotlight on endogenous angiotensin 1-7*

## Dissertation

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*Für meine Familie*

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# 1 Introduction

## 1.1 Receptors - How cells communicate and receive signals.

The cell is the smallest functional unit of life (Alberts 2022). The human body alone is made up of a multitude of different types of cells. When cells of the same type join together to form a union, they are called a tissue, and a functional combination of different tissues is called an organ. The human organism is thus characterized by a multidimensional interaction of these cells, tissues and organs (Kierszenbaum and Tres 2020).

Despite their diverse specifications, cells share a basic blueprint. They are all surrounded by a phospholipid bilayer called the plasma membrane. The plasma membrane has one critical feature: its semi-permeability. It allows only hydrophobic substances to pass through, while polar and large molecules cannot, creating a clear barrier between “inside and outside”. The cell interior, called the cytoplasm, thus becomes a reaction environment for biochemical processes (O'Connor, C. M. & Adams, J. U. 2010; Plattner and Hentschel 2011).

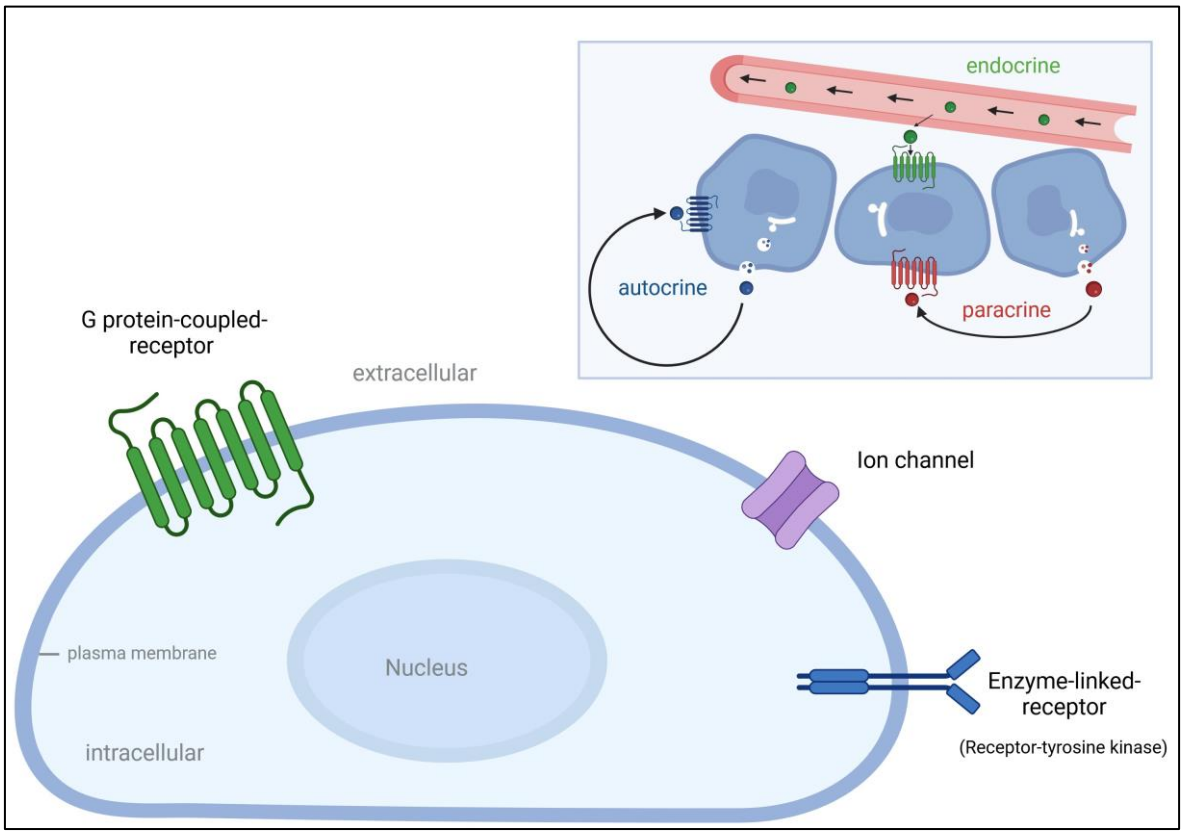


***Figure 1*: Cells communicate and receive messages:**

*Schematic representation of a cell with the characteristic plasma membrane (dark blue), which forms a boundary between the intracellular and extracellular space. The three types of transmembrane receptors are shown: a G protein-coupled receptor (left), an ion channel (middle), and an enzyme-coupled receptor (right).*

*Inlay: Illustration of how signaling molecules (messengers) reach their target cells by different routes and distances: endocrine (long distances, e.g., bloodstream), paracrine (neighboring cells, short distances), autocrine (self-stimulation of the cell). Figure was created with "BioRender.com".*

Although each cell is a self-contained, self-sustaining entity, it needs to communicate with its environment to survive (Lim et al. 2015). For this reason, cells constantly exchange information. This flow of information can be thought of as an exchange of messages, which are carried by messengers (signaling molecules). The messengers are as diverse as the messages themselves. They are mostly biochemical molecules of different classes, ranging from small molecules (e.g., biogenic amines, nucleotides) to large molecules such as peptides and proteins. In addition, cells can also detect mechanical stimuli and even light particles (Cooper and Hausman 2009). The exchange of information via messengers can take place over short distances, e.g. between two neighboring cells (neurotransmitters), as well as over long distances via the bloodstream (hormones) or lymph fluid (O'Connor, C. M. & Adams, J. U. 2010; Kramer 2016).

In order to receive and process the transported messengers, cells are equipped with signal receivers, proteins called **receptors** (from the Latin recipere = to receive, to take in) (Figure 1).

Since most signaling molecules cannot cross the cell membrane due to their size and polarity, most receptors are located in the cell membrane, i.e., they are transmembrane receptors. Membrane receptors are the key component for detecting and processing the received information. They are divided into two classes (Koeppen et al. 2018). The group of ionotropic membrane receptors are ion channels. Metabotropic membrane receptors, on the other hand, activate either protein kinases (enzyme-coupled receptors) or a G protein (*G protein-coupled receptors*) (Figure 1). The latter represent the largest family of membrane receptors and will be the focus of the following section.

## 1.2 GPCRs (G protein-coupled receptors).

The class of G protein-coupled receptors (GPCRs) can unquestionably be described as a protein superfamily. With more than 800 known genes, each encoding a GPCR, it not only represents one of the most diverse and comprehensive protein families in the human genome but also by far the largest group of membrane receptors (Lander et al. 2001). More than half of the total number of GPCRs can be assigned to the olfactory system. They function as olfactory receptors, specifically recognizing odorants as messengers (Pierce et al. 2002). A

messenger substance that is specifically bound by its receptor is referred to as its *ligand* (from the Latin ligare = to bind). While approximately one hundred GPCRs have no known endogenous ligands (orphan GPCRs), the other members of the GPCR family impress with their diversity of recognized ligands (Yang et al. 2021; Insel et al. 2019). These range from peptides and proteins (peptide hormones, chemokines, etc.), neurotransmitters (norepinephrine, dopamine, etc.), nucleotides, and fatty acids to sensory stimuli such as odors, individual tastes (bitter and sweet), and even light in the form of photons (Wacker et al. 2017). From this repertoire of ligands and associated receptors, it is easy to derive the key roles that GPCRs play in a wide variety of physiological systems in our body. Due to this central position from both a physiological and pathophysiological point of view, GPCRs are of the most intense interest and focus of drug discovery. More than 30% of all approved drugs act on or through GPCRs, and the number is increasing (Sriram and Insel 2018; Hauser et al. 2018; Santos et al. 2017; Hauser et al. 2017).

Despite the impressive abundance of ligands and involvement in diverse physiological processes, early molecular cloning and sequencing approaches of these receptors revealed that they follow a consistent structural principle with conserved motifs (Figure 2) (Dixon et al. 1986; Murphy et al. 1991; Erlandson et al. 2018).

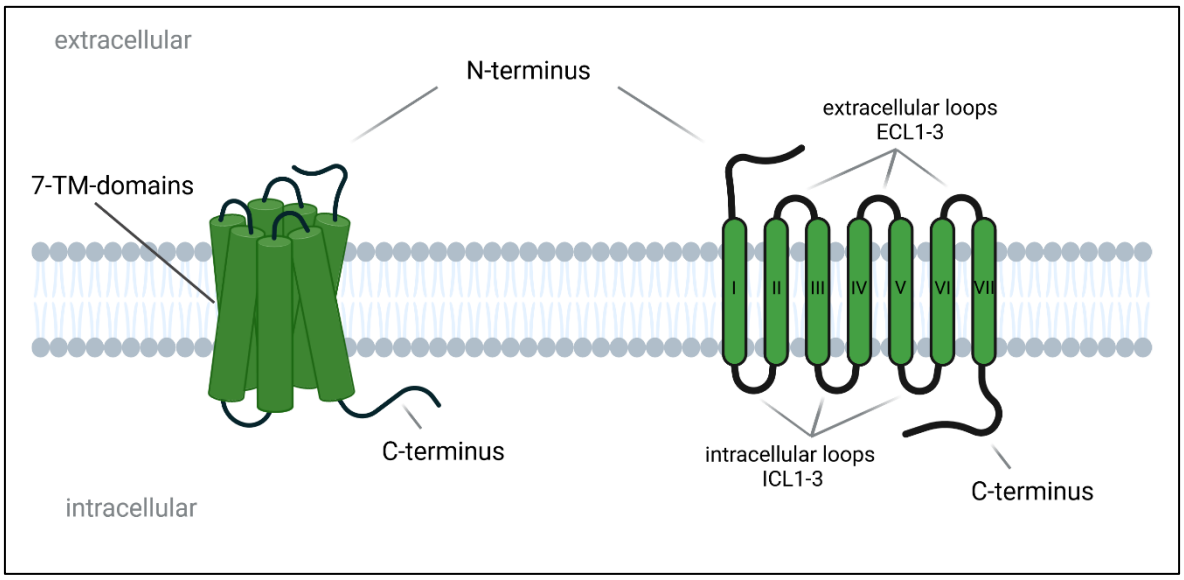


***Figure 2: Structure of a class A GPCR:***

*The protein spans the phospholipid bilayer (gray) of the plasma membrane seven times, forming three intracellular and three extracellular loops. The N-terminus is extracellular, while the C-terminus projects intracellularly. Figure was created with "BioRender.com".*

As membrane proteins (consisting of a chain of amino acids), all GPCRs share the property that they are not randomly embedded in the membrane, but that their chain spans the plasma membrane exactly seven times (Figure 2). These seven domains adopt a helical structure and are the reason why GPCRs are also called heptahelical transmembrane receptors or seven-transmembrane domain receptors (7-TM receptors) (Pierce et al. 2002). The beginning of the amino acid chain (N-terminus) is always extracellular, followed by the seven helices that penetrate the plasma membrane. The helices are interconnected by loops. This results in three extracellular and three intracellular loops (ECL1-3 and ICL1-3). The GPCR structure is then completed by the inwardly directed end of the amino acid chain, the C-terminus (Figure 2).

Human GPCRs can be classified into five families based on their phylogenetic relationship: Rhodopsin-, secretin-, glutamate-, adhesion-, and frizzled/taste2- family (Fredriksson et al. 2003). Among these, the rhodopsin family is the largest and most widely studied group. The following applies mainly to this family.

An endogenous ligand from the extracellular environment binds its GPCR at a specific binding position, the so-called *orthosteric* binding site. This binding induces a conformational change in the GPCR, which then enters an active state. In a very simplified view, the GPCR can be thought of as being in equilibrium between an inactive and an active state (two-stage model) (Leff 1995; Park 2012). Thus, the receptor exhibits a certain basal activity. By binding an activating ligand (**agonist**), the GPCR is stabilized in its active state and the equilibrium is consequently shifted towards the active stage (Figure 3 (A)).

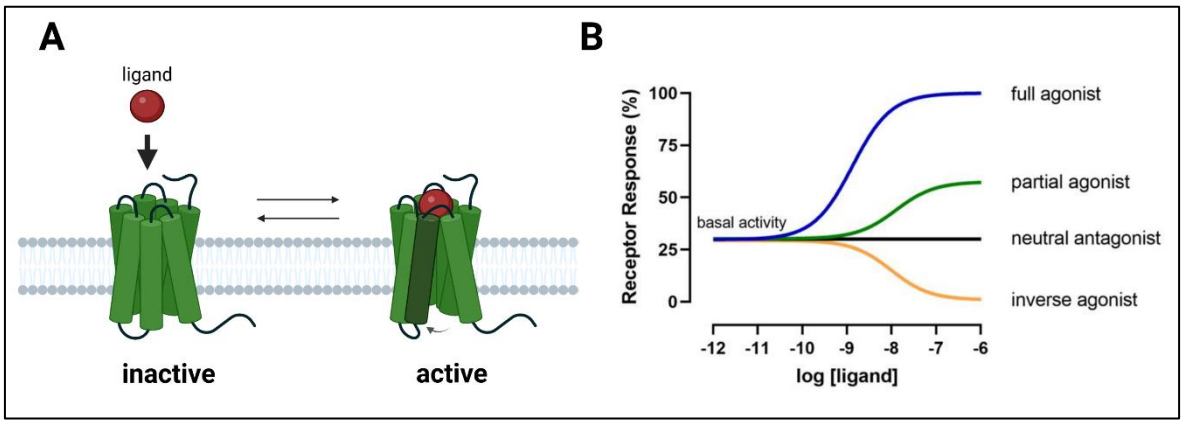


***Figure 3: Two stage activation model of a GPCR:***

*(A) Binding of the ligand (here agonist) leads to a conformational change of the GPCR, characterized by the outward movement of TM6 as shown in dark green, shifting the equilibrium to the activated state of the*

*receptor. (B) Examples of different equilibrium states: Neutral antagonists do not affect basal activity. Agonists shift the equilibrium to the active state, while inverse agonists do the opposite. Partial agonists do not achieve the maximum shift of full agonists. Figure was created with "BioRender.com".*

An inverse agonist, on the other hand, shifts the equilibrium in favor of the inactive state and therefore decreases basal activity. Whereas a neutral antagonist competes with the previously mentioned ligand types for the receptor binding site and exerts no influence on basal activity (Figure 3 (B)) (Weis and Kobilka 2018). The two-stage model provides only a minimalized insight into how ligands mediate their pharmacological effects. However, the conformations adopted by GPCRs are more complex and heterogeneous than a single model suggests. Rather, it is thought that different GPCR-conformations can be adopted depending on the ligand and that these contribute to the versatility of GPCR activation (Wootten et al. 2018; Wingler and Lefkowitz 2020).

*How can we imagine the activation of a GPCR?*

When an agonist occupies the orthosteric binding site, which in most GPCRs protrudes pocket-like into the apical part of the transmembrane region, a rearrangement of the amino acid residues within the helix bundle occurs (Zhou et al. 2019). The conformational change initiated in the orthosteric center is thus propagated across the helical bundle towards the intracellular receptor side, where it shows its greatest extent. In this process, the outward movement of transmembrane helix 6 (TM6) represents the hallmark of GPCR activation and ensures the opening of a cleft on the intracellular side of the GPCR (Figure 3 (A), dark green) (Rosenbaum et al. 2009). This cleft then allows the GPCR to interact with its partner and namesake in the membrane, the **G protein**.

G proteins act as signal mediators (signal transducers). They play a central role in this process by transmitting the incoming signal from the GPCR to the intracellular effector proteins and thus initiating a downstream signaling cascade (Syrovatkina et al. 2016). This family of proteins will be discussed in more detail below.

## 1.3 G proteins (guanosine triphosphate-binding proteins).

G proteins are so named because of their special characteristic: they bind guanosine nucleotides, in particular GDP and GTP (guanosine diphosphate and guanosine triphosphate). In general there are two types of G proteins that belong to this protein family; monomeric and heterotrimeric G proteins (Wennerberg et al. 2005; Gilman 1987).

Monomeric G proteins consist of a single peptide strand and are therefore referred to as small G proteins. Heterotrimeric G proteins are composed of three subunits and reflect the type of G protein with which GPCRs associate (Figure 4).

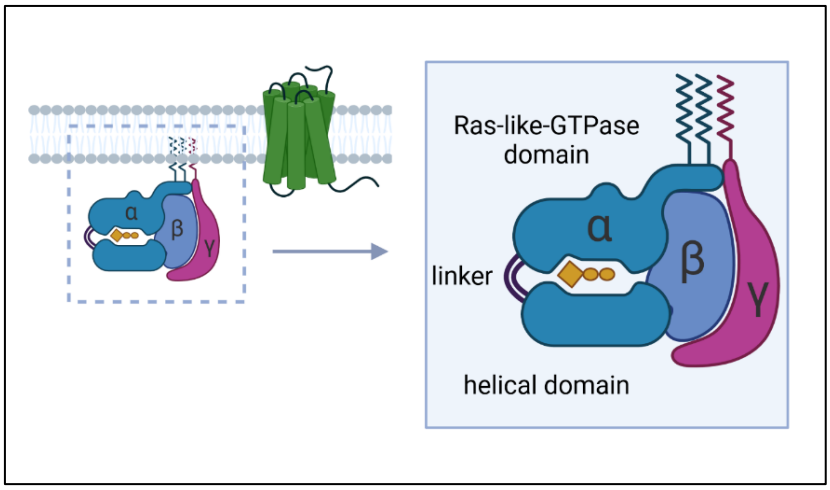


***Figure 4: Structure of a heterotrimeric G protein:***

*The G protein is bound to the membrane by its lipid anchors and associated with the GPCR (left). Enlargement of the inactive heterotrimeric structure consisting of the GDP-loaded Gα-subunit (turquoise with orange GDP) and the Gβ- and Gγ- subunits (blue, purple). See text for details. Figure was created with "BioRender.com".*

It is composed of three subunits: Gα (alpha), Gβ (beta), and Gγ (gamma). Membrane anchors at the α- and γ-subunit place most heterotrimeric G proteins at the plasma membrane, facilitating later coupling with the GPCR (Zhang and Casey 1996). The Gα-subunit binds a guanosine nucleotide (GDP or GTP) and consists of two domains. The first is the so-called GTPase domain (Ras-like domain). It acts as an enzyme capable of hydrolyzing GTP to GDP. Thus, it plays an important role in the inactivation of the Gα-subunit (see later G protein cycle). Two linkers connect the GTPase domain to the second domain, the α-helical domain. Exactly between these two domains, the nucleotide is firmly embedded (Lambright et al. 1994). The Gβ- and Gγ-subunits together form a functional unit in vivo, they are tightly linked and are also referred to as the Gβγ-dimer or Gβγ-complex (Clapham and Neer 1997; Sondek et al. 1996). Both the Gα-subunit and the Gβγ-dimer have the ability to independently modulate a wide variety of effectors upon activation of the heterotrimeric G protein (Syrovatkina et al. 2016; Lin and Smrcka 2011).

Heterotrimeric G proteins undergo a classical activation cycle after GPCR activation (Figure 5) (Oldham and Hamm 2008; Milligan and Kostenis 2006).

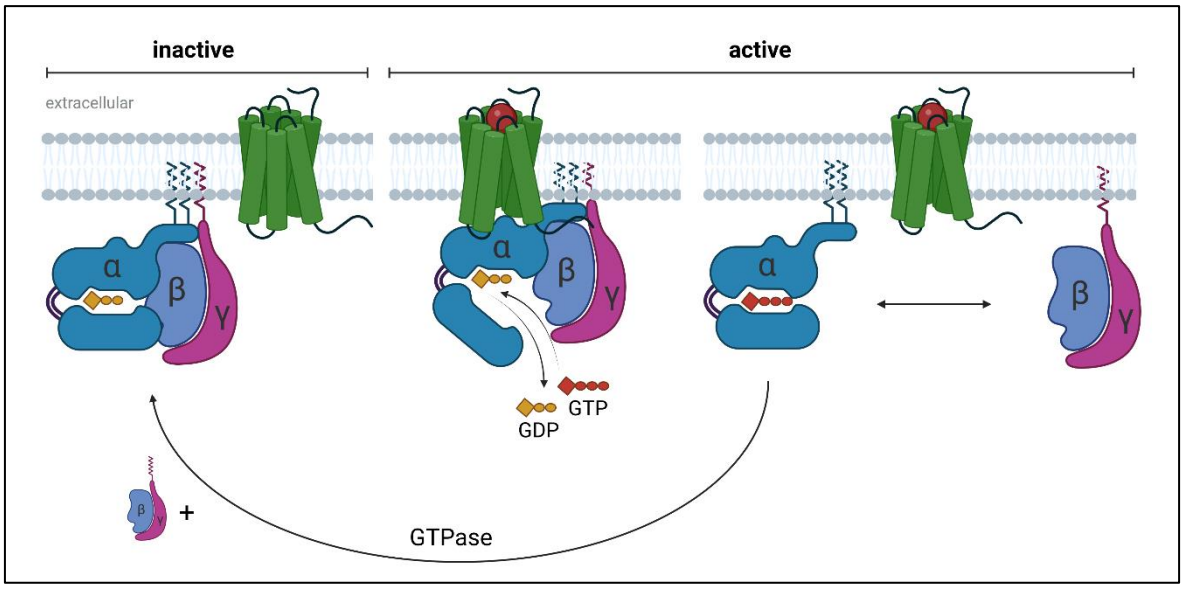


***Figure 5: G protein activation/inactivation cycle:***

*See text for details. Initial state of the inactive heterotrimeric G protein (left). Activation of the G protein induces the exchange (center) of GDP (orange) for GTP (red) within the Gα-subunit, leading to dissociation of the heterotrimer (right). Intrinsic GTPase activity of the Gα-subunit hydrolyzes GTP to GDP, leading to reassociation of the heterotrimer, terminating the cycle (curved arrow). Figure was created with "BioRender.com".*

The inactive G protein structure represents the starting point of the cycle. In the absence of an external stimulus, the G protein forms a heterotrimer. All three subunits are tightly bound to each other, with the Gα-subunit carrying a GDP molecule (Figure 5, left). Activation of the GPCR by agonists or constitutively then leads to a conformational change of the GPCR with a characteristic opening of an intracellular cleft within the receptor helix bundle (Figure 5, center). The heterotrimeric G protein inserts into this cleft and is activated. This results in a conformational change of the Gα-subunit, which subsequently loses its affinity for bound GDP. GDP is released into the cytosol and immediately replaced by GTP, which is more abundant in the cell (Syrovatkina et al. 2016). The agonist-GPCR complex assists in this process and functions as a nucleotide exchange factor (GEF) (Flock et al. 2015; Dror et al. 2015). GDP-GTP exchange within the Gα-subunit is characteristic of G protein activation. The now active G protein undergoes a conformational rearrangement due to the GTP binding and subsequently dissociates into the Gα-subunit and the Gβγ-dimer (Figure 5, right). The Gα- and Gβγ-subunit then interact with a wide variety of effector proteins, inducing downstream signaling cascades.

The cycle is terminated by the intrinsic GTPase property of the Gα-subunit. It hydrolyzes the GTP to GDP over time. As a result, the Gα-subunit regains its affinity for the Gβγ-dimer and reassociates into the inactive, GDP-loaded heterotrimer (Figure 5, curved arrow). The G protein is now ready for the next cycle. In this way, G proteins act as "switches" that convert the primary signal (agonist) received from the receptor (GPCR) into a secondary intracellular response. G proteins are the canonical transducers of the signaling cascade.

In humans, 16 Gα-subunit genes (encoding more than 20 isoforms) are known, as well as five Gβ- and 12 Gγ-genes (Downes and Gautam 1999). Some subunits, such as olfactory Gα-subunits, are found only in olfactory neurons and are therefore tissue-specific (Syrovatkina et al. 2016). The level of expression of each subunit may also differ between cell types. However, most of the subunits are present in many cell types (Milligan and Kostenis 2006). Heterotrimeric G proteins can be classified on the basis of homologies in the sequence of their Gα-subunits (Simon et al. 1991). The four major classes of G proteins are derived from this: **$G\alpha_s$ , $G\alpha_i$ , $G\alpha_q$ , and $G\alpha_{12/13}$**. Each of the four major classes activates/inactivates its specific effectors in the cell, thereby initiating a characteristic canonical signaling cascade (Figure 6) (Hildebrandt 1997; McCudden et al. 2005).

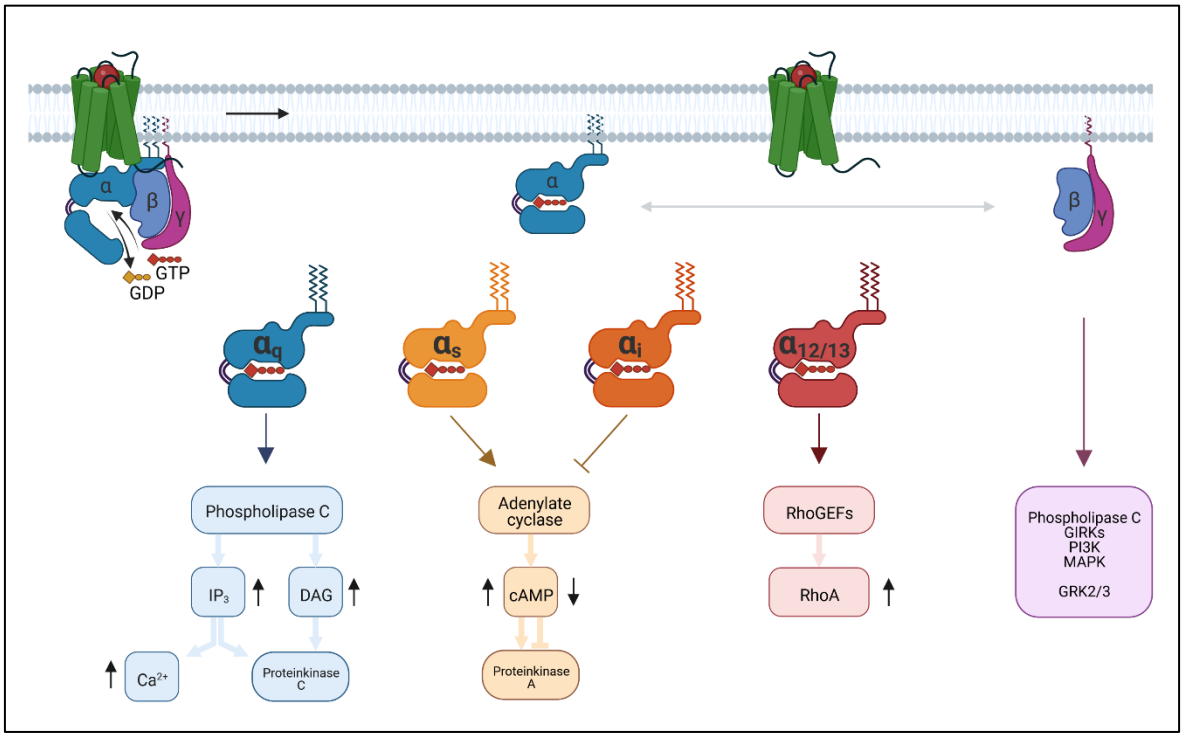


***Figure 6: The four major heterotrimeric G protein classes - $G\alpha_q$, $G\alpha_s$, $G\alpha_i$ and $G\alpha_{12/13}$:***

*Depending on its class affiliation, the active Gα-subunit interacts with specific effector proteins, thereby initiating a characteristic signaling cascade (see text for more information). Gβγ-dimers have also been reported to elicit their own signaling (shown at right). Figure was created with "BioRender.com".*

$G\alpha_s$ -class: Activated (GTP-loaded) $G\alpha_s$ members specifically activate membrane-bound adenylyl cyclases (AC). These then convert ATP into cAMP. The now increased cAMP acts on various cAMP-regulated proteins such as Epac and protein kinase A. The latter then phosphorylates a plethora of other proteins (Wettschureck and Offermanns 2005).

$G\alpha_i$ -class: It represents the antagonist of the $G\alpha_s$ -class ($G\alpha_s$ = **s**timulatory). Consequently, it has an inhibitory effect ($G\alpha_i$ = **i**nhibitory) on the adenylyl cyclases and thus reduces the cAMP concentration (Sunahara et al. 1996)

$G\alpha_q$ -class: The β-isoforms of phospholipase C (PLC-β) are activated by its members. PLC-β specifically cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacylglycerol (DAG) and inositol triphosphate ($IP_3$). $IP_3$ then binds to its receptors on the endoplasmic reticulum (ER), resulting in the release of calcium ions from the ER into the cytoplasm. DAG and $IP_3$ stimulate protein kinase C, which modulates other components of the signaling cascade by phosphorylation (Rhee 2001; Nishizuka 1995).

$G\alpha_{12/13}$ -class: By stimulating specific nucleotide exchange factors (Rho-GEFs), they regulate the activity of the small GTPase RhoA (Longenecker et al. 2001; Buhl et al. 1995; Strathmann and Simon 1991).

In addition to these four major Gα classes, Gβγ-dimers also communicate with intracellular effectors and can elicit signaling cascades. Gβγ-effectors are quite diverse and include ion channels (e.g. GIRKs and $Ca_v$ 2), adenylyl cyclases, phospholipases (PLC-β), phosphatidylinositol3-kinase (PI3K), and mitogen-activated protein kinases (MAPK) (Khan et al. 2013; Clapham and Neer 1993). Gβγ-dimers also bind the G protein-coupled receptor kinases 2/3 (GRK2/3). In this way, these kinases are recruited to the membrane and can then phosphorylate GPCRs (see 1.4.1) (Pitcher et al. 1998). In contrast to the more than 800 known GPCR genes, it is striking that they are matched by a comparatively small number of G proteins. This is partly due to evolutionary reasons, and as a consequence, one G protein

class can be activated by several GPCRs (Flock et al. 2017). At the same time, a GPCR can couple multiple G protein classes, with certain G protein classes being preferred depending on the receptor (Hermans 2003).

Various approaches are available to study specific G protein signaling pathways. For example, gene editing methods can be used to specifically modify or knock out genes of Gα-protein families to study their involvement in individual signaling processes. The discovery of natural inhibitors for individual G protein families has also greatly aided the study of G protein signaling pathways. Two highly specific inhibitors for the $G\alpha_i$- and $G\alpha_q$-classes are known and have been used as tools in this thesis. They will be briefly presented in an excursus:

### *1.3.1 Excursus: Pertussis toxin (PTX) and FR900359 (FR).*

***Tools for the targeted investigation of $G\alpha_i$- and $G\alpha_q$- signaling pathways:***

*Pertussis toxin* (PTX) is a protein (exotoxin) released by Bordetella pertussis bacteria (Carbonetti 2010). It belongs to the group of AB toxins, which are equipped with a catalytic A domain (active unit) and a pentamer of five B domains (binding unit). This binding unit systematically recognizes and binds to carbohydrate structures on the surface of cells, causing endocytotic uptake of the toxin into the cell (Mangmool and Kurose 2011). Once there, the A domain catalyzes the release of an ADP-ribosyl residue from cellular NAD and transfers it to a cysteine residue on the $G\alpha_i$ -subunit. As a result, the $G\alpha_i$ -subunit is no longer able to couple to its GPCR (Keen et al. 2022; Burns 1988; Katada and Ui 1982). The communication of the GPCR with the $G\alpha_i$ -subunit is disrupted, which means that it cannot fulfill its inhibitory function on the adenylyl cyclases. PTX thus becomes a critical virulence factor of the B. pertussis bacterium. It is partly responsible for the symptoms of pertussis (whooping cough), such as spasmodic cough and vomiting, but also for systemic leukocytosis (Wood and McIntyre 2008). Today, there are only a few cases of pertussis (whooping cough) due to vaccinations, some of which contain attenuated PTX (Trollfors et al. 1995; Carbonetti 2007).

PTX is a highly specific inhibitor (except for $G\alpha_z$) of all $G\alpha_i$ family members, making it the optimal tool to study $G\alpha_i$-signaling pathways and/or their involvement (Keen et al. 2022; Katada et al. 1983).

*FR900359 (FR)* is a cyclic depsipeptide, first isolated in the late 1980s from a methanolic extract of the tropical evergreen plant Ardisia crenata (Fujioka et al. 1988; Hermes et al. 2021a; Hermes et al. 2021b). At that time the first structural and bioactivity studies of FR were conducted (Miyamae et al. 1989) before it was forgotten for more than 20 years. It was rediscovered in 2010 in a screen of plant extracts looking for inhibitors of an intestinal hormone receptor (cholecystokinin type 1 receptor) (Nesterov et al. 2010). Depsipeptides are special peptides that contain not only peptide bonds but also ester bonds in their structure (Gold 2019). Interestingly, depsipeptides are usually not metabolites of plant metabolism, but typical natural products of marine or bacterial origin (Hamada and Shioiri 2005). In 2003, YM254890 (YM) was the first substance to be characterized that showed a high structural similarity to FR (only two residues differed), but was isolated from bacteria living in soil (Taniguchi et al. 2003). This led to the suspicion that bacteria living in symbiosis with the plant Ardisia crenata were the actual FR producers. This was confirmed in 2015, when *Carlier et al.* isolated DNA from certain bacteria of the genus Burkholderia (Candidatus Burkholderia crenata) from the plant's leaf nodules and identified the potential gene cluster for FR (Carlier et al. 2016; Hermes et al. 2021a). Today, FR can be heterologously produced in other bacterial hosts using sophisticated and complex bioengineering techniques, making it accessible as a pharmacological tool (Crüsemann et al. 2018; Hermes et al. 2021b).

FR blocks $G\alpha_q$-proteins with unique potency and selectivity (Schrage et al. 2015; Patt et al. 2021; Zhang et al. 2020; Xiong et al. 2016; Reher et al. 2018). They work by docking into a cleft in the linker region between the GTPase and helical domains of the $G\alpha_q$-subunit. As a result, no unfolding and thus no exchange of GDP for GTP can take place within the $G\alpha_q$-subunit. The subunit remains in its inactive (GDP-loaded) state and thus all $G\alpha_q$-signaling pathways are blocked (Nishimura et al. 2010; Kostenis et al. 2020).

The importance of PTX and FR as pharmacological tools is even more impressive considering the current lack of (comparable) inhibitors for the $G\alpha_s$ - and $G\alpha_{12/13}$ -class (Li et al. 2020).

## 1.4 Desensitization/Internalization: Not only G proteins interact with GPCRs.

Interestingly, in spite of continuous or excessive stimulation, a GPCR-mediated cell response is rapidly attenuated (Lefkowitz 1993). This process is called desensitization and protects cells from persistent overactivation. Desensitization typically begins with the

phosphorylation of the receptor (Stadel et al. 1983) which can be caused by the aforementioned protein kinases A and C of the $G\alpha_s$- and $G\alpha_q$-pathways (*heterologous phosphorylation)* (Hausdorff et al. 1990; Willets et al. 2003; Benovic et al. 1985). On the other hand, in addition to G protein coupling, GPCR activation (constitutive or ligand-stimulated) directly recruits specific kinases, the G protein-coupled receptor kinases (**GRKs**) (Homan and Tesmer 2014; Benovic et al. 1986). GRKs compete with G proteins for the binding site of the active receptor and phosphorylate it at characteristic serine- and threonine-residues of its C-terminal end and/or within its ICL domains (Benovic et al. 1991). This type of phosphorylation induced by GRKs is referred to as *homologous phosphorylation (Gurevich and Gurevich 2020)*. Phosphorylation, in turn, increases the affinity of the receptor for other proteins, such as **arrestins** (Lohse et al. 1990; Scheerer and Sommer 2017). Arrestins bind to the receptor and ultimately prevent the coupling of further G proteins to the GPCR (**Desensitization**) (Figure 7).

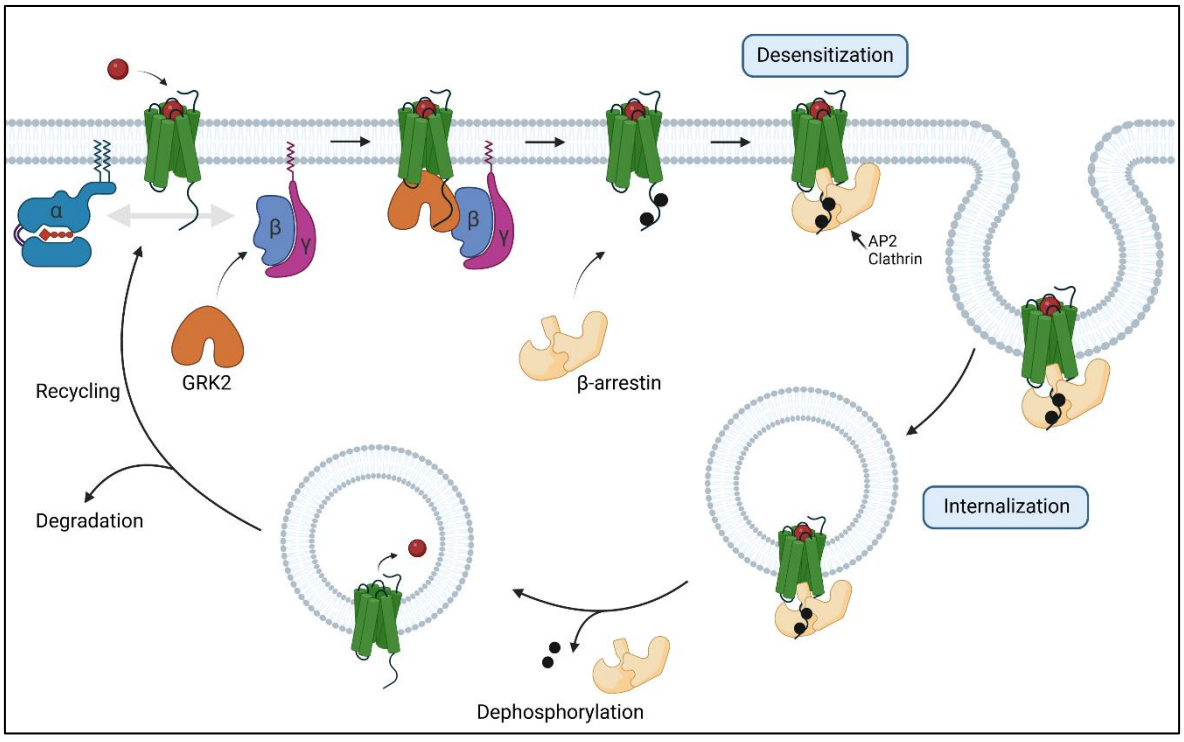


***Figure 7: Desensitization and Internalization:***

*Illustration of the internalization cycle of a GPCR such as the AT1R (see 1.5.1). Activation of the GPCR leads to heterotrimeric G protein activation and interaction with GRKs and β-arrestins. As shown, cytosolic GRK2 (orange) is recruited to the active GPCR by the released free Gβγ-subunit, leading to phosphorylation of residues in the GPCR C-tail (indicated by black dots). Phosphorylation is accompanied by binding of β-arrestins to the GPCR, preventing further G protein activation (Desensitization). β-arrestins then interact with proteins of the endocytotic apparatus (AP2/clathrin) to promote endocytosis of the GPCR (Internalization).*

*Here, the GPCR is internalized together with the β-arrestin, which is characteristic for the AT1 receptor (see 1.5.1). The internalized receptor can now either be degraded or recycled to the plasma membrane. Figure was created with "BioRender.com".*

Their name is derived from this function (to arrest = to stop, inhibit). Arrestins "arrest" the GPCR-G protein signaling cascade by blocking the intracellular binding site of the GPCR, thus preventing further G protein binding and activation (DeFea 2011). In addition, by binding to the active GPCR, arrestins undergo a conformational change that allows them to bind and recruit proteins of the endocytosis apparatus. As a result, the GPCR-arrestin complex is endocytosed into the cell (**Internalization**) (Figure 7) (Gurevich and Gurevich 2019). Both, desensitization and internalization, characterize the termination, i.e., the cessation of the ligand-induced signal-mediation. Internalized receptors can be either lysosomally degraded or recycled back to the membrane (Figure 7) (Kelly et al. 2008; Pitcher et al. 1998; Tsao and Zastrow 2000; Claing 2002). Thus, signal transduction (G proteins) and signal termination (GRKs and arrestins) occur simultaneously after GPCR activation. GRKs and arrestins are additional GPCR interaction partners playing a decisive role in signaling modulation. They will be discussed in more detail next.

### *1.4.1 GRKs (G protein-coupled receptor kinases).*

In addition to heterotrimeric G proteins and arrestins, GRKs are central to GPCR communication (Komolov and Benovic 2018). They recognize the intracellular cavity of the active GPCR and are activated by docking to it. In turn, they phosphorylate intracellular serine and threonine residues of the GPCR within its C-terminal end or intracellular loop 3 (ICL3) (Gurevich and Gurevich 2019). As a whole, GRKs belong to the group of serine-threonine kinases that transfer phosphate residues to hydroxyl groups with the help of ATP (Ribas et al. 2007).

The first GRK to be discovered was the rhodopsin kinase, named for the GPCR (rhodopsin) it phosphorylates, which is found in the rods of the retina (Kühn and Dreyer 1972; Kühn 1978). When the first non-visual GRK was identified in the 1980s, the β-adrenergic receptor kinase (βARK) (Benovic et al. 1986), which was able to specifically phosphorylate the β2-adrenergic receptor, it became clear that an entire GRK family must exist. Today, the family consists of seven GRK isoforms, titled GRK1-7 according to the new systematic nomenclature (Gurevich et al. 2012). The family can be divided into visual (GRK1, GRK7) and non-visual GRKs (GRK2-6). The latter are further subdivided into the GRK2- (GRK2/3)

and GRK4- (GRK4/5/6) subfamilies (Homan and Tesmer 2014; Mushegian et al. 2012). The two subfamilies differ markedly in how they are targeted to the plasma membrane and thus to the GPCR. GRK2 and GRK3 do not have a lipid anchor (unlike GRK4/5/6) and must therefore be targeted to the membrane by a different mechanism. They contain a pleckstrin homology domain (PH) that specifically recognizes the free Gβγ-subunit of the activated and dissociated heterotrimeric G protein. Since the Gβγ-subunit is membrane-bound due to its prenylated Gγ domain, GRK2 and GRK3 are thus recruited to the membrane and receptor (Koch et al. 1993; Touhara et al. 1994; Tesmer et al. 2005; Carman et al. 2000). In contrast, GRK4 family members (GRK4/5/6) do not possess a PH domain but are all membrane-bound by palmitoylation (Stoffel et al. 1998; Premont et al. 1996) and/or ionic interactions with negatively charged membrane phospholipids (Homan et al. 2013; Thiyagarajan et al. 2004).

With the exception of tissue-specific GRK1/7 (retina) and non-visual GRK4 (testis/heart), all other non-visual GRKs are ubiquitous in the human body, albeit with differential expression (Matthees et al. 2021). Thus, in principle, four GRKs (GRK2/3/5/6) modulate the signaling cascades of almost all GPCRs in the body, further highlighting their importance in the regulation of GPCR signaling (Drube et al. 2022). Their regulatory role is further emphasized by the fact that they interact with other proteins in addition to GPCRs (Gurevich and Gurevich 2019). Membrane recruitment of GRK2/3 by the Gβγ-subunit of active G proteins has already been described (see above). Conversely, it has been demonstrated that binding of GRK2/3 to Gβγ disrupts the Gβγ signaling cascade (Raveh et al. 2010; Abraham et al. 2018). Moreover, GRK2/3 can also interact with the active $G\alpha_q$-subunit. In this way they "capture" (figuratively speaking) active $G\alpha_q$-proteins (sequestration), leading to a reduction in the $G\alpha_q$-mediated signaling cascade (Carman et al. 1999; Tesmer et al. 2005). Other GRK-influenced proteins have also been described in the literature, which are either phosphorylated (kinase function) (Gurevich et al. 2012) or bound and thus brought into spatial proximity to other partners by GRKs (adaptor function) (Hullmann et al. 2014; Gold et al. 2013; Willets et al. 2003).

The main role of GRKs is to phosphorylate activated GPCRs. Structural data of GRKs in complex with their GPCR suggest that they recognize the same intrahelical cleft as the G protein at the active GPCR (insertion of their N-terminus) (Komolov et al. 2017; He et al. 2017). However, the exact mode of binding and activation remains controversial (Cato et al.

2021). Once GRKs bind and phosphorylate the GPCR, the receptor's signaling function is limited but not yet completely blocked (Sibley et al. 1986; Lohse et al. 1992). It requires the binding of another GPCR interaction partner, the **arrestin** protein, which then leads to uncoupling of the GPCR from the G protein (desensitization) (Benovic et al. 1987; Gurevich and Gurevich 2019).

### *1.4.2 Arrestins*

The discovery of this GPCR interaction partner has also its origin in the visual system. First described and identified in the 1970s as the so-called retinal S-antigen, it was thought to be involved in the development of a specific ocular dermatitis (uveo-retinitis) (Dorey and Faure 1977). Around the same time another group of researchers found a 48-kDa protein (48K-protein), which was able to bind to light-activated photoreceptor membranes (Kühn 1978). It has been shown that the S-antigen and the 48K-protein are one and the same protein (Pfister et al. 1985), which plays a crucial role in the silencing of retinal GPCRs (rhodopsins) (Wilden et al. 1986). The S-antigen (48K-protein) competes with the retinal G protein (transducin) for the phosphorylated rhodopsin (GPCR) and thus prevents transducin-mediated phototransduction (Krupnick et al. 1997; Wilden 1995). This property gave rise to the name of the protein family: **arrestins** (to arrest = to stop, inhibit) (Zuckerman and Cheasty 1986).

S-antigen and 48K-protein, respectively, were later renamed arrestin-1 in an effort to achieve uniform nomenclature (Sterne-Marr et al. 1993). Arrestins are listed here in chronological order according to the date of their first cloning. Arrestin-1 was the first member of the family. Today, the family consists of four arrestins (Table 1).

***Table 1: Arrestins and their synonyms:***

| ***Designation*** | ***Synonyms (Aliases)*** | ***described by*** |
|---|---|---|
| Arrestin-1 | retinal S-antigen, 48K-protein, rod-arrestin | Dorey and Faure et al.<br>Wilden et al/Kühn et al. |
| Arrestin-2 | **β-arrestin1**, β-arrestin | Lohse et al. |
| Arrestin-3 | **β-arrestin2**, hTHY-ARRX | Attramada et al, Rapoport et al,<br>Sterne-Marr et al. |
| Arrestin-4 | cone-arrestin, X-arrestin | Craft et al.<br>Murakami et al. |

Analogous to arrestin-1, there are several aliases for the individual arrestins. This is due to the fact that individual arrestins or their encoding genes have been cloned simultaneously and independently by several groups. They were often named after the original tissue (or the cDNA library derived from it) or the receptor at which they were found. For example, **β-arrestin** (arrestin-2) owes its name to its original description at the **β**2-adrenergic receptor. *Lohse et al.* were the first group to clone this protein and show in experiments that it preferentially binds the β2-adrenergic receptor instead of retinal rhodopsin (Lohse et al. 1990; Lohse et al. 1992). β-arrestin was the first non-visual arrestin found. Shortly thereafter, three groups (see Table 1) independently cloned the second non-visual arrestin, **β-arrestin2** (arrestin-3) (Sterne-Marr et al. 1993; Rapoport et al. 1992; Attramadal et al. 1992). The β-arrestin (arrestin-2) therefore had to be retroactively renamed **β-arrestin1**. The family was completed by another visual arrestin (arrestin-4), initially isolated from the cones of the retina (Table 1) (Craft et al. 1994; Murakami et al. 1993).

Thus, four arrestins control the regulation of hundreds of GPCRs in the human body. Moreover, if we disregard the visual arrestins (arrestin1/4), which are restricted to retinal photoreceptors, two non-visual arrestins (arrestin2/3) remain for all other GPCRs (Gurevich and Gurevich 2019).

The two non-visual arrestins (arrestin2/3) are mostly referred to in the literature as **β-arrestin1** and **2**. In the further course of this work, this naming will therefore be maintained. β-arrestin1 and β-arrestin2 are ubiquitously expressed (Nogués et al. 2018), meaning that they are present in virtually every cell, although their expression levels can vary depending on the tissue or cell type (Matthees et al. 2021). Arrestins have a high degree of sequence homogeneity and share a similar molecular structure (Figure 8, left) (Peterson and Luttrell 2017).

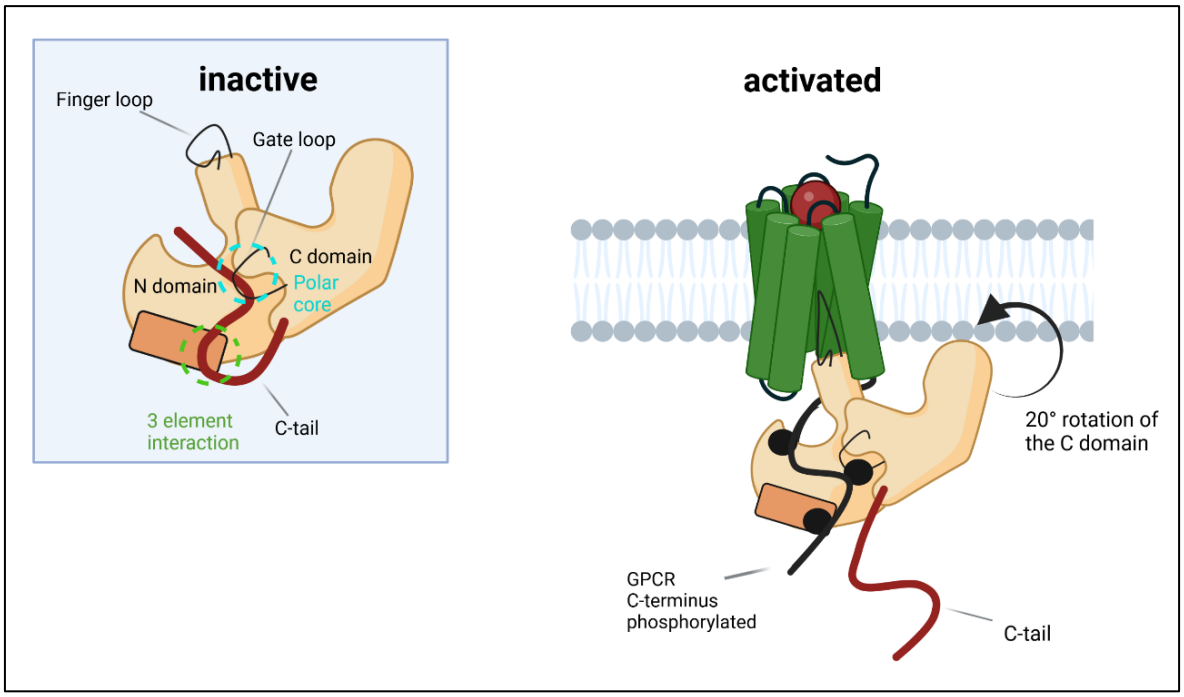


***Figure 8: Arrestin structure inactive and activated:***

*Figure based on Peterhans et al. (Peterhans et al. 2016)*: *Basal state of the arrestin molecule (left): arrestins are two-domain molecules with N- and C-domains. The relative orientation of the domains is stabilized by two central elements at which the N- and C-domain interact (polar core and three-element interaction site (green and turquoise). The C-terminus (red) of the arrestin participates in both elements and is located in a notch on the N-domain. (B) Binding of arrestin to the active and phosphorylated receptor results in disruption of the two elements and release of the C-tail. This is followed by a conformational rearrangement, resulting in a 20° rotation of the domains relative to each other. 20° rotation and C-terminal release are hallmarks of arrestin activation, which may release binding sites for interaction partners (Scheerer and Sommer 2017). Figure was created with "BioRender.com".*

Through intramolecular interactions, the protein spatially forms two domains, the N-domain and the C-domain, which are in contact through a hinge region. The C-tail of the molecule is located in a cleft on the N-domain and later plays a critical role in arrestin activation. Before being activated, arrestins must first bind to the active and phosphorylated receptor (Figure 8, right). Therefore, arrestins contain elements that recognize the phosphorylation and the active conformation of the GPCR. This model is also known as the "two-sensor model" (Chen et al. 2018). A "phospho-sensor", consisting of positively charged dimples on the N-domain, interacts specifically with the negative phosphate residues of the phosphorylated receptor (Zhou et al. 2017). It mediates the binding of the arrestin to the phosphorylated GPCR. This is followed by the binding of the second, so-called "activation sensor" (Shukla et al. 2014). This region, also known as the finger loop, recognizes the

intrahelical cleft of the active GPCR, inserts and thus prevents further G protein binding (Figure 8) (Chen et al. 2018; Kang et al. 2015).

The dual binding mode of the arrestin is characterized by a high affinity for the GPCR and is also referred to as the core complex. It represents the classical homologous desensitization accompanied by blockade of further GPCR signaling (Wilden 1995; Gurevich and Gurevich 2019). It should be mentioned that for some receptors, the binding of arrestin can also be observed by only one of the two mechanisms (Drube et al. 2022). For example, it is reported that arrestins only bind to the phosphate residues of a GPCR without insertion of the finger loop (hanging complex), thus still allowing potential G protein coupling (Thomsen et al. 2016; Cahill et al. 2017; Nguyen et al. 2019). Conversely, arrestin binding can also occur at unphosphorylated receptors (Drube et al. 2022). Nevertheless, the dual mechanism seems to be the conventional binding mode for arrestins to the active GPCR (Gurevich and Gurevich 2019).

However, desensitization of the GPCR does not end the inhibitory effect of arrestins on the GPCR signaling cascade. Upon coupling to the GPCR, arrestins themselves undergo a conformational change characterized by the release of their C-terminus (C-tail) and a 20° rotation of the N- and C-domains relative to each other (Figure 8, right) (Zhuo et al. 2014; Shukla et al. 2013; Latorraca et al. 2018). This is generally considered to be the hallmark of arrestin activation. In the case of non-visual arrestins (β-arrestins), the now free C-terminus additionally contains binding sites for proteins of the endocytosis apparatus (AP-2 and clathrin), which initiate the internalization of the receptor (see Figure 7) (Goodman et al. 1996; Laporte et al. 1999; Kim and Benovic 2002). The β-arrestin either diffuses away from the GPCR before internalization (class A receptor) or is internalized together and tightly bound to the receptor (class B receptor) (see Figure 7) (Zhang et al. 1999; Oakley et al. 2000). Once internalized the GPCR is no longer available for its external ligand. Desensitization and internalization are impressive examples of how β-arrestins interrupt the GPCR signaling cascade and protect the cell from overstimulation. β-arrestins represent the key players in “switching off” the receptor.

In addition to exposing regions for proteins of the endocytic machinery, β-arrestins also contain binding sites that can be recognized by a variety of other proteins (Chen et al. 2018; Peterson and Luttrell 2017). For β-arrestin1 and 2, more than a hundred interaction partners bound by active and/or inactive β-arrestins have been described (Xiao et al. 2007). In this

way, they act as adaptor proteins, a function that allows them to act as scaffolds, bringing proteins of a cascade into spatial and temporal proximity and thus modulating the course of the signaling cascade (Gurevich and Gurevich 2014).

Taken together, four ubiquitous GRKs (GRK2/3/5/6) and two non-visual arrestins (β-arrestin1/2) coordinate and modulate more than 800 GPCRs in the human body (Drube et al. 2022). They are critical regulators of the GPCR-G protein signaling cascade.

We now have an understanding of the function of a GPCR and its key interactors. Furthermore, we have highlighted the central role of GPCRs in the control and coordination of important physiological systems. One such system is the focus of this thesis and the following chapter.

## 1.5 RAS - Renin-Angiotensin System

The renin-angiotensin system (RAS) or renin-angiotensin-aldosterone system (RAAS) plays a key physiological role in the regulation of our body's circulatory system (Lüllmann et al. 2016). On the one hand, it effectively controls blood volume through its function as the main regulator of sodium and water balance. On the other hand, it has a direct effect on the vascular system by modulating vascular tone and integrity. Both effects (increase in blood volume and vascular tone) contribute decisively to the maintenance of normal blood pressure (Figure 9) (Karnik et al. 2015; Lüllmann et al. 2016).

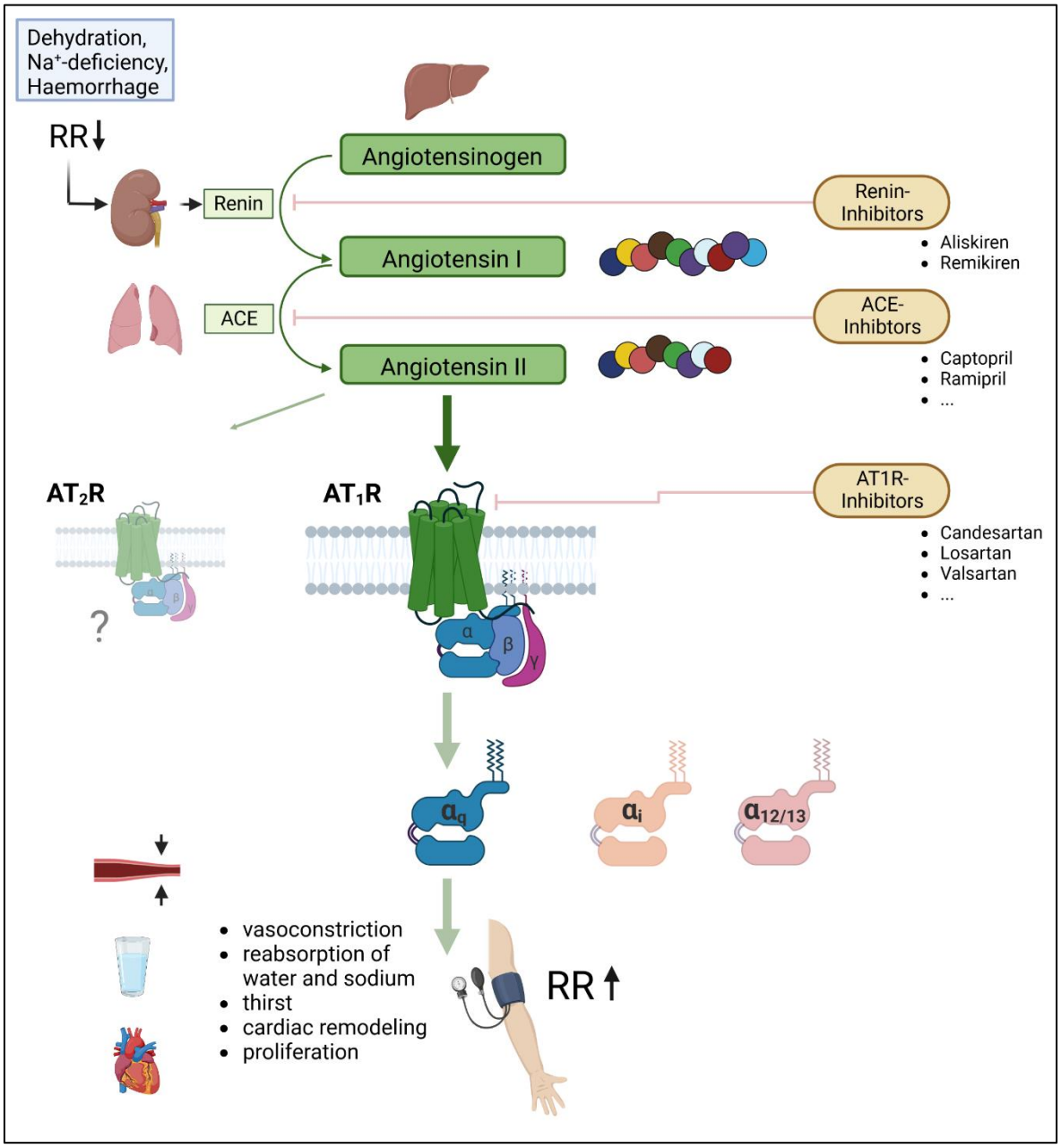


***Figure 9: Classical, canonical RAS cascade:***

*The formation of the main effector, the AngII octapeptide, is formed by a proteolytic cascade via two enzymatic cleavages. Renin, originating from the kidney, cleaves angiotensinogen to AngI. In case of pathological overactivation of the RAS, renin can be inhibited by renin inhibitors such as aliskiren (Staessen et al. 2006). AngI is finally converted to AngII by the angiotensin-converting enzyme (ACE), which is mainly expressed in the lung. Pharmacological intervention at this level is provided by the class of drugs known as ACE inhibitors. Examples are ramipril, enalapril, etc. (Kester 2012). AngII binds to the AT1R after formation by ACE and mediates its classical effects such as elevation of blood pressure and water- and electrolyte-reabsorption. AT1 receptor blockers ("sartans") inhibit the interaction of AngII with the receptor. Figure was created with "BioRender.com".*

The system involves a peptide cascade triggered by a drop in blood pressure or blood volume (or sodium levels). This stimulates specialized cells of the juxtaglomerular apparatus in the kidney, which then release the protease renin into the bloodstream (Figure 9, left). This is where renin meets the precursor protein angiotensinogen secreted by the liver. Renin cleaves the ten amino acid long angiotensin I (decapeptide) from angiotensinogen. Angiotensin I itself is biologically inactive and is in turn cleaved by another enzyme in the cascade, the angiotensin converting enzyme (ACE), to the eight amino acid long (octapeptide) **angiotensin II (AngII)**. Angiotensin II represents the main effector of the cascade (Figure 9) (Ferrario 2006). AngII binds to its main receptor, a GPCR called angiotensin II type 1 receptor (**AT1R**), on the target cells and mediates through this receptor the classical RAS effects such as vasoconstriction and water-/sodium-reabsorption. The resulting increase in blood pressure then counteracts the initiation of the cascade (the decrease in blood pressure). The AngII/AT1R axis is also referred to as the classical, canonical RAS signaling axis (Ferrario and Mullick 2017).

Therefore, the maintenance of the balance of this axis is of great importance for the hemodynamic stability and the regular physiology of the renal and cardiovascular systems (Higuchi et al. 2007; Pacurari et al. 2014; Karnik et al. 2015). An imbalance of the AngII/AT1R axis leads to fatal pathological changes. For example, sustained overactivation of the AT1R leads to persistently elevated blood pressure and vascular remodeling, which can ultimately cause diseases such as arterial hypertension, thrombosis, atherosclerosis, heart failure, diabetic nephropathy, and many others (Ferrario 2006; Muñoz-Durango et al. 2016; Pacurari et al. 2014; Karnik et al. 2015). For this reason, drugs have been developed that specifically inhibit the RAS. These drugs act at the three levels of the system to prevent either the generation of AngII or its interaction with the AT1R (Figure 9, right). For example, AT1 receptor blockers (so-called "sartans" such as losartan, candesartan, etc.) prevent the

binding of AngII to the AT1R and thus signaling. “Sartans” are antagonists or inverse agonists at the AT1R (Michel et al. 2013; Ishimitsu et al. 2010; Unal and Karnik 2014).

In addition to this endocrine (systemic) RAS, in which AngII circulates through the bloodstream, other local RAS, also called tissue RAS, have been described (Paul et al. 2006; Karnik et al. 2015). Here, AngII is produced locally and acts directly on neighboring cells (paracrine) or on the cell itself (autocrine) (see also Figure 1). In addition to tissues and organs such as the heart, adipose tissue, kidney and pancreas, the existence of a brain RAS and its involvement in some CNS diseases is suspected (Rocha et al. 2021; Mohite et al. 2020; Cassis et al. 2008; Leung and Chappell 2003; Sadoshima et al. 1993). The RAS thus plays a central role not only in blood pressure regulation, but also in the maintenance of organ function (Muñoz-Durango et al. 2016).

AngII mediates its effects through GPCRs on the target cells, the angiotensin II receptors. In humans, there are two types of angiotensin II receptors: AT1R and AT2R (angiotensin II type 1 and type 2 receptors, respectively). While both bind AngII with high affinity, they share a low sequence homology of 34% (Singh and Karnik 2016). The clear physiological function of AT2R is still controversial, as it does not exhibit classical GPCR signaling at the cellular level (Porrello et al. 2009; Turu et al. 2006). Moreover, its expression decreases progressively after birth, so that only low levels of the receptor can be detected in adults (Ozono et al. 1997).

The AT1R, on the other hand, is the main receiver of the AngII signal. It forms with AngII the AngII/AT1R axis and occupies the key position in the RAS. It will be discussed in more detail below.

### *1.5.1 Angiotensin II type 1 receptor (AT1R)*

The AT1R is found in a large number of tissues in the human body. These include the heart, kidney, and blood vessels, as well as other cell types of the endocrine, hepatic, and neuronal systems (Gasparo et al. 2000). In the human genome, there is exactly one gene (*AGTR1)* on chromosome 3 that encodes the AT1R. Rodents have two genes (*Agtr1a* and *Agtr1b*). The AT1a- and AT1b-receptors encoded by these genes differ in tissue distribution and transcriptional regulation, but are pharmacologically and functionally identical (Gasparo et al. 2000; Kakar et al. 1992; Chen et al. 1997).

The vast majority of all classical RAS effects are mediated by the AT1R (AngII/AT1R axis) (Dinh et al. 2001; Audoly et al. 2000). Activation of the receptor by AngII leads to the characteristic effects on blood pressure and water balance, resulting in effects such as vasoconstriction (vasculature), increase in cardiac contractility (heart), electrolyte- and water-reabsorption (kidney), aldosterone release (adrenal gland), CNS effects such as thirst (brain), and activation of other blood pressure regulating systems (sympathetic and vasopressin systems) (Singh and Karnik 2016; Gasparo et al. 2000). As mentioned above, permanent AT1R overactivation has enormous pathological consequences within the cardiovascular system. These include prolonged increased vascular tone, cardiac remodeling and hypertrophy, altered vascular reactivity and platelet activation as well as vascular inflammation (Forrester et al. 2016; Jurewicz et al. 2007; Putnam et al. 2012; Kim and Iwao 2000). Taken together, AT1R overstimulation promotes the development of diseases such as heart failure, hypertension, atherosclerosis, thrombosis, renal insufficiency and insulin resistance (Capric et al. 2021; Singh and Karnik 2016; Tóth et al. 2018b). AT1 receptor antagonists (see above) have therefore been the cornerstone of treating these diseases for decades (Ferrario and Mullick 2017; Michel et al. 2013; Ishimitsu et al. 2010; McMurray et al. 2010).

The AT1R is a *GPCR*, which belongs to the rhodopsin subfamily within the GPCR superfamily (Tóth et al. 2018b). All general tree features of this family are found in the structure of the 359 amino acid long receptor. It possesses the typical extracellular N-terminus, the intracellular C-terminus, and the intervening seven transmembrane domains (7TM) connected by three intracellular- and extracellular-loops (ICL1-3/ECL1-3) (Figure 10).

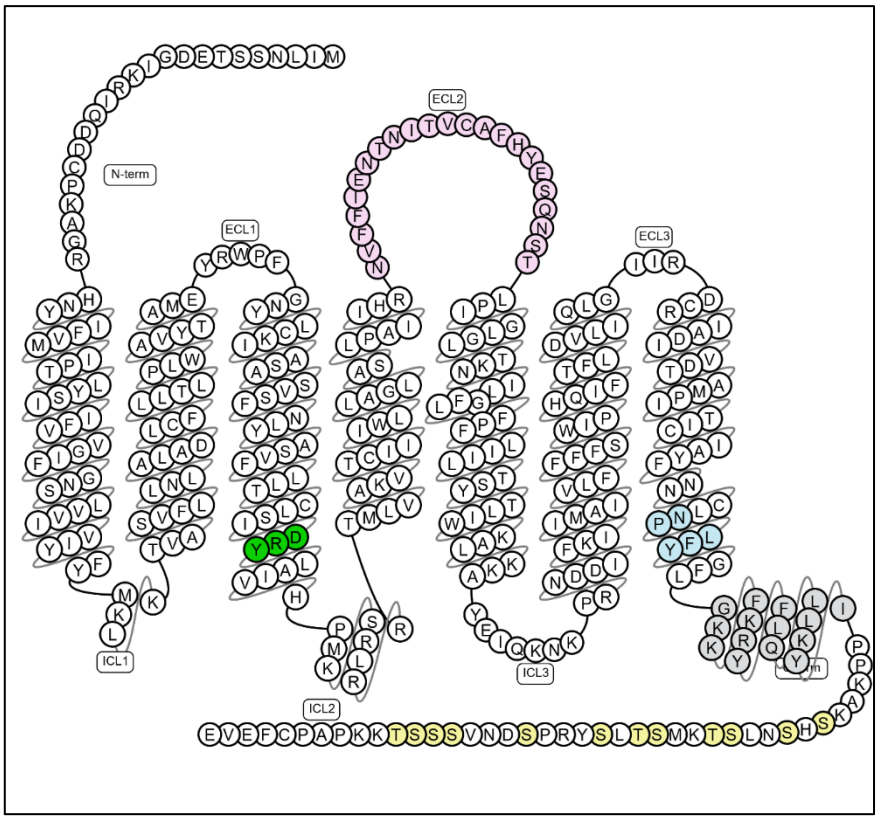


***Figure 10: Structure of the human AT1R (from GPCRdb):***

*Snake plot of the 359 amino acid long receptor showing the characteristic seven transmembrane helices connected by ICL1-3 and ECL1-3. Important motifs within the AT1R are highlighted: ECL2 with epitope for autoantibodies (pink), important activation sites such as the DRY and NPXXY motifs (green and light blue), helix VIII (gray) and the serin-/threonine-residues as important phosphorylation sites for protein kinases such as GRKs (yellow).*

Structural insights into the AT1R have increased tremendously over the last 30 years. Initially, a plethora of mutagenesis studies (targeted mutation of individual amino acids in the AT1R and subsequent study of the biological consequences) provided broad insights into the mechanisms of AngII binding, activation and internalization of the receptor (Inoue et al. 1997). The knowledge of the structural aspects of the AT1R-ligand interaction was further improved by the first crystal structures of the inactive AT1R in combination with the antagonist ZD7155 or the inverse agonist olmesartan (Zhang et al. 2015b; Zhang et al. 2015a). Shortly thereafter, another breakthrough was the successful crystallization of the active AT1R coupled to the agonist (AngII or AngII-analog) (Wingler et al. 2019; Wingler et al. 2020).

The AT1R has a unique binding pocket that extends from the receptor core (central residues in TMs 2/3/7) to the extracellular domains (Figure 11).

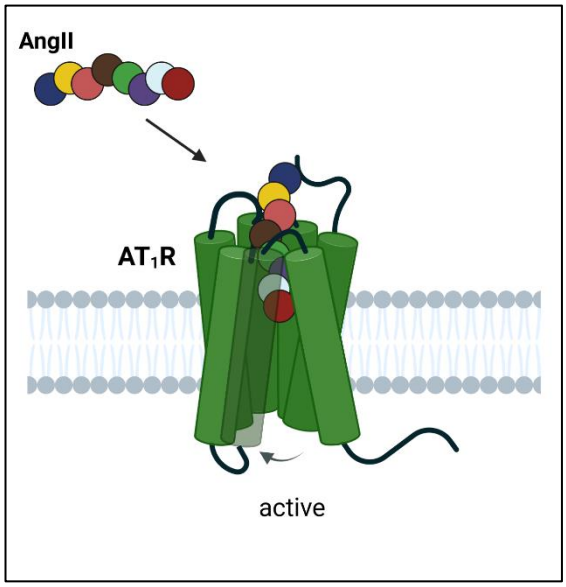


***Figure 11: Schematic illustration of the AngII-AT1R interaction:***

*Angiotensin II inserts its C-terminal phenylalanine residue (red sphere) deeply into a binding pocket of the AT1R. Interaction of the aromatic phenylalanine with central elements in the AT1R is responsible for full activation of the AT1R (see text). Figure was created with "BioRender.com".*

In the active AT1R, the AngII peptide inserts its C-terminus, the amino acid phenylalanine (Figure 11, red sphere), into the base of the pocket, while its N-terminus projects extracellularly and interacts with ECL2 and the N-terminus of the AT1R. The interaction of the phenylalanine of AngII with the receptor at the base of the binding pocket is thought to be responsible for full activation of the AT1R (Wingler and Lefkowitz 2020). The active AT1R is characterized by classical GPCR activation features, such as the movement of TM6 (see Figure 11 and 3). A rearrangement of the two sequence motifs DRY (TM3) and NPXXY (TM7) (see Figure 10, green and light blue), typical for activated GPCRs, can also be detected in the active AT1R (Wingler et al. 2020; Wingler and Lefkowitz 2020; Wingler et al. 2019).

The AT1R has characteristic features. Its ECL2 is particularly prominent and is considered an epitope for autoantibodies in pre-eclampsia and rheumatic diseases (Xia and Kellems 2013; Unal et al. 2012; Yue et al. 2022). The C-terminus of the receptor forms a helical structure, also known as helix VIII (Figure 10, grey). In contrast to other GPCRs, this helix does not have a palmitoylation site (Escribá et al. 2007), is extremely flexible (Wingler et al. 2019), and interacts with certain anionic membrane lipids, thus influencing the AT1R conformation (Hirst et al. 2015).

The C-terminus contains numerous serine- and threonine-residues (Figure 10, yellow) that are phosphorylated by protein kinases (GRKs and PKC) and is thus essential for the regulation of receptor desensitization and internalization (Hunyady et al. 1994b; Smith et al. 1998).

*AT1R Signaling:*

AngII binding leads to activation of the AT1R and recruitment of heterotrimeric G proteins. After GDP-GTP exchange, the heterotrimer dissociates as described into the Gα- and Gβγ-subunits, which then elicit the downstream signaling cascade by coupling to effector proteins (Figure 12 and 6).

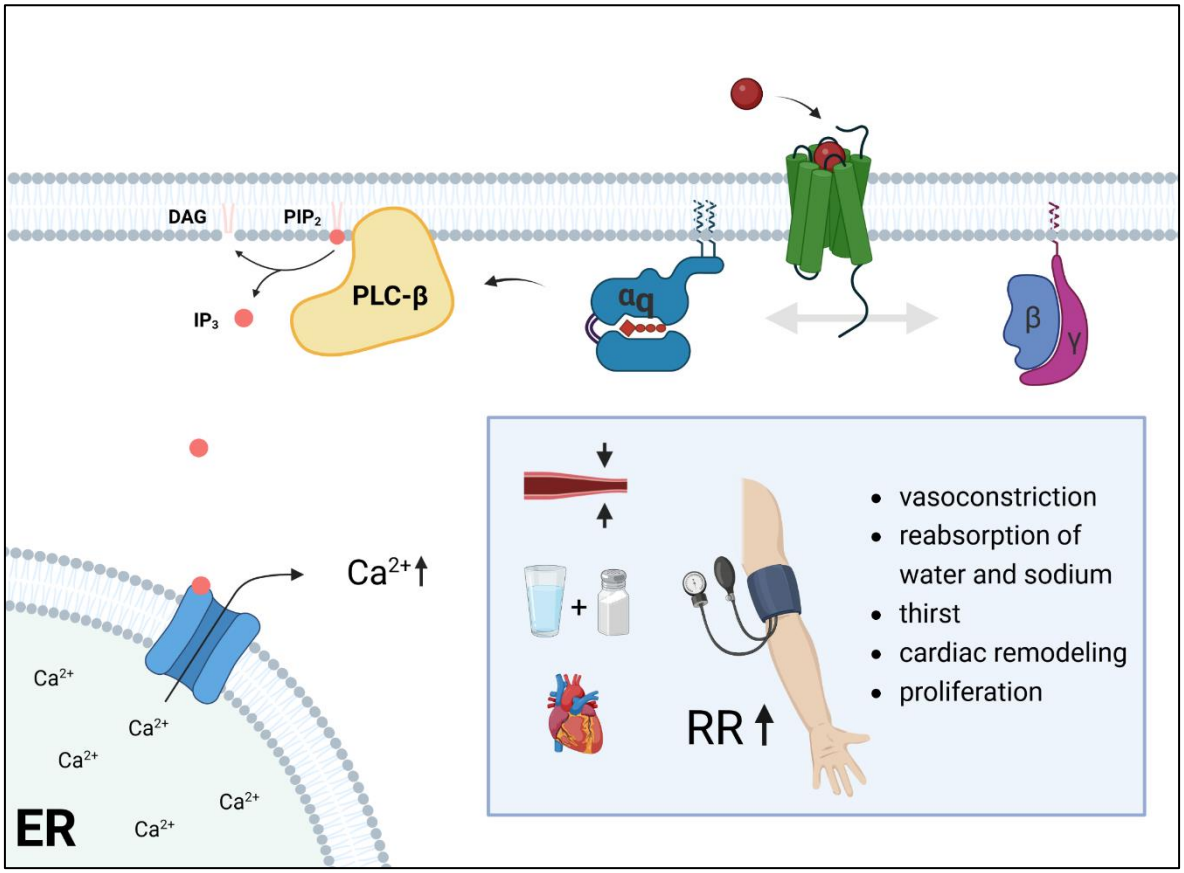


***Figure 12: AngII-AT1R-Gα$_q$ axis:***

*AngII stimulation of the AT1R predominantly leads to the activation of heterotrimeric G proteins of the Gα$_q$-family, which are mainly responsible for the classical outcomes of the RAS (blue box). Figure was created with "BioRender.com".*

The AT1R can interact with not only one but several G protein classes including Gα$_q$ , Gα$_i$ and Gα$_{12/13}$, of which Gα$_q$-proteins are preferentially activated (Saulière et al. 2012). Activation of Gα$_q$ results in PLC-β-mediated hydrolysis of $PIP_2$ to DAG and $IP_3$ (see also Figure 6 and 9). $IP_3$ releases calcium ions from intracellular stores, which through modulation of numerous calcium-dependent proteins, controls biochemical processes such as contraction of vascular and cardiac myocytes, secretion of second messengers, etc. (Figure 12) (Hunyady and Catt 2006). DAG, formed alongside $IP_3$, activates a number of PKC

isoforms, alone or in concert with $IP_3$, which in turn phosphorylate and regulate various target structures (Tóth et al. 2018b). In the AngII-AT1R axis, $G\alpha_q$-mediated physiological effects play a predominant role; therefore, in a broader sense, it is also referred to as the *AngII/AT1R/G$\alpha_q$* axis (Figure 12). In addition to the classical activation by the endogenous ligand AngII, the AT1R can also be activated by autoantibodies or mechanical stretch (Xia and Kellems 2013; Hong et al. 2017; Schleifenbaum et al. 2014; Hunyady and Turu 2004). Furthermore, interactions of the AT1R with other receptors, such as dimerization and transactivation, have been described (Nishimura et al. 2016; AbdAlla et al. 2000; Gyires et al. 2014; Forrester et al. 2016; Tóth et al. 2018b).

AT1R activation is immediately followed by the aforementioned desensitization and internalization processes (see Figure 7). GRKs of the GRK2- and GRK5- families recognize the active AT1R and phosphorylate it at its C-terminus (homologous phosphorylation). It is discussed whether the two GRK families lead to different phosphorylation patterns at the AT1R-C-terminus (barcode theory, see below), each inducing different β-arrestin conformations (Smith and Rajagopal 2016). Also, PKCs activated by other receptors (Tóth et al. 2018a) or by the AT1R-$G\alpha_q$ pathway, phosphorylate the C-terminus (heterologous phosphorylation) (Qian et al. 1999). After phosphorylation, β-arrestin1 or -2 bind to the AT1R, blocking further activation of heterotrimeric G proteins (desensitization) and subsequently initiating clathrin-mediated endocytosis (internalization). The interaction of the phosphorylated AT1R-C-terminus with the β-arrestin protein is so strong that the receptor is internalized with the bound β-arrestin (class B receptor) (see Figure 7) (Oakley et al. 2000; Wei et al. 2004). Inside the cell, the AT1R-β-arrestin complex is either lysosomally degraded (down-regulation) or returned to the plasma membrane after a certain period of time (recycling) (see Figure 7) (Tóth et al. 2018b).

However, β-arrestins recruited to the AT1R do not only fulfill desensitization and internalization tasks, but they also have the ability to bind a diverse repertoire of proteins (scaffold function) (Kendall and Luttrell 2009). Known binding partners are kinases of the MAPK family (mitogen-activated protein kinases), which have substantial impact on cell growth and proliferation. A classic example is the ERK1/2 cascade (extracellular signal-regulated kinases 1/2) (Figure 13).

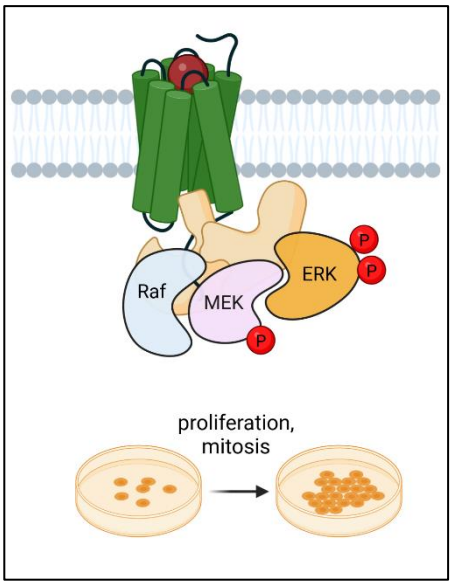


***Figure 13: β-arrestins can interact with ERK1/2 cascade members:***

*Illustration of β-arrestin coupled to the AT1R after AngII treatment. β-arrestins can act as a scaffold and bind a variety of proteins; here all three members (RAF, MEK, ERK) are bound by β-arrestin. The number of phosphates indicates the serial kinase cascade from left to right. Figure based on (Gurevich and Gurevich 2019). Figure was created with "BioRender.com".*

In this cascade, the three kinases (c-Raf, MEK1/2 and ERK1/2) are connected in series (Jean-Charles et al. 2017). Activation of c-Raf via MEK1/2 ultimately leads to phosphorylation and activation of ERK1/2, which can then trigger "mitogenic" effects (Luttrell et al. 2001). β-arrestins are reported to bind all three kinases (scaffold function) and thus influence the cascade (Gurevich and Gurevich 2019).

### *1.5.2 β-arrestin-dependent signaling at the AT1R*

Upon AngII stimulation, the AT1R activates heterotrimeric G proteins that initiate their specific signaling cascades. G proteins act as signal transducers of the generated cell response. Simultaneously recruited β-arrestins can modulate the G protein-elicited signal and, most importantly, initiate the termination of the signaling cascade by desensitizing and internalizing the receptor (Hunyady and Catt 2006).

In this classical GPCR-G protein signaling principle, G proteins are the sole signal transducers. In the early 2000s, the field of research changed dramatically, as β-arrestins were no longer ascribed only scaffolding and internalization functions, but β-arrestins themselves were thought to function as signal transducers (Rankovic et al. 2016; Smith and Rajagopal 2016). This phenomenon, termed *β-arrestin-dependent signaling* (β-arrestin-biased signaling), is characterized by the fact that upon GPCR activation, β-arrestins can initiate their own signaling cascades independent of G proteins. Due to its physiological and pharmacological relevance, the AT1R is one of the most studied GPCRs with regard to β-arrestin-biased signaling (Turu et al. 2019). In 2002, *Seta et al.* found that an AT1R mutant that had been described as unable to couple G proteins (due to mutations in its ICL2) was still able to activate the ERK1/2 cascade after AngII stimulation. They concluded that AngII may induce signaling mechanisms at the AT1R that are independent of G proteins (Seta et

al. 2002). “G protein-independent” signaling was further investigated using AT1R mutants that were still capable of recruiting β-arrestins, but did not show any detectable G protein activation (Gáborik et al. 2003). One of these mutants, and the most important one, is AT1R-DRY, in which the highly conserved DRY motif of the AT1R is altered (Asp125/Arg126 to Ala) (see also Figure 10) (Ohyama et al. 1992; Ohyama et al. 2002). AngII stimulation of this mutant did not show measurable G protein activation, but still resulted in β-arrestin recruitment and activation of the ERK1/2 cascade (Wei et al. 2003). Furthermore, siRNA-mediated knockdown of β-arrestin2 abolished the ERK1/2 response, leading to the conclusion that β-arrestins initiated the ERK1/2 cascade. This β-arrestin-dependent, G protein-independent ERK1/2-activation thus created a novel signaling paradigm in which β-arrestins and G proteins independently mediate their own signaling cascades upon GPCR stimulation (Lefkowitz et al. 2006).
ERK1/2-activation subsequently became the preferred measure for studying β-arrestin-dependent signaling (Gurevich and Gurevich 2018).

The new paradigm of β-arrestin-biased signaling was even further expanded. With the simultaneous discovery of so-called “**biased ligands**”, a whole new field of research in molecular pharmacology has been developed (Turu et al. 2019; Lefkowitz and Shenoy 2005; Lefkowitz 2007). Originally, ligands were characterized as agonists, inverse agonists, and antagonists (see 1.2) (Weis and Kobilka 2018). Here, the classical “balanced” agonist causes G proteins and β-arrestins to be activated to the same extent after GPCR binding (Figure 14 (B)).

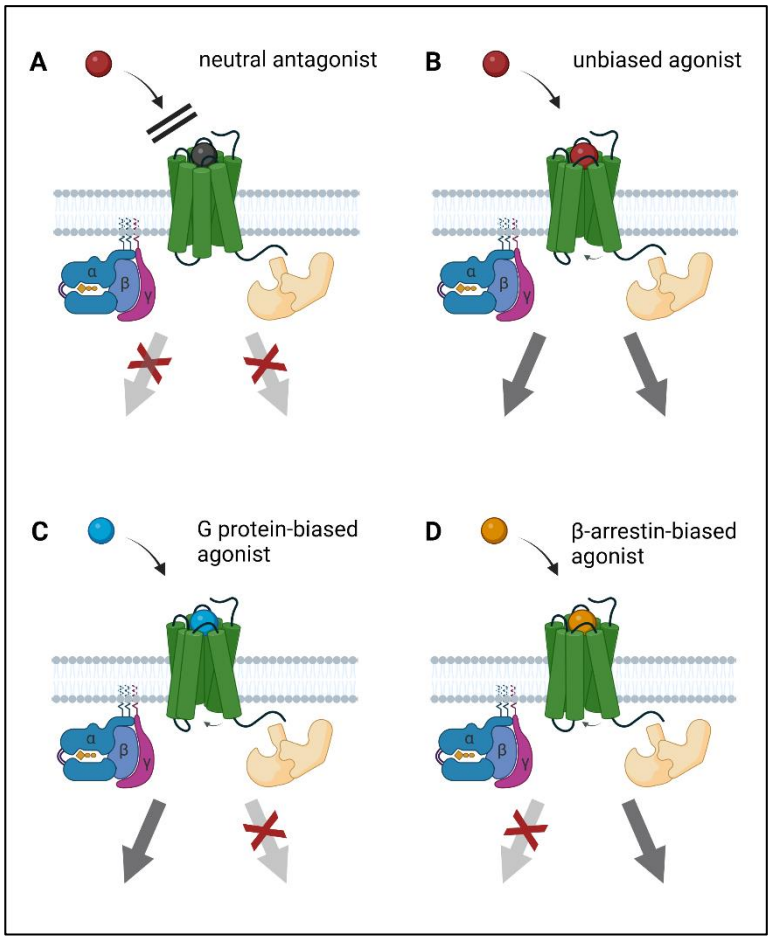


***Figure 14: Biased agonism at the AT1R:***

*(A) Neutral antagonists, such as some AT1R blockers, occupy the AT1R binding site for AngII but do not activate the receptor. (B) Unbiased agonists (such as AngII) activate the AT1R, accompanied by a balanced G protein and β-arrestin activation. (C) G protein-biased ligands are thought to promote an AT1R-conformation that favors G protein activation with less or no β-arrestin activation. (D) β-arrestin-biased ligands at the AT1R (such as TRV120027 = TRV027, SII, Ang1-7) are reported to induce β-arrestin-mediated signaling cascades without or less G protein activation. Figure modified from Whalen et al. (Whalen et al. 2011).*

*Figure was created with "BioRender.com".*

Biased ligands instead lead to the activation of either G proteins (G protein-bias) or β-arrestins (β-arrestin-bias) at the same GPCR (Figure 14 (C and D)). They thus "bias" the initiated signaling cascade in favor of a particular transducer. This ligand phenomenon is also known as **functional selectivity** or **biased agonism** (Chang and Bruchas 2014; Smith and Rajagopal 2016; Jean-Charles et al. 2017).

### *Excursus: What theories underlie biased agonism?*

GPCRs are highly dynamic proteins that are able to adopt multiple conformations (Wingler and Lefkowitz 2020). Depending on the nature of the ligand, the equilibrium of the receptor-state can be shifted towards a particular conformation, which may then differ in its interaction with heterotrimeric G proteins or β-arrestins (*theory of differential GPCR conformations*) (Weis and Kobilka 2018). Recently obtained crystal structures of the AT1R in association with either the "balanced" endogenous agonist AngII or β-arrestin-biased

ligands showed that AngII induced stronger rearrangements in the AT1R than the β-arrestin-biased agonists (Wingler et al. 2020). In addition, the interaction with β-arrestins is affected by the phosphorylation of the AT1R-C-terminus by GRKs and protein kinase C (Drube et al. 2022). The AT1R forms a stable binding to β-arrestin (class B receptor) (Oakley et al. 2000). However, the phosphorylated serine-/threonine-residues of the C-terminus determine not only the binding strength but also the subsequent conformational change of the recruited β-arrestin (Zhou et al. 2017; Mayer et al. 2019). This gave rise to the so-called *barcode theory*, which states that the phosphorylation pattern of the receptor can induce different conformations in the β-arrestin protein, which in turn may lead to different functional outcomes (Matthees et al. 2021; Yang et al. 2015; Lee et al. 2016; Tobin et al. 2008; Butcher et al. 2011). Expression level, tissue distribution, and temporal and spatial localization of the kinases thus affect the resulting receptor phosphorylation, adding another level of complexity to the system (Matthees et al. 2021). The theory of differential GPCR conformations induced by biased ligands and the barcode theory cannot be considered separately, but are interdependent processes (Turu et al. 2019).

In addition to the "non-G protein activating" AT1R mutants mentioned above, β-arrestin-biased agonists became the tool of choice to explore G protein-independent, β-arrestin-mediated signaling (Takezako et al. 2017). In the case of the AT1R, one such β-arrestin-biased agonist was designed and synthesized: the AngII-analog **S**ar$_1$-**I**le$_4$-**I**le$_8$-AngII (**SII**) (Figure 15) (Holloway et al. 2002). Compared to AngII, three amino acids have been substituted. While position 1 is replaced by the protease-stable amino acid sarcosine (Sar$_1$), positions 4 and 8 are each changed to an isoleucine (Ile$_4$ and Ile$_8$). Most importantly, SII has a critical mutation at position 8, the C-terminal phenylalanine of AngII (Figure 15, highlighted in red). This has been shown to be the key residue for G protein activation by the AT1R (Balakumar and Jagadeesh 2014). For SII, no G protein activation could be detected at the AT1R, but the peptide still led to the recruitment of β-arrestins to the receptor and the activation of the ERK1/2 cascade (Wei et al. 2003). Notably, reported β-arrestin-dependent ERK1/2-activation by SII resulted in a slower and more persistent ERK1/2-phosphorylation that was confined to the cytosol compared to unbiased AngII (Ahn et al. 2004a; Aplin et al. 2007a, 2007b). Thus, SII became a widely used tool to study β-arrestin-

dependent, G protein-independent signaling mechanisms at the AT1R (Lefkowitz and Shenoy 2005; Hansen et al. 2008).

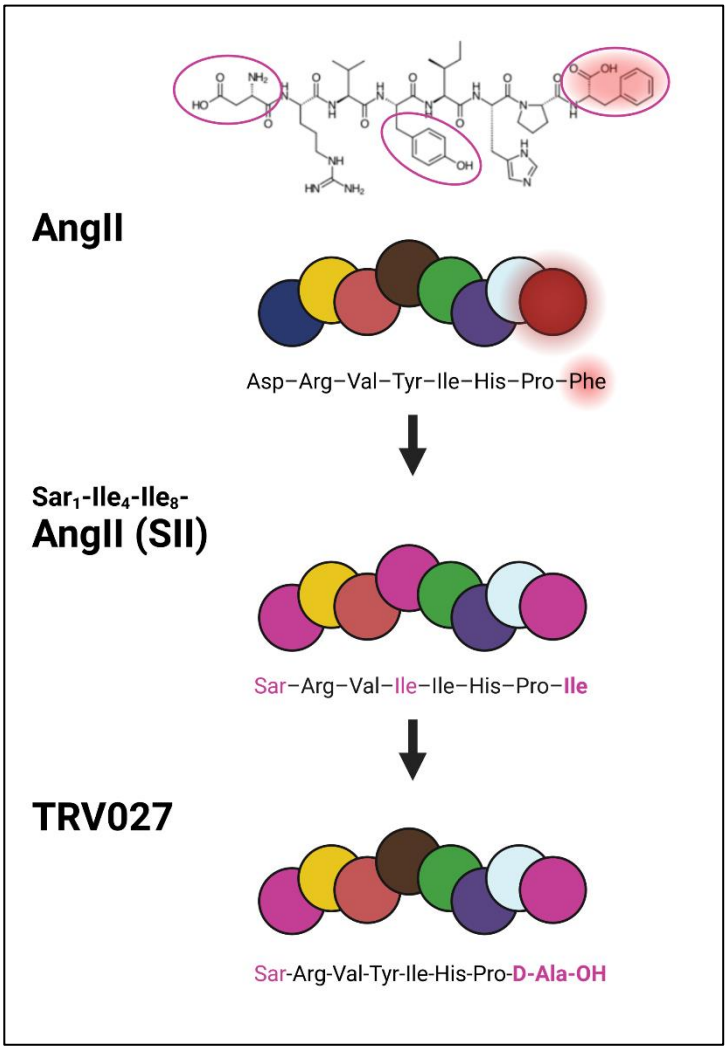


***Figure 15: Structure of AngII and the synthetic β-arrestin-biased analogs SII and TRV027:***

*AngII structure (top); the pink ovals indicate the mutated amino acids in the SII analog (middle). While the change at position 1 (aspartate to sarcosine) confers protection against proteases and increases affinity, all β-arrestin-biased analogs lack the bulky aromatic phenylalanine (highlighted in red) of AngII at position 8. This residue is critical for AT1R/Ga$_q$ -activation. SII and TRV027 have smaller residues there. This has been described to change the ligand property towards β-arrestin biased agonism. Figure was created with "BioRender.com".*

β-arrestin-dependent signaling examined by SII was described to not only differentially activate the ERK1/2 cascade, but also lead to distinct phosphorylation events and protein synthesis downstream of the AT1R (Xiao et al. 2010; Dewire et al. 2008). In addition, SII has been reported to induce its own inotropic and lusitropic effects and to promote intact proliferation in mouse cardiomyocytes (Rajagopal et al. 2006; Aplin et al. 2007a). Taken together, these studies defined a novel role for β-arrestins as independent signal transducers in the heart with beneficial cardioprotective signaling properties (Rakib et al. 2021).

Overactivation of the AngII/AT1R/G$\alpha_q$ axis of the RAS promotes the development of cardiovascular diseases including arterial hypertension, atherosclerosis, and cardiac hypertrophy (see 1.5.1). As mentioned above, AT1R blockers have become standard of care in the treatment of arterial hypertension and heart failure (Michel et al. 2013). While AT1R-

blockers prevent AngII binding to the receptor and thus do not allow any activation (see Figure 14 (A)), β-arrestin-biased ligands instead would still be able to elicit β-arrestin-mediated effects with no G protein involvement. Thus, SII has paved the way for the development of novel β-arrestin-biased peptides at the AT1R with possible new therapeutic potential to prevent detrimental $G\alpha_q$-activation while still allowing for cardioprotective β-arrestin-mediated signaling. Based on the SII structure, new β-arrestin-biased ligands for the AT1R were synthesized with higher affinity for the receptor and a comparable pharmacological profile (TRV027, TRV023) (Figure 15) (Rajagopal et al. 2011; Strachan et al. 2014; Tarigopula et al. 2015; Boerrigter et al. 2012). To date, the β-arrestin-biased compounds TRV027 and TRV023 have replaced the original SII as main tool for the study of β-arrestin-dependent signaling via the AT1R (Rankovic et al. 2016; Takezako et al. 2017; Smith and Rajagopal 2016; Jean-Charles et al. 2017). Regarding their structure, the β-arrestin-biased peptides share with SII that they have rather small residues instead of the aromatic phenylalanine in position 8 compared to endogenous AngII (see Figure 15). Because the phenylalanine in AngII is critical for AT1R and subsequent G protein activation (see 1.5.1), changes here are thought to be causative for a β-arrestin-biased ligand characteristic (Miura et al. 1999; Holloway et al. 2002; Balakumar and Jagadeesh 2014).

All β-arrestin-biased AngII analogs listed above are of synthetic origin. Fascinatingly, our body produces an *endogenous, natural* AngII-analog that combines the above characteristics: **Angiotensin 1-7 (Ang1-7).**

The Ang1-7 peptide is the centerpiece of an alternative branch of the renin-angiotensin system, often referred to as the *alternative RAS.*

## 1.6 Alternative RAS - Focus on angiotensin 1-7.

The renin-angiotensin system described above involves a typical peptide cascade in which the decapeptide AngI (Ang1-10) is released from the long precursor protein angiotensinogen by proteolytic cleavage. Finally, under ACE catalysis, AngI is converted to the octapeptide AngII (Ang1-8), the major effector of the classical RAS, which then binds to and activates its receptor, the AT1R. AngII and AT1R together form the classical ACE/AngII/AT1R axis, which is responsible for the characteristic effects of AngII on blood pressure and water-salt homeostasis (Gasparo et al. 2000).

While AngII was long thought to be the only biologically active metabolite of the cascade, evidence for other partially bioactive peptides within the RAS has increased over the last two decades (Santos 2014; Rukavina Mikusic et al. 2021). These peptides are mostly derived from AngI (Ang1-10) and AngII (Ang1-8), respectively, by alternative enzymatic routes (Santos et al. 2018). In this context, the term "alternative RAS" was coined, and Ang1-7 was identified as the midpoint of a second axis that exists in parallel to the classical AngII/AT1R pathway. Among other enzymes, Ang1-7 is predominantly formed from AngII by the carboxypeptidase ACE2 (angiotensin converting enzyme 2) which represents the critical interface between the classical and alternative RAS (Figure 16) (Patel et al. 2016).

***Figure 16: Classical RAS and alternative RAS (next page):***

*Angiotensin converting enzyme 2 (ACE2) represents the interface between the classical- and alternative-RAS axis. Ang1-7 (bold) is the main effector of the alternative RAS and is predominantly formed from AngII by ACE2. Other pathways of this peptide cascade also lead to Ang1-7 formation, such as direct formation from AngI by the peptidases: neprilysin (NEP), thimet oligopeptidase (TOP) and prolyl endopeptidase (PEP). In addition, an indirect pathway is the generation of Ang1-9 by ACE2 followed by ACE conversion to Ang1-7. The effects of Ang1-7 are mainly related to its actions through the MAS receptor, but the AT2R and MrgD have also been associated with Ang1-7. Ang1-7 was later shown to also act through the AT1R (bold arrow), but with unique signaling properties (see below). The alternative RAS, with Ang1-7 as the main effector, is able to counter-regulate a detrimental over-activated classical RAS by exerting cardioprotective, anti-fibrotic, vasodilatory and anti-inflammatory effects (Ferreira and Santos 2005; Santos et al. 2005; Ferrario 2011; Kucharewicz et al. 2000; Simões e Silva et al. 2013). Figure inspired by (Medina and Arnold 2019; Patel et al. 2016; McKinney et al. 2014). Figure was created with "BioRender.com".*

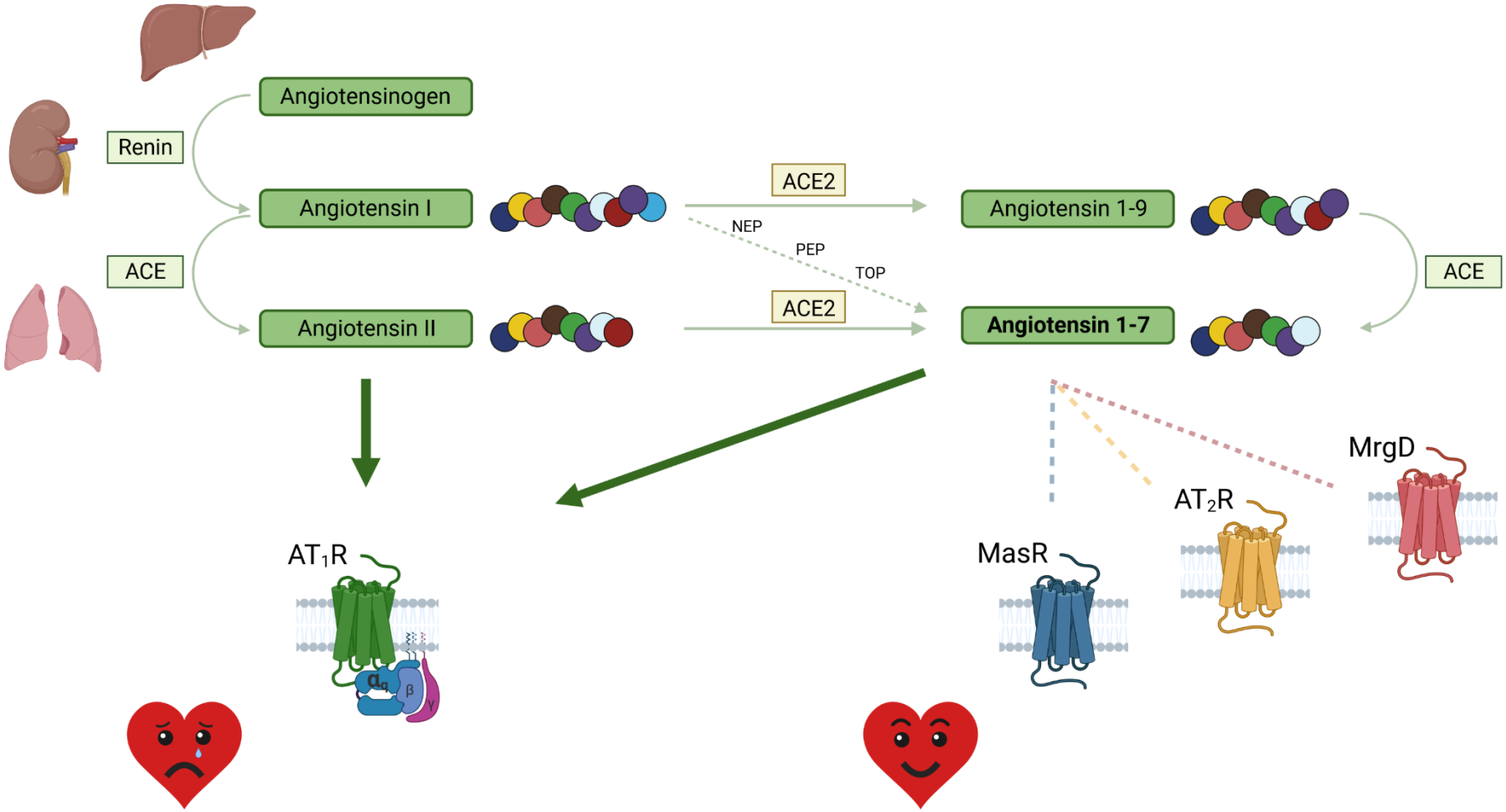


***Figure 16: alternative RAS axis***

Ang1-7 was first reported in the literature in 1968 as an inactive degradation product of AngII (Yang et al. 1968). Cardiovascular effects of the peptide, suggesting its own biological activity, were not discovered until 20 years later by *Campagnole-Santos et al.* (Campagnole-Santos et al. 1989). The identification of ACE2 as the major Ang1-7-forming enzyme and the subsequent discovery of the MAS receptor (see Appendix) as a potential endogenous receptor for Ang1-7 established the name of a new, *alternative RAS*, also called the ACE2/Ang1-7/MAS receptor axis (Santos et al. 2018; Santos 2019). Although the MAS receptor was originally proposed as the endogenous receptor for Ang1-7 (Santos et al. 2003), recent studies have failed to demonstrate a clear interaction of Ang1-7 with this receptor (see Appendix for more information) (Gaidarov et al. 2018). Considering that there are reports of additional receptors (AT2R and MrgD) that may be involved in the actions of Ang1-7, the complexity of the alternative RAS axis becomes even more apparent (see Figure 16) (Gembardt et al. 2008; Solinski et al. 2014; Souza et al. 2004; Ohshima et al. 2014). However, the IUPHAR Nomenclature Committee has previously decided not to list Ang1-7 as an endogenous ligand for these receptors due to the lack of complete conclusive data (Karnik et al. 2017). As a result, the characterization of potential Ang1-7 receptors is ongoing.

As mentioned above, the heptapeptide Ang1-7 is the core of the alternative ACE2/Ang1-7 axis. Of particular importance, the actions of Ang1-7 have been shown to be beneficial to the cardiovascular system (Figure 16) (Loot et al. 2002; Mori et al. 2014; Patel et al. 2012). Anti-hypertensive, anti-thrombotic, anti-fibrotic and anti-inflammatory effects are just a few of the many favorable properties reported for Ang1-7 in the literature (Medina and Arnold 2019; Iwai and Horiuchi 2009). Strikingly, these cardiovascular effects can counteract the detrimental consequences of an overactivated classical RAS, as seen in hypertension and other diseases (Bader 2013; Tesanovic et al. 2010; Bradford et al. 2010). For this reason, the alternative RAS is also considered to be the vaso/cardio-protective arm of the RAS, with Ang1-7 counterbalancing the potentially deleterious effects of the AngII/AT1R axis (Figure 16) (Iusuf et al. 2008; Zimmerman and Burns 2012).

The importance of an orderly interplay between the classical and the alternative axis of the RAS could not be more evident at the present time: The severe acute respiratory syndrome (SARS)-like coronavirus (SARS-Cov2) docks onto the ACE2 enzyme and uses it to enter its host cell (Figure 17) (Shang et al. 2020; Hoffmann et al. 2020). In this way, the virus

downregulates the presence of ACE2 enzymes on the cell surface (Figure 17). Due to the lack of free ACE2 enzymes, less AngII is converted to Ang1-7, resulting in increased AngII levels and decreased Ang1-7 (Rysz et al. 2021). This imbalance toward increased inflammatory, deleterious AngII and scarcity of protective Ang1-7 is associated with a more severe outcome and worse prognosis in Covid-19 (Issa et al. 2021; Carpenter et al. 2022; Henry et al. 2021; Rysz et al. 2021).

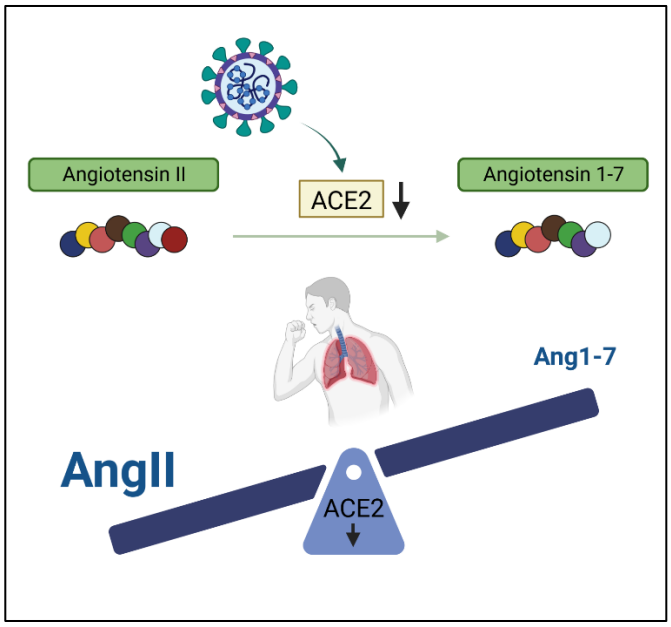


***Figure 17: Dysregulation of the RAS in Covid-19:***

*ACE2 deficiency due to viral infection leads to imbalance between potentially harmful AngII and protective Ang1-7 (Almutlaq et al. 2021). Figure was created with "BioRender.com".*

Despite the well-documented biological effects of Ang1-7 on a variety of organs in the human body, relatively little is known about the underlying molecular pharmacology of the peptide (Jiang et al. 2014). Only a few publications have focused on Ang1-7 signaling using cell-based approaches with controversial results (see above for the MAS receptor) (Santos et al. 2003; Gaidarov et al. 2018; Tirupula et al. 2014; Karnik et al. 2017).

Ang1-7 is so named because it differs from AngII (Ang1-8) only in the absence of the C-terminal amino acid phenylalanine (Figure 18) (Santos et al. 2018). As outlined earlier, this phenylalanine residue plays a crucial role in the AngII-mediated activation of the AT1R (Regoli et al. 1974; Noda et al. 1995; Miura et al. 1999). The synthetic analogs SII and TRV027 are modified with a smaller residue at this exact position (see Figure 15). Notably, these modifications are reported to confer a distinct signaling profile to the peptides that does not lead to classical G protein activation, but rather to β-arrestin recruitment and β-arrestin-dependent signaling (Violin et al. 2010; Violin et al. 2014). Furthermore, beneficial β-arrestin-dependent signaling, and the non-activation of deleterious $G\alpha_q$-proteins were

associated with the cardioprotective effects of SII and TRV027 (Rajagopal et al. 2006; Boerrigter et al. 2012; Boerrigter et al. 2011; Tarigopula et al. 2015).

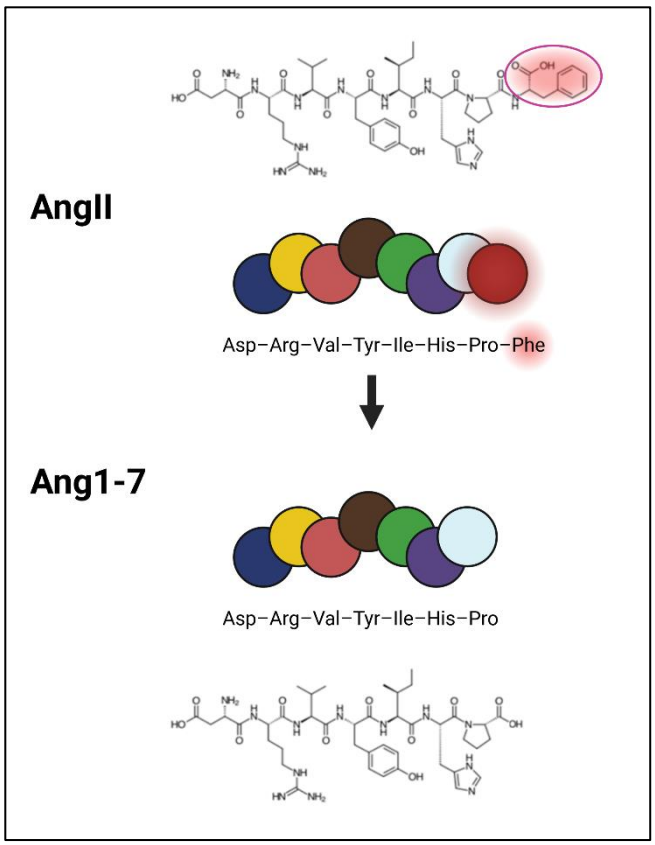


***Figure 18: Comparison of the structure of AngII and Ang1-7:***

*Ang1-7 (bottom) lacks the C-terminal aromatic phenylalanine of AngII (highlighted in red), which is critical for the AT1R-G protein activation and has been described as crucial for changing the ligand behavior toward β-arrestin-biased agonism (compare also with Figure 15). Figure was created with "BioRender.com".*

Based on the structural similarity of Ang1-7 to the synthetic peptides SII and TRV027, two groups set out to investigate whether the natural Ang1-7 could also exhibit β-arrestin-dependent signaling properties at the AT1R (Teixeira et al. 2017; Galandrin et al. 2016). Ang1-7 was shown to bind to the AT1R and compete with AngII for the receptor (Galandrin et al. 2016). Strikingly, Ang1-7 stimulation of the AT1R resulted in significant β-arrestin recruitment and ERK1/2-phosphorylation (Teixeira et al. 2017), without any detectable canonical G protein activation (Teixeira et al. 2017; Galandrin et al. 2016). Consequently, Ang1-7 was identified as an *endogenous* β-arrestin-biased agonist at the AT1R, leading to β-arrestin-dependent, but G protein-independent ERK1/2-activation. Moreover, this unique signaling profile of promoting β-arrestin-dependent signaling without activating the potentially deleterious $G\alpha_q$ axis is thought to contribute to the multiple beneficial cardiovascular effects of Ang1-7 (Figure 19) (Silva et al. 2020; Paz Ocaranza et al. 2020).

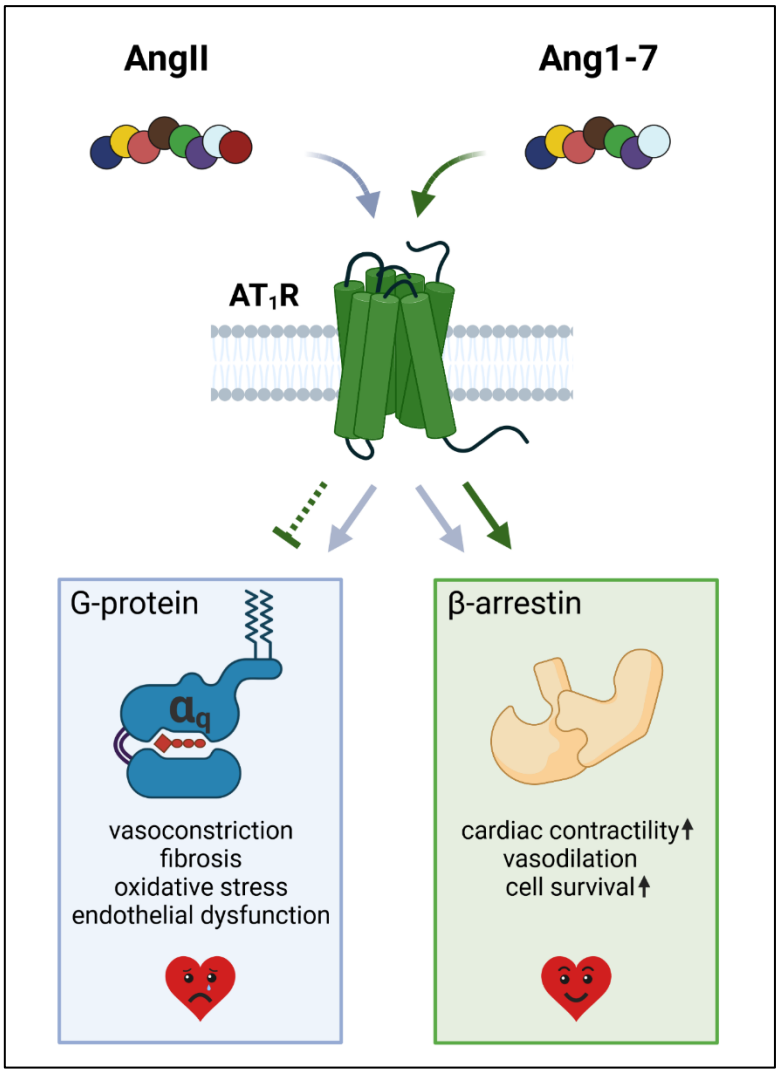


***Figure 19: The described mode of action of Ang1-7 at the AT1R:***

*Ang1-7 counteracts the deleterious AngII/AT1R/G$\alpha_q$ axis by (i) competing with AngII for the receptor, (ii) not activating potentially deleterious G$\alpha_q$-proteins, and instead (iii) inducing cardioprotective β-arrestin-mediated signaling. Figure was created with "BioRender.com".*

In recent years, however, findings from our group and others in the field have challenged the concept of G protein-independent, but β-arrestin-dependent signaling:

*The controversy over β-arrestin-biased signaling:*

Biased agonism at GPCRs has created a completely new pharmacological concept in which the nature of the ligand can lead to the selective activation of a specific signal transducer (functional selectivity) (see Figure 14). In this ligand bias paradigm β-arrestins and G proteins are considered to be independent signal transducers that elicit their own signaling cascades (Lefkowitz et al. 2006; Lefkowitz and Shenoy 2005; Rajagopal et al. 2006). In

addition, the separation of G protein vs. β-arrestin signaling has given rise to a novel therapeutic approach in which a biased ligand can be used to promote the desired effects while avoiding the undesired ones. Drug screening for biased ligands has therefore gained momentum for several years, focusing on GPCRs with central (patho-)physiological roles in the human body. A separate company specializing in the search for biased ligands as drug candidates, Trevena Inc., was founded in 2007. However, it was not until 13 years later that Trevena Inc. received the first and only approval to date for a biased agonist, oliceridine (TRV130/Olinvyk®), an opioid analgesic (Lambert and Calo 2020; Mullard 2020). Oliceridine was designed to act as a $G\alpha_i$-protein-biased agonist at the μ-opioid receptor (MOR), favoring the G protein-dependent analgesic effects while avoiding β-arrestin-mediated side effects (e.g., respiratory depression) (Dewire et al. 2013; Bohn et al. 1999).

However, this postulated mode of action was later challenged by other groups who found that opioid side effects are not a consequence of β-arrestin-dependent signaling (Kliewer et al. 2020). Furthermore, the improved side effect profile of oliceridine was shown to be due to its low intrinsic efficacy on all signaling pathways rather than a biased agonist mechanism (Gillis et al. 2020). This is in line with our group's recent publication on carvedilol, a beta-adrenergic receptor blocker (βAR) used in heart failure and after myocardial infarction (Benkel et al. 2022; Maack et al. 2000). Previously, carvedilol was considered a prototype β-arrestin-biased ligand at the $\beta_2$AR (Rankovic et al. 2016), and its ability to promote favorable β-arrestin signaling was suggested as a reason for its superiority over other β-blockers (Wisler et al. 2007). *Benkel et al.* found that instead of β-arrestin-biased signaling, all signaling cascades were driven by a low level of intrinsic heterotrimeric G protein activation (Benkel et al. 2022).

The new studies on oliceridine and carvedilol have shed more light on the detailed molecular mode of action of already approved drugs and further challenged the concept of β-arrestin-mediated signaling. Moreover, these findings follow several studies in recent years that took advantage of latest molecular pharmacological tools (like CRISPR/Cas9-technology etc.) that increasingly have questioned β-arrestins as independent signal transducers. For example:

(i) β-arrestins have been demonstrated to be dispensable for the initiation of $\beta_2$-adrenergic receptor signaling to ERK1/2, the traditional readout for β-arrestin-mediated signaling (O'Hayre et al. 2017; Luttrell et al. 2001)

(see 1.5.2 and Figure 13).

(ii) *Alvarez-Curto et al.* showed that ERK1/2-phosphorylation downstream the free fatty acid receptor 4 (FFA4) was completely $G\alpha_q$-dependent but not affected by the absence of β-arrestins (Alvarez-Curto et al. 2016).

(iii) Finally, our group was able to perform a comprehensive multi-GPCR study to distinguish between G protein-mediated and β-arrestin-mediated outcomes using HEK293 cell lines depleted of either active Gα proteins ("zero functional G") or β-arrestins ("zero arrestin") by CRISPR/Cas9 genome editing and pharmacological inhibition (Grundmann 2018). Of note, in the absence of active G proteins, neither phosphorylation of ERK1/2, nor cell morphological changes were detectable upon GPCR stimulation (Grundmann 2018). β-arrestin-mediated effects were shown to require the presence of active G proteins, suggesting that β-arrestins are not able to act as independent signal transducers.

As its name suggests, TRV027 is also a Trevena Inc. compound and, as mentioned above, has been developed as a synthetic β-arrestin-biased ligand at the AT1R (Figure 15 and 1.5.2). Its ability to promote β-arrestin-biased signaling at the AT1R has been widely associated with cardioprotective effects in animal models (Kim et al. 2012). Noteworthy, TRV027 is currently enrolled in the National Institute of Health ACTIV (**A**ccelerating **C**ovid-19 **T**herapeutic **I**nterventions and **V**accines) trial and is being investigated as a potential therapeutic agent in Covid-19 therapy. The idea is to counteract the Ang1-7 deficiency caused by loss of ACE2 and mimic the Ang1-7 effects (Figure 17): Namely, prevent AngII overstimulation of the classical RAS by binding to the AT1R and inducing the beneficial β-arrestin-mediated signaling instead of activation of the deleterious $G\alpha_q$-protein axis.

Taken together, this once again underscores the prominent role of the endogenous Ang1-7 peptide in our organism. Even more, given the controversy in the field, it makes Ang1-7 the ideal subject to better understand its intriguing, if still enigmatic, mode of action. For this reason, we decided to investigate whether the natural Ang1-7 peptide is a true $G\alpha_q$-protein-independent, β-arrestin-biased agonist at the AT1R and how it might contribute to its widely reported beneficial effects (on the cardiovascular system and in Covid-19).

# 2 Materials

## 2.1 Cell culture

### *2.1.1 Cell culture media - preparation and compositions*

***Table 2: Cell media/buffer and solutions***

| *Name* | *Company* | *Reference Number* |
|---|---|---|
| BSA (Bovine Serum Albumin), fatty acid free | Sigma-Aldrich | # A6003 |
| DMEM (1X) | Thermo Fisher Scientific | # 11965092 |
| OptiMEM™ | Thermo Fisher Scientific | # 31985062 |
| FBS/FCS (fetal bovine/calf serum) | PAN Biotech GmbH | # P30-3702 |
| G418 (100 mg/ml) | PAN Biotech GmbH | # P06-17200 |
| Penicillin/Streptomycin | PAN Biotech GmbH | # P06-07050 |
| Trypsin 0.05%/EDTA 0.002% in PBS | PAN Biotech GmbH | # P10-023100 |
| HBSS | Thermo Fisher Scientific | # 14175129 |
| HEPES (*N*-2-Hydroxyethylpiperazine-*N*'-2-ethane sulphonic acid) | Carl Roth GmbH & Co. KG | # HN77.4 |
| UltraPure™ DNase/RNase-Free Distilled Water | Thermo Fisher Scientific | # 10977049 |
| Gibco™ PBS (10X), pH 7.4 | Thermo Fisher Scientific | # 70011044 |
| S.O.C. Medium | Thermo Fisher Scientific | # 15544034 |

**Standard (HEK293) Medium:**

| *Constituent* | *Volume [ml]* | *Final concentration* |
|---|---|---|
| DMEM | 445 | |
| Fetal Bovine Serum | 50 | 10% |
| Penicillin-Streptomycin | 5 | 100 U/ml penicillin<br>0.1 mg/ml streptomycin |

Antibiotics and FBS were stored at -20°C and added to a full bottle of DMEM (500 ml) in the indicated amounts. The mixture was then stored at 4°C.

**Selection/Selective Medium:**

| *Constituent* | *Volume [ml]* | *Final concentration* |
|---|---|---|
| DMEM | 442.5 | |
| Fetal Bovine Serum | 50 | 10% |
| Penicillin-Streptomycin | 5 | 100 U/ml penicillin<br>0.1 mg/ml streptomycin |
| G418 | 2.5 | 0.5 mg/ml |

G418 was added to the standard medium and has been used to grow stable cell lines carrying the appropriate G418 resistance gene.

**Starvation Medium**

| *Constituent* | *Volume [ml]* | *Final concentration* |
|---|---|---|
| DMEM | 495 | |
| Penicillin-Streptomycin | 5 | 100 U/ml penicillin<br>0.1 mg/ml streptomycin |

Starvation Medium is standard medium without FBS, used when cell-based assays require starvation of cells.

### *2.1.2 Other buffers and solutions*

Purified water from a Milli-Q® Water System was used to prepare all solutions and buffers. Sterile UltraPure™ water was used for cell and molecular biology experiments.

**Assay buffer (HBSS + HEPES 20 mM)**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| HBSS | 490 ml | |
| HEPES (1M) | 10 ml | 20 mM |

HEPES (1M) was mixed with HBSS and stored at room temperature. Bovine serum albumin (BSA) (final concentration 0.01/0.1%) was added to the assay buffer as indicated in "Methods" to saturate free binding sites on plastic surfaces to prevent non-specific peptide binding.

**Competent bacteria buffer 1**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| $CH_3COOK$ | | 30 mM |
| $CaCl_2$ x $2H_2O$ | | 100 mM |
| $MnCl_2$ | | 50 mM |
| Glycerol | | 15% (v/v) |
| $dH_2O$ | ad 50 ml | |
| HCL | | used for pH adjustment pH 5.8 |

The solution was adjusted to pH 5.8 followed by sterile filtration (0.2µm).

**Competent bacteria buffer 2**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| RbCl | | 10 mM |
| $CaCl_2$ x $2H_2O$ | | 75 mM |
| MOPS | | 10 mM |
| Glycerol | | 15% (v/v) |
| $dH_2O$ | ad 50 ml | |
| HCL | q.s. | used for pH adjustment pH 6.8 |

The solution was adjusted to pH 6.8 and then filter-sterilized (0.2µm).

**Ethanol 70%**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| Ethanol absolute 96% | 730 ml | 70% |
| $dH_2O$ | ad 1000 ml | |

The mixture was stored at room temperature and used for plasmid DNA purification.

**HEPES (1M)**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| HEPES | 23.8 g | 1 M |
| $dH_2O$ | ad 100 ml | |
| NaOH (1M) | q.s. | used for pH adjustment between pH 7.2-7.4 |

The pH was adjusted, and the solution was sterilized by membrane filtration and stored at -20°C in 10 ml aliquots.

**LB (lysogeny broth) agar**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| Agar | 15 g | 1.5% |
| LB medium | ad 1000 ml | |

After adding the agar to the LB medium, the mixture was autoclaved and stored at room temperature. See “Methods” for further preparation of LB agar plates.

**LB medium (Lennox)**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| Tryptone | 10 g | 1% |
| Yeast extract | 5 g | 0.5% |
| NaCl | 5 g | 0.5% |
| $dH_2O$ | ad 1000 ml | |
| NaOH | q.s. | used for pH adjustment pH 7.4 |

The components were dissolved, and the pH was adjusted to 7.4. The solution was then sterilized by autoclaving and stored at room temperature.

**Paraformaldehyde 4% (PFA)**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| Paraformaldehyde | 8 g | 4% |
| PBS (10X) | 20ml | |
| $dH_2O$ | ad 180 ml | |
| NaOH (1M) | q.s. | used to properly dissolve PFA |

Paraformaldehyde was added to 100 ml $dH_20$ and heated to 60°C under a laminar air flow safety cabinet. NaOH was applied dropwise to obtain a clear solution. A mixture of 20 ml PBS (10X) and 80 ml $dH_2O$ was then added. The solution was subsequently stored in aliquots at -20°C.

**Phosphate-buffered saline (PBS 1X)**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| **PBS tablet:** | | |
| Potassium chloride | | 2.68 mM |
| Sodium chloride | | 140 mM |
| Sodium phosphates | | 10 mM |
| $dH_2O$ | ad 500 ml | |

The solution was sterilized by autoclaving and stored at room temperature.

**Poly-D-Lysine (PDL) solution**

| *Constituent* | *Amount* | *Final concentration* |
|---|---|---|
| Poly-D-lysine | 5 mg | 0.1 mg/ml |
| $dH_2O$ | ad 50 ml | |

Poly-D-lysine was dissolved in $dH_2O$, followed by sterile filtration, and then stored at 4°C.

## 2.2 Cell lines

***Table 3: Cell lines***

| *Name* | *Source* | *ID* |
|---|---|---|
| HEK293 (wild-type (wt)/parental) | A. Inoue lab | N/A |
| **Δ**Gα s/olf/q/11/12/13/z HEK293 (Δseven) | A. Inoue lab | N/A |
| **Δ**arr2/3 HEK293 (Δarr) | A. Inoue lab | N/A |
| HEK-T | G. Schulte lab | N/A |
| HEK293 wt HA-ratAT1aR | E. Kostenis lab | N/A |
| HEK293 Δseven HA-ratAT1aR | E. Kostenis lab | N/A |
| HEK293 Δarr HA-ratAT1aR | E. Kostenis lab | N/A |
| HEK293 Δarr SNAP-ratAT1aR | E. Kostenis lab | N/A |

## 2.3 Chemicals, peptides, and recombinant proteins

***Table 4: Chemicals, peptides, and recombinant proteins***

| *Name* | *Source/Company* | *Reference Number* |
|---|---|---|
| A23187 | Sigma-Aldrich | # C7522 |
| Agar | Fluka | # 34250 |
| Ampicillin | Carl Roth GmbH & Co. KG | # K029.1 |
| Angiotensin 1-7 | Sigma-Aldrich | # A9202 |
| Angiotensin II | Sigma-Aldrich | # A9525 |
| Bovine Serum Albumin (BSA), fatty acid free | Sigma-Aldrich | # A6003 |
| $CaCl_2$ x $2H_2O$ (calcium chloride dihydrate) | Carl Roth GmbH & Co. KG | # A119.1 |
| $CH_3COOK$ (potassium acetate) | Carl Roth GmbH & Co. KG | # T874.2 |
| Coelenterazine | Carbosynth Limited | # EC14031 |
| D-(+)-Glucose | Sigma Aldrich | # 16301 |
| DMSO (Dimethyl sulfoxide) | AppliChem GmbH | # A3672 |
| EGF (Epidermal Growth Factor, human) | Sigma-Aldrich | # E9644 |
| Ethanol 96% (Ethanol absolute) | KMF | # 08-205 |
| Fluorescein | Sigma Aldrich | # 46955 |

| | | |
|---|---|---|
| FR900359 (FR) | G. König lab | N/A |
| Furimazine<br>NanoBiT™ Nano-Glo® Substrate | Promega | # N1573 |
| Gibco™ PBS (10X), pH 7.4 | Thermo Fisher Scientific | # 70011044 |
| Glycerol | Merck | # 1.04092.2500 |
| HEPES<br>2-(4-(2-Hydroxyethyl) piperazin-1-yl) ethane-sulfonic acid | AppliChem | # A1069.0500 |
| Hydrochloric acid (HCl) 37% | KMF | # 08-721 |
| KCl (potassium chloride) | Carl Roth GmbH & Co. KG | # 6781.1 |
| $MnCl_2$ x $H_2O$ (manganese-II-chloride monohydrate) | Carl Roth GmbH & Co. KG | # 4320.3 |
| MOPS (3-(*N*-Morpholino)-propane sulphonic acid) | Carl Roth GmbH & Co. KG | # 6979.4 |
| $Na_2HPO_4$ (disodium hydrogen phosphate) | Sigma Aldrich | # NIST2186II |
| NaCl (sodium chloride) | Carl Roth GmbH & Co. KG | # P029.3 |
| NaOH (sodium hydroxide) | Grüssing GmbH | # 12156 |
| Paraformaldehyde (PFA) | Carl Roth GmbH & Co. KG | # 0335.2 |
| Pertussis toxin (PTX) | Thermo Fisher Scientific | # PHZ1174 |
| Phosphate-Buffered Saline (PBS) tablets | Thermo Fisher Scientific | # 18912014 |
| Poly-D-Lysine (PDL) | Sigma Aldrich | # P6407 |
| Poly-Ethylene-Imine (PEI) (linear, $M_w$ 2,500) | Polysciences | # 24313-2 |
| RbCl (rubidium chloride) | Carl Roth GmbH & Co. KG | # 4471.4 |
| SNAP-Surface® 649 | New England Biolabs | # S9159S |
| Tag-lite® SNAP-Lumi4®-Tb | CisBio International | # SSNPTBD |
| Tryptone | Carl Roth GmbH & Co. KG | # 8952.1 |
| Yeast extract | Carl Roth GmbH & Co. KG | # 2363.3 |

## 2.4 Plasmids

***Table 5: Plasmids***

| *Name* | *Source* | *ID* |
|---|---|---|
| pcDNA3.1(+) | E. Kostenis lab | # 1218 |
| HA-ratAT1aR-pcDNA3.1 | E. Kostenis lab | # 1418 |
| SNAP-ratAT1aR-pcDNA3.1 | E. Kostenis lab | # 1363 |
| AT1R-SmBiT-pCAGGS | A. Inoue lab | # 1539 |
| $G\alpha_q$-pcDNA3.1 | E. Kostenis lab | # 728 |
| $G\alpha_{i2}$-pcDNA3.1 | A. Inoue lab | # 1528 |
| $G\alpha_{o1}$-pCAGGS | A. Inoue lab | # 1559 |
| GRK2-pcDNA3.1 | C. Hoffmann lab | # 1706 |
| β-arrestin2-pcDNA3.1 | E. Kostenis lab | # 505 |
| β-arrestin1-lgBiT-pCAGGS | A. Inoue lab | # 1511 |
| β-arrestin2-lgBiT-pCAGGS | A. Inoue lab | # 1512 |
| β-arrestin1-GFP-pcDNA3.1 | E. Kostenis lab | # 952 |
| β-arrestin2-GFP-pcDNA3.1 | E. Kostenis lab | # 519 |
| PTX-S1-pCAGGs | A. Inoue lab | # 1464 |
| Mas-GRK3ct-Nluc-pcDNA3.1 | K. Martemyanov lab | # 1599 |
| $G\beta_1$-Venus $_{(156-239)}$ -pcDNA3.1 | K. Martemyanov lab | # 1601 |
| $G\gamma_2$- Venus $_{(1-155)}$ -pcDNA3.1 | K. Martemyanov lab | # 1602 |

## 2.5 Commercial Assay Kits

***Table 6: Commercial assay Kits***

| *Name* | *Company* | *Reference Number* |
|---|---|---|
| Advanced phospho-ERK (Thr202/Tyr204) cellular kit HTRF® | CisBio International | # 64AERPEH |
| Phospho-ERK (Thr202/Tyr204) cellular kit HTRF® | CisBio International | # 64ERKPEH |
| Total ERK cellular kit HTRF® | CisBio International | # 64NRKPEH |
| FLIPR® Calcium 5 Assay kit | Molecular Devices | # R8186 |
| HTRF-IP One dynamic 2 kit | CisBio International | # 62IPAPEC/62IPAPEJ |

| | | |
|---|---|---|
| NucleoBond Xtra Midi Plus | Macherey-Nagel | # 740412.50 |
| innuPREP Plasmid Mini Kit | Analytik Jena | # 845-KS-5040250 |

## 2.6 Equipment

***Table 7: Equipment***

| ***Name*** | ***Source*** | ***ID*** |
|---|---|---|
| Axiocam 503 mono | Carl-Zeiss AG | # 426559-0000-000 |
| AxioObserver Z.1 | Carl-Zeiss AG | N/A |
| Block thermostat QBD2, 2 blocks | Buch & Holm A/S | # 9852308 |
| Cell Counter | Corning | # 6749 |
| Corning® Epic® whole cell biosensor | Corning | N/A |
| CyBio®-SELMA Semi-automatic pipettor (384 and 96 channels) | Analytik Jena | # OL7001-26/-216<br># OL7001-26/-213 |
| E4™ XLS, electronic single-channel pipette | Mettler Toledo | # 17014486 |
| Eppendorf 5415D Centrifuge | Eppendorf SE | # 5425 000.219 |
| Eppendorf 5810 Centrifuge | Eppendorf SE | # 5810 000.424 |
| Eppendorf BioPhotometer® D30 | Eppendorf SE | # 6133000001 |
| Eppendorf Research® Plus Pipette 100-1000 µl | Eppendorf SE | # 3123000020 |
| Eppendorf Research® Plus Pipette 10-100 µl | Eppendorf SE | # 3123000047 |
| Eppendorf Research® Plus Pipette 0.5-10 µl | Eppendorf SE | # 3123000063 |
| FlexStation® 3 Multi-Mode Microplate Reader | Molecular Devices | # Flex3 |
| Fluid aspiration system | VacuuBrand GmbH & Co. KG | N/A |
| Freezer (liquid nitrogen) | Chart BioMedical Ltd. | # MVE 815P-190 |
| Freezer: Heraeus® Herafreeze® | Thermo Fisher Scientific | N/A |
| Heracell™ 240i $CO_2$ Incubator | Thermo Fisher Scientific | # 51032876 |
| Inverted microscope CKX31 | Olympus Corporation | # CKX31SF |
| JP Selecta™ Precisdig Water bath | J.P. Selecta S.A. | # 6001196 |
| Mili-Q® Water System | Merck KGaA | N/A |

| | | |
|---|---|---|
| Mithras² LB 943 Monochromator and Filter Multimode Reader | Berthold Technologies GmbH & Co. KG | # 56600-001 |
| Multipette® E3 | Eppendorf SE | # 4986 000.017 |
| Neubauer-Counting Chamber 0.1 mm | Paul Marienfeld GmbH & Co. KG | # 640130 |
| Objective Plan-Apochromat 20x/0,8 | Carl-Zeiss AG | # 440640-9903-000 |
| Objective Plan-Apochromat 63x/1,4 Oil DIC M27 | Carl-Zeiss AG | # 420782-9900-799 |
| Peltier-cooled incubator | Memmert GmbH & Co. KG | # IPP110 |
| PHERAstar® FSX Microplate Reader | BMG Labtech | N/A |
| PIPETMAN™ Classic P1000 | Gilson Inc. | # F123602 |
| PIPETMAN™ Classic P20 | Gilson Inc. | # F123600 |
| PIPETMAN™ Classic P200 | Gilson Inc. | # F123601 |
| Pipette Controller, accu-jet® pro | Brand GmbH & Co. KG | # 26300 |
| Sartorius Balances (TE64, TE6101) | Sartorius AG | # TE64<br># TE6101 |
| SevenEasy™ S20 pH meter | Mettler Toledo | N/A |
| Thermo scientific Herasafe™ Class II Biological Safety Cabinet | Thermo Fisher Scientific | # 51022712 |
| Thermomixer® 5355 comfort R | Eppendorf SE | N/A |
| Transferpette®-12 electronic DE-M | Brand GmbH & Co. KG | # 705454 |
| Varioclav® | HP Medizintechnik GmbH | N/A |
| Vortex | Heidolph Instruments GmbH & Co. KG | N/A |
| VWR™ Galaxy Mini Centrifuge | VWR International GmbH | # 37000-930 |

## 2.7 Consumables

***Table 8: Consumables***

| ***Name*** | ***Source*** | ***ID*** |
|---|---|---|
| µ-slide 8 well | ibidi GmbH | # 80826 |
| 384-well fibronectin-coated Epic® biosensor plates | Corning | # 5042 |
| 384-well, small-volume microplate | Greiner Bio-One | # 784075 |
| Axygen Microvolume pipette tips 10 µl | Axygen | # T-300-R-S |

| | | |
|---|---|---|
| Cell culture flask 175 $cm^2$ | Corning | # 431079 |
| Cell culture flask 25 $cm^2$ | Corning | # 430168 |
| Cell culture flask 75 cm2 | Sarstedt AG & Co. KG. | # 83.3911.002 |
| Combitips®advanced | Eppendorf SE | # 0030089405 (0.1 ml)<br># 0030089413 (0.2 ml)<br># 0030089421 (0.5ml)<br># 0030089430 (1.0 ml)<br># 0030089650 (2.0 ml)<br># 0030089456 (5.0 ml) |
| Corning® 100 mm TC-treated Culture Dish | Corning | # 430293 |
| Corning® 384-well Clear Round Bottom | Corning | # 3657 |
| Corning® 60 mm TC-treated Culture Dish | Corning | # 430166 |
| Corning® 96-well Clear Flat Bottom | Corning | # 3596 |
| Corning® 96-well Clear Round Bottom | Corning | # 3795 |
| Corning® 96-well Flat Clear Bottom Black | Corning | # 3603 |
| Corning® 96-well Solid White Flat Bottom | Corning | # 3917 |
| Costar® 50 ml Reagent Reservoir | Corning | # 4870 |
| Costar® multi-well plates | Corning | # 3506 (6-well)<br># 3512 (12-well)<br># 3527 (24-well)<br># 3548 (48-well) |
| CryoPure 2.0 ml tubes. | Sarstedt AG & Co. KG | # 72.379 |
| CyBio® TipTray 384/25 µl | Analytik Jena | # OL3800-26-513-N |
| CyBio® TipTray 96/250 µl | Analytik Jena | # OL3800-26-559-N |
| Disposable filter unit 0.2 µl | GE Healthcare GmbH | # FB30/0.2 CA-s |
| Falcon® 96-well sterile (white, flat, bottom) | Corning | # 353296 |
| FlexStation® Pipet Tips 96-well | Molecular Devices | # 9000-0912 |
| Marienfeld Superior™ Coverslips | Paul Marienfeld GmbH & Co. KG | # 10249911 |
| Parafilm®M | Bemis Company | # P7668 |

| | | |
|---|---|---|
| Pipette tips 200µl | Sarstedt AG & Co. KG | # 70.760.002 |
| Pipette tips, 1000µl | Sarstedt AG & Co. KG | # 70.762. |
| Ritips® Dispenser tips 0.1 ml | Ritter GmbH | N/A |
| SafeSeal microtubes | Sarstedt AG & Co. KG | # 72.706.400 (1 ml)<br># 72.695.400 (2 ml)<br># 72.701.400 (5 ml) |
| Screw cap tubes | Sarstedt AG & Co. KG. | # 62.554.502 (15ml)<br># 62.547.254 (50 ml) |
| Serological pipettes | Sarstedt AG & Co. KG | # 86.1252.001 (2 ml)<br># 86.1253.001 (5 ml)<br># 86.1254.001 (10 ml)<br># 86.1685.001 (25 ml)<br># 86.1256.001 (50 ml) |

## 2.8 Bacterial strains

***Table 9: Bacterial strains***

| ***Name*** | ***Source*** | ***ID*** |
|---|---|---|
| DH5α Competent Cells | Thermo Fisher Scientific | # 18265-017 |
| XL1-Blue Competent Cells | Stratagene | # 200130 |

## 2.9 Software and Algorithms

***Table 10: Software and Algorithms***

| ***Name*** | ***Source*** | |
|---|---|---|
| BioRender | bioRender | https://www.biorender.com/ |
| Citavi 6.14 | Swiss Academic Software GmbH | https://www.citavi.com/en |
| Epic Autoalign/Imager Lab View 2009 9.0.1f2 | Perkin Elmer | N/A |
| ImageJ | National Institutes of Health | http://imagej.nih.gov/ij/ |

| | | |
|---|---|---|
| MARS 3.32 | BMG Labtech | https://www.bmgLabtech.com/de/microplate-reader-software/ |
| Microsoft 365 V2306 | Microsoft Corporation | https://www.microsoft.com/de-de/microsoft-365 |
| MikroWin® 2000 V5.22 | Berthold Technologies GmbH & Co KG | https://mikrowin-2000.software.informer.com/ |
| PheraStar FSX Reader Control V5.41 | BMG Labtech | https://www.bmgLabtech.com/de/microplate-reader-software/ |
| Prism 9.2.0 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| SnapGene 7.0.1 | SnapGene | https://www.snapgene.com/?referrer=SnapGene |
| SoftMax® Pro 5.4.3 Software | Molecular Devices | https://de.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software |
| Zeiss Zen blue edition 3.3.89.00000 | Carl Zeiss AG | https://www.zeiss.com/microscopy/de/produkte/software/zeiss-zen.html |

## 2.10 Data collection and analysis

Commercial software provided by the device manufacturers was used for all data collection. In detail, PheraStar FSX Reader Control V5.41 (PHERAstar FSX, BMG Labtech), SoftMax® Pro 5.4.3 (FlexStation® 3, Molecular Devices), MikroWin2000 V5.22 (Mithras² LB 943, Berthold Technologies), Epic Autoalign/Imager Lab View 2009 (Epic® Corning® biosensor, Perkin Elmer version: 9.0.1f2) and Zeiss Zen blue edition (Carl Zeiss AG, Version: 3.3.89.00000).

The following commercially available software was used to analyze the data: MARS 3.32 (BMG Labtech) and MikroWin® (Berthold Technologies). Data were processed using Microsoft Excel and statistically analyzed using Graph Pad Prism 9.2.0 software. All experiments were performed at least three times, as indicated in the figure legends, unless otherwise noted. Representative experiments are expressed as mean ± standard error (SD), quantifications are mean ± SEM (standard error of the mean).

For clarity, kinetic recordings are shown with the above SD, unless otherwise noted. Concentration Response Curves (CRCs) were fitted to either a three-parameter logistic

function (with a fixed Hill slope of 1) or to a four-parameter logistic function using GraphPad Prism 9.2.0. Data were analyzed using analysis of variance (ANOVA) or two-tailed Student's t-test. Specific statistical analyses are described in the figure legends and in the Methods section.

# 3 Methods

## 3.1 Cell biological methods

### *3.1.1 Cell culture conditions*

All cells were grown in incubators at 37°C in a 5% $CO_2$, 96% humidity atmosphere. Cell media and solutions were warmed to 37°C in a water bath prior to use. To ensure aseptic conditions, a laminar airflow safety cabinet was used for all cell treatments and cell-based experiments. HEK293 cells (wild-type, Δseven, Δarr) and HEK-T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FCS) and with 1% of a mixture of penicillin (100 U/ml) and streptomycin (0.1 mg/ml). In addition, the selective antibiotic G418 (500 µg/ml) was added, when stable transfected HEK293 cells were cultured.

### *3.1.2 Passaging of cells*

Depending on the growth or proliferation rate, cells were passaged when they reached approximately 95% confluence. Cell medium was then aspirated, and the cells were washed once with PBS before trypsin (Trypsin-EDTA 0.05%) was added to allow the cells to detach. The trypsin reaction was stopped by adding cell medium (medium/trypsin ratio 4:1), and the desired number of cells was either transferred back to the flask or to a new flask.

### *3.1.3 Cryopreservation and defrosting of cells*

Cryopreservation was performed for storage and later use of the cells. Slow cooling of the cells is important to avoid the formation of ice crystals, which can damage the cell membrane. Prior to cooling, cells were detached as described above and resuspended in cell medium followed by centrifugation (800 rpm for 4 min). The resulting cell pellet was then resuspended in freezing medium (FCS supplemented with 10% DMSO) and transferred to cryogenic vials at a concentration of 1-2 $x10^6$ cells. The vials were placed on a cryo-rack insulated by a Styrofoam box and placed in a -80°C freezer to allow the cells to cool slowly. The next day, the cryo-vials were placed in a liquid nitrogen tank (-196°C) for long-term storage.

Rapid warming of the cryo-vials in a 37°C water bath was used to revitalize the respective cell lines, followed by immediate transfer to prewarmed cell medium (5-10 ml) containing tubes. The cells were then centrifuged (800 rpm, 3 min), the supernatant aspirated, and the pellet resuspended in fresh pre-warmed cell medium, followed by transfer of the cell

suspension to 25 $cm^2$ flasks. The cells were then maintained at 37°C in a 5% $CO_2$ atmosphere. Routine polymerase chain reaction (PCR) testing for mycoplasma contamination was performed on supernatants from all cell lines.

### *3.1.4 Cell counting*

The number of cells in a given cell suspension was determined using either a *Neubauer* counting chamber (hemocytometer) or the Corning® Cell Counter. Cells were detached as described above and resuspended in cell medium. A small amount of the suspension was then transferred to an Eppendorf tube and immediately before counting, the suspension was homogenized by vortexing and 10 µl was pipetted onto either a *Neubauer* chamber or the glass slide for the Corning® Cell Counter, both covered with a coverslip that creates a defined volume between the slide and the coverslip. If cell density was high, appropriate predilution was performed prior to counting.

In the case of the Neubauer chamber, the cell count was determined as follows: Cell density [cells/ml] = count x dilution factor x $10^4$.

Before counting cell suspensions with the Corning® Cell Counter, the size range for each cell line and the focus of the camera were set. Cell counts were performed in at least three different areas of the slide and then averaged.

### *3.1.5 Poly-D-lysine coating of cell culture plates and dishes*

Poly-D-lysine (PDL) coating of plastic and glass surfaces facilitates cell adherence and growth in dishes and plates used for cell culture and cell-based experiments. An appropriate volume of PDL (0.1 mg/ml) was added to completely cover the cell culture surfaces and then incubated at 37°C for 1 hour. The PDL was then removed, and the surfaces were washed three times with sterile PBS. Subsequently, PBS was aspirated, and the surfaces were dried under UV light in a laminar air flow safety cabinet. PDL-coated well plates were then either stored at 4°C for a maximum of 2 weeks or used directly for cell-based experiments.

### *3.1.6 Transfection of HEK293 cells with polyethylenimine (PEI)*

Polyethylenimine is a highly charged cationic polymer that effectively interacts with the negatively charged phosphate residues of DNA (e.g., plasmid DNA) (Boussif et al. 1995). The resulting DNA/PEI aggregates are now slightly positively charged on the outside and can attach to negative residues on the plasma membrane of mammalian cells and are then taken into the cell by endocytosis. Once inside the cell, the DNA is released from the

aggregate by osmotic effects and can then be transcribed in the nucleus (González-Domínguez et al. 2019; Rudolph et al. 2000).

Transfection with PEI reagent can be performed in adherent format (DMR assays) or in suspension (other assays). The procedure described here is an example of adherent transient transfection in DMR assays (see other assays for suspension method details). Further information (DNA amounts, constructs) can also be found in the respective assays.

One day prior to transfection, 2 x$10^6$ wild-type HEK293 cells were seeded in 10 cm dishes and cultured overnight (37°C, 5% $CO_2$) to reach 70-80% subconfluence. Transfection with PEI was then performed according to following protocol (PEI/DNA ratio 3:1): Two Eppendorf tubes were filled with 300 µl OptiMEM (for 10 cm dishes). In one tube the DNA plasmids used were dissolved (for DMR: 5.8µg HA-ratAT1R + 0.2µg pcDNA3.1), while in the other tube PEI was dissolved in three times the amount of total DNA (for DMR: 6µg x 3 = 18µg). The PEI solution was then pipetted into the DNA solution and the mixture was incubated for 15 minutes at room temperature. The PEI/DNA mixture was then added dropwise to the cells seeded the day before and the cells were maintained at 37°C and 5% $CO_2$. Experiments were performed 24 and 48 hours after transfection.

### *3.1.7 Generation of stably expressing HA-ratAT1R and SNAP-ratAT1R HEK293 cells*

HEK293 cells (wild-type, Δseven, Δarr) were maintained in standard cell medium in 10 cm petri dishes 48 hours after transfection with the DNA plasmid (pcDNA3.1) encoding the HA-ratAT1 receptor. Analogous SNAP-ratAT1R-expressing Δarr cells were obtained by transfection with pcDNA3.1-based SNAP-ratAT1R DNA. To control for sufficient transfection, the corresponding non-transfected HEK293 cells were plated. Cells were then harvested and different dilutions (1:2, 1:5, 1:10) of each cell line were prepared. The cells were then seeded on 10 cm petri dishes and grown for the next two weeks in standard cell medium supplemented with the selective antibiotic G418 (working concentration 750 µg/ml), which was replaced every three days. Surviving cells (pooled stable transfectants) were then cultivated to reach confluence and maintained in 75 $cm^2$ cell culture flasks or frozen. For stably expressing HA-ratAT1R HEK293 cells (wild-type, Δseven and Δarr) a subsequent individual clone isolation was performed using limited dilution cloning. Therefore, stably expressing pooled clones were detached and diluted in G418 supplemented standard medium (750 µg/ml) at a concentration of 1 cell/well and then plated in PDL-coated

transparent 96-well plates. Individual clones were identified by light-microscopy and then grown in selective medium, that was renewed every second day. When individual cell clone colonies had reached confluence in the plate, they were transferred to the next larger plate until they were ready to be cultured in 75 $cm^2$ flasks. Stably expressing single clones of HA-ratAT1R (wild-type, Δseven, Δarr) were then maintained in selective medium or frozen. Functionality and the best expressing clone were then determined by ELISA and DMR (data not shown).

## 3.2 Cell-based experiments

### *3.2.1 NanoBiT: Protein-Protein Interaction experiments*

β-arrestin1/2 recruitment experiments were conducted using the NanoLuc® Binary Technology (NanoBiT®) as previously reported in the literature (Dixon et al. 2016). In brief, 24 hours before measurement a transient transfection was performed by following transfection scheme: DNA-plasmids encoding either for human AT1R-SmBiT (0.25 µg) or lgBiT-β-arrestin-1 and -2 (1.25 µg), respectively (ratio 1:5 SmBiT/lgBiT) were dissolved in 150 µl OptiMEM and the final DNA amount of 3 µg was then set by filling with empty vector DNA (pcDNA3.1). Next, 150 µl OptiMEM containing 9 µg PEI transfection reagent was applied to the DNA mixture and the preparation was incubated for 15 minutes. Meanwhile 8 $x10^5$ cells were detached and diluted in 3 ml culture medium. The DNA/PEI-mixture was then added to the cell suspension, that was subsequently plated onto 6 cm petri dishes and kept in the incubator (37°C, 5% $CO_2$) over night. The following day cells were detached, centrifuged, and washed once with HBSS + HEPES. After another centrifugation step, the cell pellet was resuspended in 4 ml assay buffer (HBSS + HEPES, supplemented with 0,01% BSA) and FR (1 µM) was added, when $G\alpha_q$-inhibition was applied. Cells were then seeded into white, non-coated, 96 well plates using 80 µl per well (corresponding 4 $x10^4$ cells/well). Cells were subsequently treated with 50 µM coelenterazine (20 µl/well, assay buffer) and the plates were stored protected from light at room temperature for 1.5 hours. For monitoring luminescence, plates were transferred into a PheraStar FSX multiplate reader and a baseline read of six time points was carried out. Ligands were then added, and alterations in luminescence, depicted as RLU, were recorded for at least 30 minutes.

### *3.2.2 HTRF-based phospho-ERK1/2- and total-ERK1/2-phosphorylation experiments*

To determine changes in the accumulation of phospho-ERK1/2 and total ERK1/2 protein, the advanced phospho-ERK1/2 (Thr202/Tyr204) and total ERK1/2 cellular kits (CisBio) were used according to the manufacturer's protocol.

Briefly, for experiments with HEK293 cells transiently expressing the rat HA-AT1aR and other proteins of interest as indicated (β-arrestin1/2; $G\alpha_q$, $G\alpha_{i2}$, $G\alpha_o$, and GRK2), wild-type HEK293 cells (parental HEK293 cells) or genome-edited HEK293 cells depleted of functional Gα-protein subunits other than the $G\alpha_i$ -family (Δseven) were treated 48 hours before measurement according to the following transfection scheme: For the 10 cm dish approach, 3 x$10^6$ wild-type (wt)- or Δseven-HEK293 cells were transfected in suspension. Therefore, 8 µg of total plasmid DNA was dissolved in 500 µl OptiMEM, with 4 µg rat HA-AT1R and 1 µg each for additional transfection with plasmid DNA encoding the proteins of interest. The remaining amount of DNA was filled with empty vector (pcDNA3.1). Subsequently, PEI (24 µg) diluted in 500 µl OptiMEM was added to the DNA mixture, followed by an incubation period for 15 minutes at room temperature. The PEI/DNA-mixture was then added to the cell suspension (3 x$10^6$ cells in 9 ml standard medium) and the cells were seeded on 10 cm petri dishes and maintained overnight (37°C and 5% $CO_2$). The next day, the cells were detached and transferred to 96-well poly-D-lysine-coated microtiter plates at a density of 5 x$10^4$ cells per well. This step is also the first step in the use of Δarr cells, which already stably express the rat HA-AT1aR. For PTX treatment, cells were treated with 100 ng/ml PTX at least 14 hours prior to the measurement. On the day of the experiment, the cells were starved for 4 hours with starvation medium (75 µl/well) and then treated with 25 µl starvation medium, supplemented with BSA 0.4% without (buffer control) or with Ang1-7, AngII (4x concentrated to give final concentrations of 30 µM Ang1-7 or 100 nM AngII, respectively). After stimulation at the indicated times, the supernatant was discarded by inverting the plate, and the adherent cells in the wells were lysed by adding 40 µl of lysis buffer per well using a Cybio® Selma semi-automatic pipette (Analytik Jena AG). After addition of the lysis buffer, the plates were incubated for 1 hour on an orbital shaker (at room temperature) and then stored overnight at -20°C. The next day, lysates were transferred to white 384-well plates and antibody mixtures from either the advanced phospho-ERK1/2 or total ERK1/2 detection kits were added. The standard phospho-ERK1/2 kit was used only in preliminary experiments (as indicated). Lysates were then incubated for

4 hours (advanced phospho-ERK1/2 antibodies) or 24 hours (total ERK1/2 antibodies) at room temperature with protection from light. Time-resolved FRET signals (excitation 320 nm) were detected using the Mithras² LB 943 multimode reader (Berthold Technologies). ERK1/2 (pERK) and total ERK1/2-phosphorylation is expressed as a fold increase over the corresponding basal levels (fob), which were calculated using Graph Pad Prism 9.2.0 software.

### *3.2.3 Label-free Dynamic Mass Redistribution (DMR) assay*

Dynamic mass redistribution (DMR) measurements were performed as previously described in detail (Schröder et al. 2010; Schröder et al. 2011; Meyrath et al. 2020). Briefly, for experiments using wild-type HEK293 cells transiently expressing the AT1 receptor, 1.8 x$10^6$ cells were seeded on 10 cm petri dishes and grown to reach 60-80% confluence. Subconfluent cells were then transiently transfected with pcDNA3.1-based plasmids encoding either the rat HA-AT1aR or not (empty vector) and incubated overnight (37°C and 5% $CO_2$). The following day, cells were transferred to a 384-well Epic® biosensor plate at a density of 1.8 x$10^4$ cells and, if indicated pertussis toxin (PTX) was added at a concentration of 100 ng/ml once the cells were attached. HEK293 cells stably expressing the rat HA-AT1aR (wild-type or Δarr) were seeded directly to the 384-well Epic® biosensor plate (1.8 x$10^4$ cells) and PTX was added in the same manner as described above. The next day, the cells were washed twice with Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES and 0.1% BSA (wash buffer). After a final wash, FR900359 (1 µM) was added if indicated. The plate was subsequently placed in the Epic® DMR reader and allowed to equilibrate at 37°C for 1 hour to stabilize the baseline. Angiotensin peptides diluted in the wash buffer were then applied to the biosensor plate using the semi-automated pipettor CyBio® Selma and ligand-induced DMR alterations were recorded for at least 3600 s at 37°C. Raw data were processed and analyzed with the GraphPad Prism 9.2.0 software.

### *3.2.4 Gβγ-GRK3 protein-interaction assay*

Real-time detection of G protein activation was performed using a BRET-based Gβγ-GRK3ct protein interaction assay analogous to the protocol of *Masuho et al. (Masuho et al. 2015b; Masuho et al. 2015a)*. Briefly, HA-ratAT1aR stably expressing HEK293 cells, genome-edited to lack all functional Gα-protein subunits except the $G\alpha_i$ -family (Δseven), were transfected in suspension as follows: 2.8 x$10^6$ trypsinized cells were treated with a transfection mixture consisting of the transfection agent polyethylenimine (PEI) and the

pcDNA3.1-based plasmid DNA encoding $G\alpha_q$ (0.8 µg), $G\beta_1$-$Venus_{(156-239)}$ (0.4 µg), $G\gamma_2$-$Venus_{(1-155)}$ (0.4 µg), masGRK3ct-NLuc (0.4 µg) and PTX-S1 (0.4 µg). PTX-S1 co-transfection was used to block residual $G\alpha_i$-proteins in the cells, while empty vector (pcDNA3.1) was used to equalize the total amount of DNA to 5µg. The cells were then seeded onto 10 cm petri dishes and incubated overnight at 37°C in a 5% $CO_2$ atmosphere. At 24 hours after transfection, the cells were detached, washed once with Hank's Balanced Salt Solution (HBSS) supplemented with HEPES, and then transferred (in the same buffer) to a 96-well plate at 8 $x10^4$ cells per well. Nano-Glo® luciferase substrate (Promega) was subsequently applied to the cells and the plate was transferred to a PHERAstar FSX microplate reader (BMG Labtech) for detection. After 3 minutes, a baseline BRET reading was performed for five measurement points, followed by the addition of increasing concentrations of the angiotensin peptides. The detected BRET signals were calculated as the ratio of the acceptor signal Venus-Gβγ (535 ± 30 nm) to the donor signal GRK3ct-Nluc (475 ± 30 nm), subtracting the last BRET ratio before agonist stimulation.

### *3.2.5 HTRF-based $IP_1$-accumulation detection*

IP1 accumulation was determined using the CisBio HTRF IP-One assay kit (CisBio, Codolet, France) according to the manufacturer's protocol with the subsequent variations: HEK293 cells stably expressing the AT1R (rat HA-AT1aR) were suspended in stimulation buffer (CisBio, LiCl supplemented buffer, to block further $IP_1$ degradation) and plated in a 384-well plate at 3 $x10^4$ cells per well. Increasing concentrations of Ang1-7 and AngII in stimulation buffer supplemented with BSA (final conc. 0.1%) were then added to the cells, followed by an incubation at 37°C for 1 hour. After agonist stimulation, cells were lysed and exposed to the detection antibody (anti-$IP_1$-cryptate (donor) and d2-labeled $IP_1$ (acceptor)) for 1 hour at room temperature. HTRF ratios were subsequently measured using the Mithras$^2$ multimode plate reader LB943 (Berthold Technologies, Bad Wildbad, Germany). The HTRF ratios were later converted to $IP_1$ concentrations, expressed in [nM], using the standard curve generated from the manufacturer's standard solutions.

### *3.2.6 $Ca^{2+}$ mobilization assay*

HEK293 cells stably expressing the AT1 receptor (rat HA-AT1aR) or not (parental HEK) were seeded at a density of 9 $x10^4$ cells in poly-D-lysine precoated flat 96-well black plates with a clear bottom and cultured overnight. The next day, media was aspirated, and cells were loaded with FLIPR® Calcium 5 dye (Molecular Devices, Sunnyvale, CA, USA) and

incubated for 1 hour at 37°C and 5% $CO_2$. For $G\alpha_q$-protein inhibition, FR (1 µM) was added to the calcium dye. Next, 100 µl of HBSS supplemented with HEPES and BSA (final assay concentration 0.1%) was added to each well and the plate was transferred to a FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). After a basal fluorescence reading of 20 seconds, the compounds were added (50 µl/well) and fluorescence was recorded for another 90 seconds. In all experiments, 5 µM of A23187 was used as a viability control. The increase in $[Ca^{2+}]$ is shown as RFU over time corrected by the minimum RFU measured prior to agonist stimulation.

### *3.2.7 Diffusion-enhanced resonance energy transfer (DERET)*

Real-time measurement of agonist-mediated internalization of the SNAP-AT1 receptor was performed using the DERET method as described previously (Ward et al. 2011; Roed et al. 2014; Benkel et al. 2022). Briefly, 1.8 $x10^6$ wild-type HEK293 cells (parental HEK) were transfected in suspension with a mixture of 9 µg polyethylenimine (L-PEI, 1 mg/ml), 0.25 µg plasmid DNA encoding for either the SNAP-ratAT1aR or SNAP-CD86 and 2.75 µg empty vector (pcDNA3.1). Cells were then plated on 6 cm petri dishes and incubated overnight at 37°C and 5% $CO_2$. The next day, cells were harvested, resuspended in cell media, and subsequently seeded into poly-D-lysine (PDL)-coated white flat-bottomed 96-well plates (1 $x10^5$ cells/well) and incubated overnight (37°C, 5% $CO_2$). HEK293-Δarr cells stably expressing the SNAP-ratAT1aR or not (Δarr) were seeded directly into 96-well plates at a density of 1 $x10^5$ cells. On the day of measurement, cell medium was aspirated, and cells were starved for 2 hours with DMEM without FCS (starvation medium). The starvation medium was then removed and replaced with 100 nM SNAP-Lumi4®-Tb substrate (CisBio) in assay buffer (HBSS supplemented with 20 mM HEPES) and the plate incubated for 1 hour at 4°C. Excess SNAP-Lumi4-Tb was removed, followed by three washes with assay buffer and the subsequent addition of fluorescein in the assay buffer (final concentration 50 µM). The cells were then stimulated with the angiotensin peptides and the plate was placed for DERET measurement into a PHERAstar FSX multimode reader (BMG Labtech), which allows simultaneous detection of donor (Lumi4-Tb, 620 nm) and acceptor (fluorescein, 520 nm) fluorescence after excitation at 337 nm. Recordings were taken for at least 60 minutes at 37°C. Detected DERET signals were calculated using MARS data analysis software (BMG Labtech) as the ratio of Lumi4-Tb (donor-) signal to fluorescein (acceptor-) signal and recorded for at least 60 minutes at 37°C. By using GraphPad Prism 9.2.0, ratios were later fitted to a one phase association model.

### *3.2.8 Fluorescence microscopy*

The transient expression of SNAP-AT1R in HEK293 cells was achieved in the same way as described for DERET (see 3.2.7). 24 hours prior to measurement, SNAP-AT1R-expressing wild-type HEK293 cells were seeded at a density of 1.5 x$10^5$ cells/well into PDL-coated 8-well plates (Ibidi) and cultured overnight at 37°C and 5% $CO_2$. The next day, cells were exposed to starvation medium (DMEM without FCS) for 2 hours at 37°C. After starvation, cells were incubated with SNAP-Surface® 649 (New England Biolabs) in assay buffer (HBSS supplemented with 20 mM HEPES) for 1 hour at 4°C. After removal of excess SNAP-Surface® 649, cells were washed three times with assay buffer and then treated with Ang1-7 or AngII in assay buffer for 1 or 60 minutes before fixation with 4% paraformaldehyde (PFA). Cells were then washed three times with phosphate-buffered saline (PBS) and then kept on HBSS. A Zeiss AxioObserver Z (Carl Zeiss, Jena, Germany) equipped with Apotome2.0 and Cy5 filter set was used for image acquisition. Structural images were obtained using Apotome2.0 at (20x) magnification, while the corresponding images at higher magnification (63×) were obtained using conventional fluorescence microscopy without the use of Apotome2.0. Zen blue Imaging software (Carl Zeiss, Jena, Germany) was used for subsequent image processing.

For DMR, pERK/total-ERK and $Ca^{2+}$ mobilization experiments performed with HEK293 cells transiently expressing the SNAP-AT1 receptor, the same transfection paradigm as described in 3.2.7 was used, and the cells were then treated according to the appropriate method protocols (3.2.2/3.2.3/3.2.6)

## 3.3 Molecular biological methods

### *3.3.1 Preparation of LB agar plates*

Lysogeny Broth (LB) is a rich nutrient solution used for the growth of bacteria. For preparation of LB agar plates. Sterilized LB agar was heated in a microwave (to obtain liquid) and cooled to approximately 50-60°C before the addition of ampicillin (100 µg/ml). 10 ml each was then immediately pipetted into 10 cm dishes under a laminar airflow safety cabinet. Further cooling at room temperature solidified the LB agar and the plates were either stored at 4°C or used directly.

### *3.3.2 Preparation of chemically competent bacteria (E. coli - DH5α or XL1blue)*

DH5α/XL1blue bacterial strains were grown overnight at 37°C on LB agar plates. The next day, a single colony was picked and used to inoculate 5 ml of Super Optimal Broth medium (SOB), which was kept at 37°C for 16 hours with vigorous shaking at 220 rpm. Then, for further growth, 1 ml of this propagated bacterial culture was pipetted into 500 ml SOB and maintained (37°C, 220 rpm shaking) until the optical density at 550 nm of the suspension reached 0.4-0.6. The suspension was immediately centrifuged (3000 x g, 10 min) at 4°C and the resulting pellet was resuspended in ice-cold competent bacteria buffer 1. The suspension was centrifuged again, followed by a second resuspension in ice-cold competent bacteria buffer 1. After a final centrifugation, the resulting pellet was resuspended in competent bacteria buffer 2 and the achieved suspension was aliquoted (50-100 µl) into Eppendorf tubes and quickly transferred to a liquid nitrogen tank for freezing. The aliquots were then stored at -80°C.

### *3.3.3 Transformation of chemically competent E. coli*

Bacterial transformation is a process by which exogenous DNA can be taken up by competent bacteria (e.g., DH5α and XL1blue). This mechanism can be used biotechnologically to store and amplify DNA of interest in bacteria.

Aliquots of competent E. coli (DH5α/XL1blue) were thawed on ice before 5-50 ng of DNA (e.g., plasmid DNA) was added, gently mixed, and incubated on ice for 30 minutes. Meanwhile, LB agar plates and S.O.C. medium were placed in the incubator (37°C) to warm up. After 30 minutes, the DNA/bacteria mixture was “heat shocked” at 42°C for 90 seconds and immediately placed on ice for another 90 seconds. Then 500-900 µl of pre-warmed S.O.C. medium was pipetted to the mixture and incubated for 60 minutes at 37°C with

shaking at 220 rpm. 100 µl of the mixture was then plated onto LB agar plates containing the appropriate selection antibiotic and incubated overnight at 37°C. The next day, a single bacterial clone colony was harvested and used as a stock for further colony growth (LB cultures).

### *3.3.4 Amplification of plasmid DNA with LB-cultures*

For subsequent use in mammalian cell transfection, it is important to obtain sufficient amounts of the desired plasmid DNA. To this end, single clone colonies on the LB agar plate (see above) must be amplified to provide adequate amounts of DNA for further extraction and purification.

Therefore, one transformed colony was picked from the LB agar plate and transferred to 5 ml LB medium supplemented with ampicillin (100 µg/ml) and grown for 16 hours at 37°C with shaking (220 rpm). The resulting LB bacterial culture was either used for plasmid DNA isolation using the innuPREP® Plasmid Mini Kit (see below) or grown on a larger scale. For this purpose, 150 µl of the LB bacterial culture was added to 150 ml of LB medium supplemented with ampicillin (100 µg/ml) and incubated for a further 16 hours at 37°C on an orbital shaker (220 rpm). The NucleoBond® Xtra Midi/Maxi Kit was then used to extract and purify DNA at this scale.

### *3.3.5 Isolation of plasmid DNA*

In order to isolate small amounts of plasmid DNA for analytical purposes (sequencing, etc.), the innuPREP® Plasmid Mini Kit was used. Bacterial cultures containing the plasmid DNA of interest were amplified as described above, and the DNA was then extracted and purified according to the manufacturer's protocol.

For experimental purposes (e.g., transfection of plasmid DNA), larger amounts of DNA are required, which are prepared as described above (150 ml LB culture). The isolation of the DNA was then carried out with the NucleoBond® Xtra Midi/Maxi Kit according to the protocol of the manufacturer. Plasmid DNA obtained from both isolation methods was then stored in UltraPure™ (DNAse, RNAse free distilled water) at -20°C.

### *3.3.6 Determination of plasmid DNA concentration and purity*

The concentration and purity of the isolated plasmid DNA was determined by UV light absorbance (260 nm, specific for DNA) using the Eppendorf BioPhotometer®. In addition, absorbance measurements were performed at 230 nm and 280 nm, which are indicative of

impurities (proteins or sugars, salts, etc.). The ratios of absorbance at 260 nm and 230 nm or 280 nm were calculated ($A_{260/230}$ and $A_{260/280}$). $A_{260/230}$ values between 2.0-2.2 and $A_{260/280}$ values close to 1.8 are indicative of DNA purity.

After isolation, the plasmid DNA amplified in competent bacteria was verified for the correctness of the gene of interest using next-generation sequencing (NGS). For this purpose, a small sample of the plasmid DNA was sent to EuroFins, and the sequence results obtained (FASTA format) were checked for correctness.

### *3.3.7 Cryoconservation of bacterial strains*

Transformed bacterial cultures in LB medium can be stored at -80°C with the addition of glycerol as a cryoprotectant. Therefore, the LB bacterial culture was mixed in a cryovial with sterilized 30% glycerol (1:1) and then stored in a -80°C freezer.

### *3.3.8 Design of SNAP-tagged ratAT1aR*

To fuse the ratAT1a receptor with a SNAP protein, the SNAP sequence (according to NEB and CisBio) was added in front of the ratAT1aR sequence. This resulted in an N-terminal SNAP tag of the ratAT1aR. The sequence was designed as follows (amino acid sequence):

MALPVTALLLPLALLLHAARPAAASGIDYKDDDDKAGIDAIMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAA
DAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQLAALA
GNPAATAAVKTALSGNPVPILIPCHRVVSSSGAVGGYEGGLAVKEWLLAHEGHRLGKPGLGDIQHSGGRALNSSAEDGIKRI
QDDCPKAGRHSYIFVMIPTLYSIIFVVGIFGNSLVVIVIYFYMKLKTVASVFLLNLALADLCFLLTLPLWAVYTAMEYRWPFGN
HLCKIASASVSFNLYASVFLLTCLSIDRYLAIVHPMKSRLRRTMLVAKVTCIIIWLMAGLASLPAVIHRNVYFIENTNITVCAFHY
ESRNSTLPIGLGLTKNILGFLFPFLIILTSYTLIWKALKKAYEIQKNKPRNDDIFRIIMAIVLFFFFSWVPHQIFTFLDVLIQLGVIHD
CKISDIVDTAMPITICIAYFNNCLNPLFYGFLGKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSSAKKPASCFEVE

*CD8 signal peptide*, *FLAG-Tag*, *SNAP-Tag (SNAP26b)*, *ratAT1aR*

The DNA sequence was synthesized and inserted into a pcDNA3.1(+) vector (between 5' HIndIII and XhoI 3' restriction sites at the multiple cloning site (MCS)) by GeneCust. In addition, a SalI restriction site was added directly in front of the XhoI site.

# 4 Results

As discussed in the introductory chapter, Ang1-7 has been described as a ***natural*** β-arrestin-biased ligand at the angiotensin II type 1 receptor (AT1R). This gives the compound a distinct position among the other β-arrestin-biased peptides reported at the AT1R, all of them of synthetic origin (Galandrin et al. 2016). The mode of action of Ang1-7 was originally characterized by its unique ability not to initiate canonical G protein signaling ("G protein-independent"), but instead to lead to β-arrestin-mediated phosphorylation of extracellular signal-regulated kinases ERK1/2 ("β-arrestin-dependent") (Teixeira et al. 2017). Although minor activation of G protein members of the $G\alpha_{12/13}$- and $G\alpha_{i}$-families was later reported for the Ang1-7 peptide (Namkung et al. 2018), its signaling has been characterized as not leading to activation of the potentially deleterious $G\alpha_{q}$-protein-axis (Teixeira et al. 2017; Namkung et al. 2018; Silva et al. 2020). In addition, both the non-activation of $G\alpha_{q}$-proteins and the signaling through β-arrestins are suggested to contribute to its cardioprotective effects and are currently under investigation in Covid-19 treatment (Paz Ocaranza et al. 2020).

Nevertheless, this hypothesized β-arrestin-dependent, $G\alpha_{q}$-protein-independent mode of action is at odds with recent findings of our and other groups demonstrating that β-arrestins are not required for the initiation of GPCR signaling to ERK1/2 (O'Hayre et al. 2017). Furthermore, "β-arrestin-mediated" ERK1/2-activation downstream of a GPCR has been shown to be completely dependent on the presence of active Gα-proteins (see also Introduction) (Grundmann 2018; Benkel et al. 2022; Alvarez-Curto et al. 2016). Thus, we decided to study whether the natural Ang1-7 peptide is a true $G\alpha_{q}$-protein-independent, β-arrestin-biased agonist at the AT1R and how this may contribute to its beneficial effects on the cardiovascular system.

For this purpose, we started our investigations with the most prominent property of the Ang1-7 peptide examining its ability to promote the recruitment of β-arrestins to the AT1R in our HEK293 cell system.

## 4.1 Ang1-7 mediates β-arrestin recruitment to the AT1R in a partial-agonistic manner.

To investigate the capability of Ang1-7 to induce the recruitment of β-arrestins to the AT1R, we took advantage of the Nanoluc®-Binary-Technology (NanoBiT®). This technology offers a suitable and sensitive method to determine protein-protein-interactions (PPI) in real-time (Dixon et al. 2016). It is based on a (Nano-)luciferase enzyme (Nanoluc®), that is splitted into a small (SmBiT) and a large subunit (lgBiT). SmBiT and lgBiT are fused to the respective proteins of interest. In our case, AT1R has been C-terminally tagged with SmBiT, while β-arrestin1 or 2 have been N-terminally fused to lgBiT. Once β-arrestins are recruited to the activated AT1R, SmBiT and lgBiT come close and complement each other to the full (Nano-)luciferase, which generates a bright luminescence from its substrate (Figure 20).

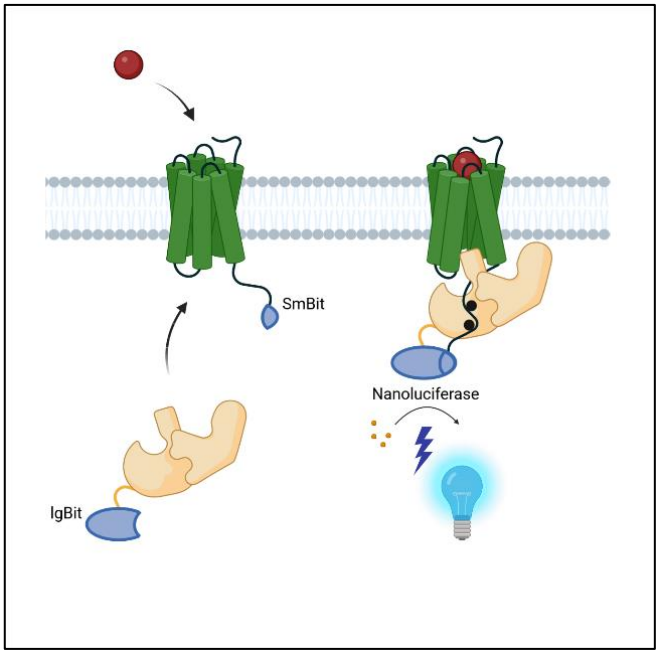


***Figure 20: (NanoBiT®)-β-arrestin recruitment assay principle**:*
*See text for details. Figure was created with "BioRender.com".*

As can be seen in Figure 21, stimulation of the AT1R by Ang1-7 resulted in a considerable concentration-dependent increase in luminescence, reflecting the recruitment of β-arrestins to the receptor.

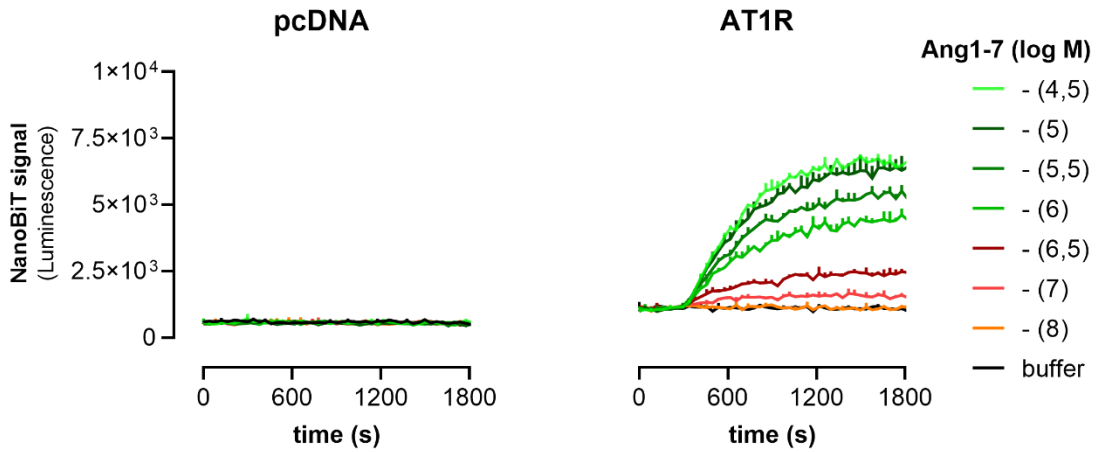

***Figure 21: Ang1-7-induced recruitment of β-arrestin1 to the AT1R:***

*Representative traces of wild-type HEK293 cells transiently expressing the AT1R-SmBiT or not (pcDNA) upon stimulation with increasing concentrations of Ang1-7 (as indicated). Cells were co-transfected with β-arrestin1-lgBiT, and the Ang1-7-promoted recruitment of β-arrestin1-lgBiT to the receptor was monitored for 30 minutes. Representative traces are mean+SD.*

For comparison, endogenous full agonist AngII was also used (Figure 22) which provoked a rapid and strong recruitment of β-arrestin1 and 2. Compared to AngII-mediated responses, we obtained efficacies of around 20-30% for Ang1-7, thus identifying Ang1-7 as a partial agonist for the recruitment of both β-arrestins to the AT1R (Figure 22 (B)).

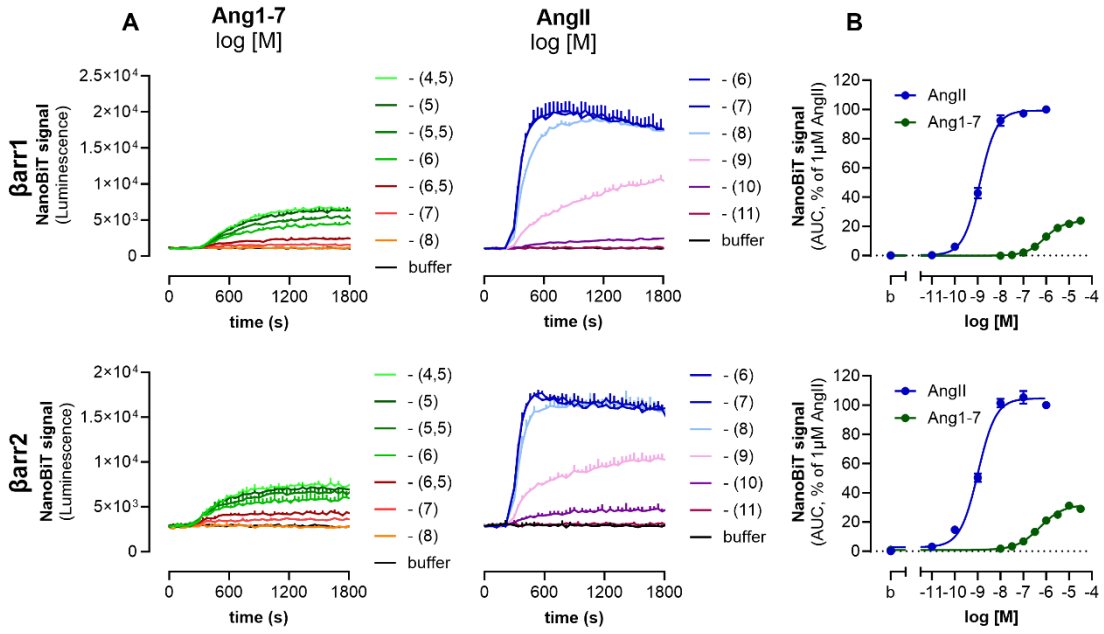


***Figure 22: Ang1-7- and AngII- provoked recruitment of β-arrestin1 or 2 to the AT1R in wt-HEK293 cells:***

*(A) Ang1-7- and AngII- promoted signals for the interaction of either β-arrestin1- (upper panel) or 2-lgBiT (lower panel) with the AT1R-SmBiT. (B) Summarized data depicted as concentration response curves of the AUC over 1800s normalized to 1µM AngII. Indicated curves consist of at least three independent experiments conducted as duplicate. Summarized data is presented as mean±SEM, while representative traces are mean+SD.*

Interestingly, differences in the generated basal luminescence for both β-arrestins could be detected. β-arrestin2 transfected HEK293 cells exhibited higher basal luminescence values than those obtained in the corresponding β-arrestin1 transfectants (Figures 22 (A) and 24 (A) - compare buffer luminescence values βarr1 vs. βarr2). Therefore, we wanted to find out if the increased luminescence was due to a higher expression of β-arrestin2 or as a result of

a “pre-recruitment” of β-arrestin2 to the AT1R. Since the lgBiT subunit shows a certain amount of intrinsic luminescence in the absence of SmBiT, this allowed us to infer possible differences in the expression of both β-arrestins. As shown in Figure 23, the augmented luminescence could not be explained by a better expression of the β-arrestin2 construct, as both β-arrestins showed a non-statistical slight difference in their basal luminescence without the AT1R (Figures 23 (A), beige traces, and 23 (B), left panel). However, when AT1R was coexpressed, basal luminescence was strongly increased in favor of β-arrestin2 (Figure 23 (A), brown traces, and 23 (B)), indicating a “pre-recruitment” or closer proximity of β-arrestin2 to the AT1R than β-arrestin1 from the beginning. This observation is in line with findings from other groups, showing β-arrestin2 to have a greater tendency to bind inactivated AT1R than β-arrestin1 (Kawakami et al. 2022).

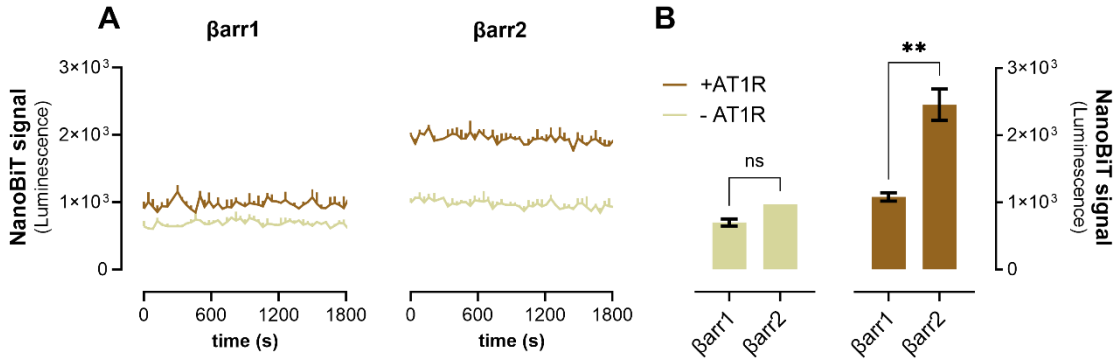


***Figure 23: Comparison of basal luminescence of both β-arrestin-lgBiT constructs with or without AT1R-SmBiT presence:***

*(A) representative experiment of wt-HEK293 cells expressing either βarrestin1- or 2-lgBiT in the presence or absence of AT1R-SmBiT. Traces shown represent the “buffer”-condition, not showing any changes in the basal luminescence over 1800s. (B) Summary, depicted as mean of buffer-luminescence over 1800s of four independent experiments for β-arrestin1±AT1R and β-arrestin2+AT1R; β-arrestin2 alone is n=1. Statistical analysis was calculated using a two-tailed, unpaired t-test with *P<0.05, **P<0.01, ns= non-significant.*

In our hands, Ang1-7 elicited recruitment of both β-arrestins to the AT1R with efficacies of around 20-30% compared to that of AngII. However, higher efficacy-values for Ang1-7 can be found in the literature (Galandrin et al. 2016; Teixeira et al. 2017; Namkung et al. 2018). Efficacy values are highly dependent on the method used. In addition, cellular composition and cellular expression of individual components also influence the extent of obtained responses. Thus, a specific HEK cell line, called HEK-**T** cells, has been widely used by other groups to measure β-arrestin recruitment. HEK-T cells owe their name to the fact that they harbor the SV40-**T**-antigen, which enables them to produce high copy numbers of plasmids

carrying an SV40 origin of replication (ORI) (Rio et al. 1985; Lin et al. 2014). Since our applied constructs harbor respective ORI, their transfection in HEK-T cells should lead to high expression levels of the proteins encoded on those plasmids. Therefore, we also conducted experiments in HEK-T cells to test whether our measured efficacies could be modified by switching the cell line.

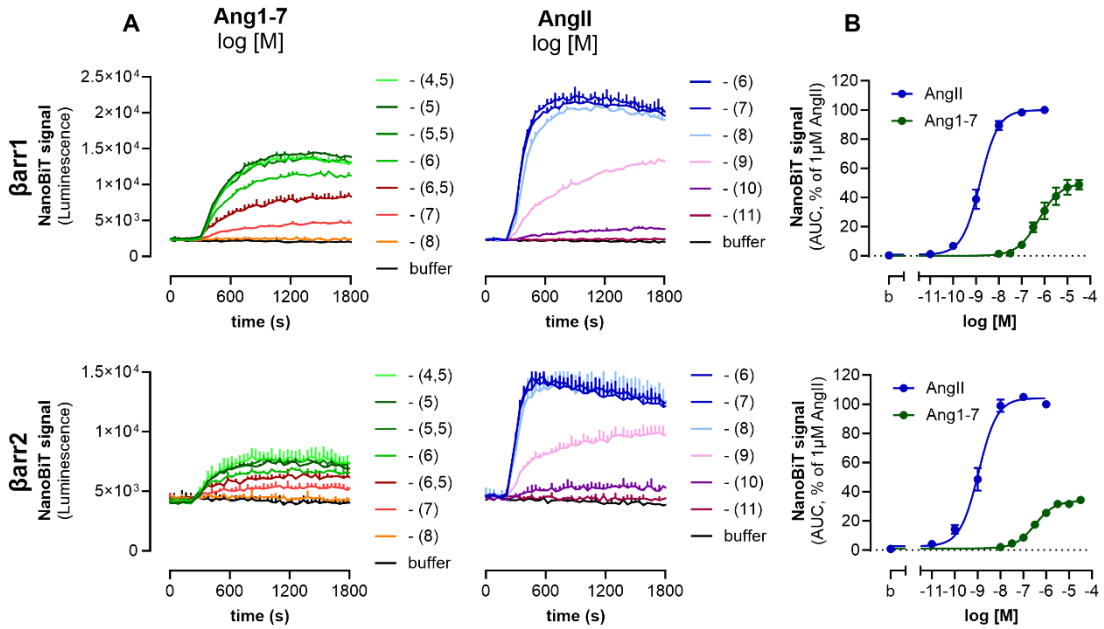


***Figure 24: Ang1-7- and AngII- provoked recruitment of β-arrestin1 or 2 to the AT1R in HEK-T cells:***

*(A) Ang1-7- and AngII- promoted signals for the interaction of either β-arrestin1- or 2-lgBiT with the AT1R-SmBiT. (B) Summarized data depicted as concentration response curves of the AUC over 1800s normalized to 1µM AngII. Indicated curves consist of at least three independent experiments conducted as duplicate. Summarized data is presented as mean±SEM, while representative traces are mean+SD.*

As displayed in Figure 24, HEK-T cells transiently expressing the AT1R-SmBiT showed consistent concentration dependent signals upon Ang1-7 activation. Both basal luminescence and maximal responses achieved for Ang1-7 were higher (Figure 24 (A)) compared to those determined in the previously used HEK293 cell line, suggesting higher protein abundance. Moreover, evaluated efficacies for Ang1-7 could also be improved in HEK-T cells for both β-arrestin isoforms, remaining around 40% compared to that of AngII (Figure 24 (B)).

Altogether, and in agreement with previous publications, our data show that Ang1-7 induces a consistent recruitment of β-arrestins to the AT1R, albeit partially agonistic compared to

the full agonist AngII. Because the quality of β-arrestin recruitment in response to Ang1-7 stimulation is similar in both HEK293 and HEK293-T cell lines, we chose to proceed our investigations with our HEK293 cell line since these cells are the parental origin of the genome-edited cells used later in the present study.

## 4.2 G proteins play a critical role in Ang1-7-induced ERK1/2-phosphorylation.

As mentioned earlier, activation of mitogen-activated protein kinases through a GPCR has been commonly used to study β-arrestin-mediated signaling, with ERK1/2-phosphorylation being the main readout (Gurevich and Gurevich 2018; Smith et al. 2018; Rankovic et al. 2016). *Teixeira et al.* found Ang1-7 to exhibit unique signaling modalities by not activating the $G\alpha_q$- and $G\alpha_{i3}$- subunit but promoting β-arrestin recruitment and ERK1/2-phosphorylation, thus characterizing Ang1-7 as a β-arrestin-biased agonist at the AT1R (Teixeira et al. 2017). Given this background and having demonstrated the ability of Ang1-7 to promote β-arrestin recruitment to the AT1R, we further investigated whether we could also detect ERK1/2-phosphorylation mediated by Ang1-7. A high time resolved FRET (HTRF®-) sandwich immunoassay was utilized, which offers a suitable method to detect signals proportional to phosphorylated ERK1/2 proteins (**pERK**) (Degorce et al. 2009). The assay principle is illustrated in Figure 25.

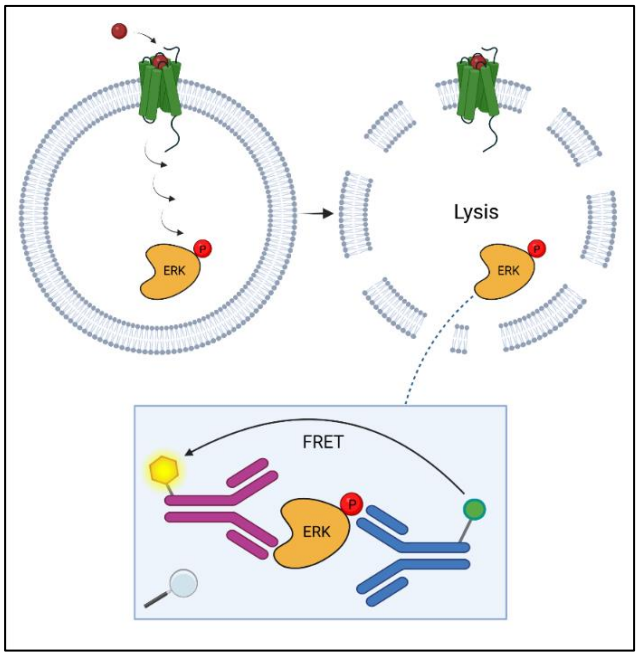


***Figure 25: ERK1/2-phosphorylation assay (pERK-assay):***

*AT1R stimulation leads to the phosphorylation of ERK1/2 kinases, which are detected by two antibodies after cell lysis. The first antibody linked with the HTRF®-donor specifically binds the phosphorylated epitope of ERK1/2 while the second antibody containing corresponding HTRF®-acceptor recognizes a binding site in close proximity. Light excitation results then in a high time resolved FRET (HTRF®) from donor to the acceptor molecule, which is proportional to phosphorylated ERK1/2 proteins. By analogy total- ERK1/2 protein is determined by two antibodies detecting unphosphorylated ERK1/2.*

*Figure adapted from CisBio-company. Figure was created with "BioRender.com".*

Initially, the *standard* phospho-ERK1/2 kit (CisBio) was used and the accumulation of pERK upon Ang1-7 and AngII treatment in wild-type HEK293 cells transiently overexpressing the AT1R was determined at different time points (Figure 26).

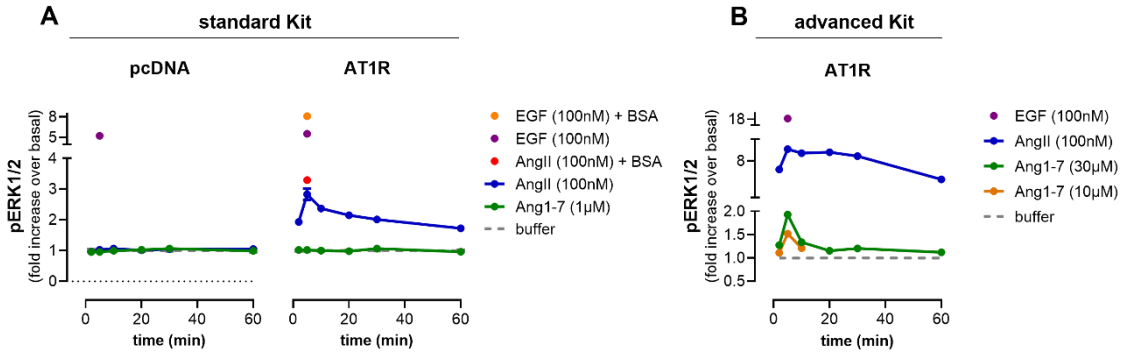


***Figure 26: Finding of optimal experimental conditions to measure Ang1-7-induced pERK responses:***

*(A) Representative ERK1/2-phosphorylation upon Ang1-7 or AngII stimulation in wild-type HEK293 cells transiently expressing the AT1R or empty vector (pcDNA). pERK responses were detected with the standard phospho-ERK1/2 kit (CisBio). Bovine serum albumin (BSA) was used at a concentration of 0.1%. (B). Further adaptations with more sensitive antibodies of the advanced-phospho-ERK1/2 kit as well as BSA supplementation and lysis buffer volume adjustment improved the measurement window for both ligands.*

As shown in Figure 26 (A), we could not detect measurable Ang1-7-promoted pERK accumulation using the standard phospho-ERK1/2 kit. Only AngII stimulation resulted in detectable pERK responses that were robust over 60 minutes and two to three times higher than basal levels. Since *Teixeira et al.* demonstrated the phosphorylation of ERK1/2 through the AT1R in response to Ang1-7 stimulation, we proceeded with the optimization of our experimental approach. One option is to add bovine serum albumin (BSA) to the assay-buffer to prevent proteins such as the angiotensin peptides from adhering to laboratory ware (96-well plates, Eppendorf tubes, etc.) (Schmitz 2011). As can be seen in Figure 26 (A), BSA supplementation resulted in a slight increase in AngII peptide-mediated pERK responses as well as in the Epidermal growth factor- (EGF-) protein control, but it could not widen the measurement window sufficiently to detect Ang1-7 responses. Another way to improve the measurement window is to use more sensitive antibodies, as provided by the CisBio *advanced*-phospho-ERK1/2 kit. Moreover, further adjustment was accomplished by reducing the amount of lysis buffer, which allowed for an optimal measurement range. After this optimization, determination of reliable and reproducible Ang1-7-mediated pERK signals was achieved, with the highest responses at a concentration of 30µM (Figure26 (B)).

In contrast to the full agonist AngII, which shows strong pERK responses that are constant for at least 30 minutes, we identified Ang1-7 to act in a weak partially agonistic manner and with a distinct kinetic profile (Figure 27). This profile is transient, with signals peaking rapidly between two and five minutes, but then declining sharply to basal levels. The kinetic profiles obtained for both angiotensin peptides were in complete agreement with previously published data (Teixeira et al. 2017).

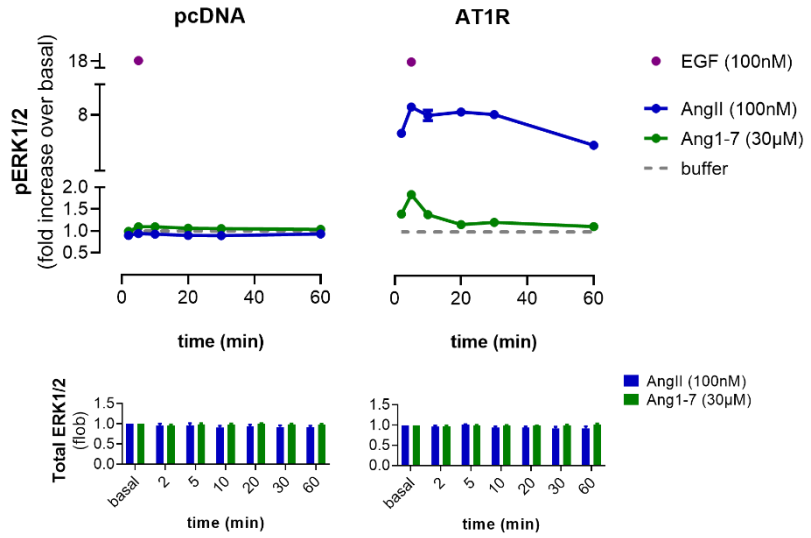


***Figure 27: ERK1/2-phosphorylation induced by Ang1-7 and AngII stimulation of the AT1R:*** *Kinetic pattern of Ang1-7-(30µM) and AngII-(100nM) stimulated wt-HEK293 cells transiently expressing the AT1R or empty vector (pcDNA) as control. Epidermal growth factor (EGF 100nM), which activates non-GPCR tyrosine kinase receptors, was used as a viability control. The data shown represent the summarization of three independent experiments performed in triplicate. Errors are expressed as mean±SEM. Bottom panel displays corresponding total ERK control values.*

Finding a suitable setup to measure reliable pERK responses for Ang1-7 allowed us to subsequently study this signal in more detail. The Ang1-7-evoked activation of ERK1/2 has been reported to be a consequence of its β-arrestin-biased agonism at the AT1R, in the absence of activation of heterotrimeric G proteins (Teixeira et al. 2017; Paz Ocaranza et al. 2020). However, as discussed earlier, the existence of G protein-independent, β-arrestin-mediated signaling from GPCRs to ERK1/2 has been challenged in the last decade. For example, *Grundmann et al.* have shown in a comprehensive study that Gα-proteins are essential for and the sole driver of GPCR-mediated ERK1/2-phosphorylation (Grundmann 2018). With this in mind, we used the selective $G\alpha_q$- (FR900359) and $G\alpha_i$- (pertussis toxin = PTX) protein inhibitors to investigate whether these G proteins are involved in Ang1-7-induced pERK accumulation at the AT1R.

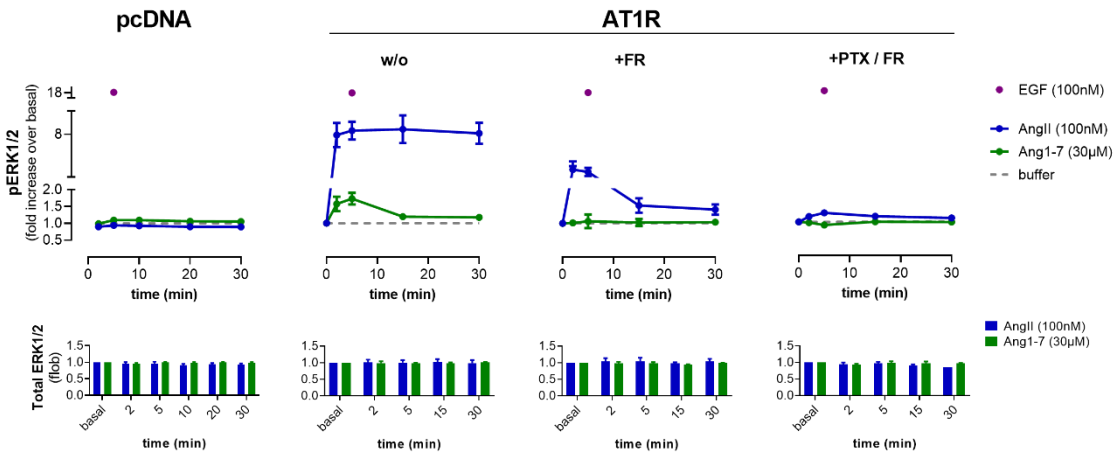


***Figure 28: Ang1-7- and AngII- promoted ERK1/2-phosphorylation in the presence of the specific Gα$_q$-(FR900359) and Gα$_i$-protein (PTX) inhibitors:***

*Kinetic pERK1/2 and totalERK1/2 profiles of wild-type HEK293 cells transiently expressing the AT1R or not (pcDNA) treated with Ang1-7 (30µM) or AngII (100nM) in the absence (w/o) or presence of the specific Gα-protein inhibitor FR (1µM) alone (+FR) or together with PTX (+PTX/FR). Data is shown as mean±SEM of at least three independent experiments, performed in triplicate.*

For the full agonist AngII, pERK accumulation was markedly reduced by FR treatment, showing the known contribution of Gα$_q$ to its signal, but significantly changing the kinetics from a sustained to a more transient profile (Figure 28, (+FR)). This profile was nearly attenuated when Gα$_i$ -proteins were simultaneously blocked with PTX (+PTX/FR), suggesting that the observed transient signal for AngII is due to Gα$_i$ activation when Gα$_q$-proteins are blocked.

Strikingly and to our surprise, inhibition of Gα$_q$ by FR900359 (FR) diminished Ang1-7-mediated ERK1/2-phosphorylation (Figure 28, (+FR)), suggesting that Gα$_q$-proteins play a central role in Ang1-7-induced ERK1/2-activation, which is in contrast to previous findings showing that Gα$_q$-proteins are little or not activated by the β-arrestin-biased Ang1-7 (Galandrin et al. 2016; Namkung et al. 2018). In addition, β-arrestins, which are endogenously expressed in our HEK293 cells, did not seem to initiate ERK1/2-phosphorylation by themselves, as there was no detectable pERK accumulation for Ang1-7 when Gα$_q$ and Gα$_i$ were blocked by their respective inhibitors (Figure 28, (+PTX/FR)).

In summary, our data suggest that G proteins play a key role in Ang1-7-induced activation of ERK1/2 through the AT1R. Even more fascinating, the Gα$_q$-protein subunit, which has

been reported not to be activated by Ang1-7, appears to be the major driver of Ang1-7-mediated ERK1/2-activation.

## 4.3 $G\alpha_q$-proteins are involved in the Ang1-7-promoted signal transduction at the AT1R.

Our observation of a central role for $G\alpha_q$ in Ang1-7-mediated pERK accumulation is apparently at odds with recent reports that Ang1-7 does not lead to canonical G protein (or at least $G\alpha_q$) activation (Galandrin et al. 2016; Teixeira et al. 2017; Namkung et al. 2018). Therefore, we were curious if we could also detect Ang1-7 activation by commonly used readouts of the AT1R-$G\alpha_q$ axis.

***Figure 29: Classical AT1R-$G\alpha_q$-signaling axis:***

*(1)-(3) show critical steps of the cascade from "upstream" to "downstream", beginning with the dissociation of the heterotrimeric $G\alpha_q$-protein (1), followed by $IP_3$ release (2) and subsequent $Ca^{2+}$ mobilization into the intracellular space (3). The blue box indicates the respective assays applied at the different levels of the cascade. Figure was created with "BioRender.com".*

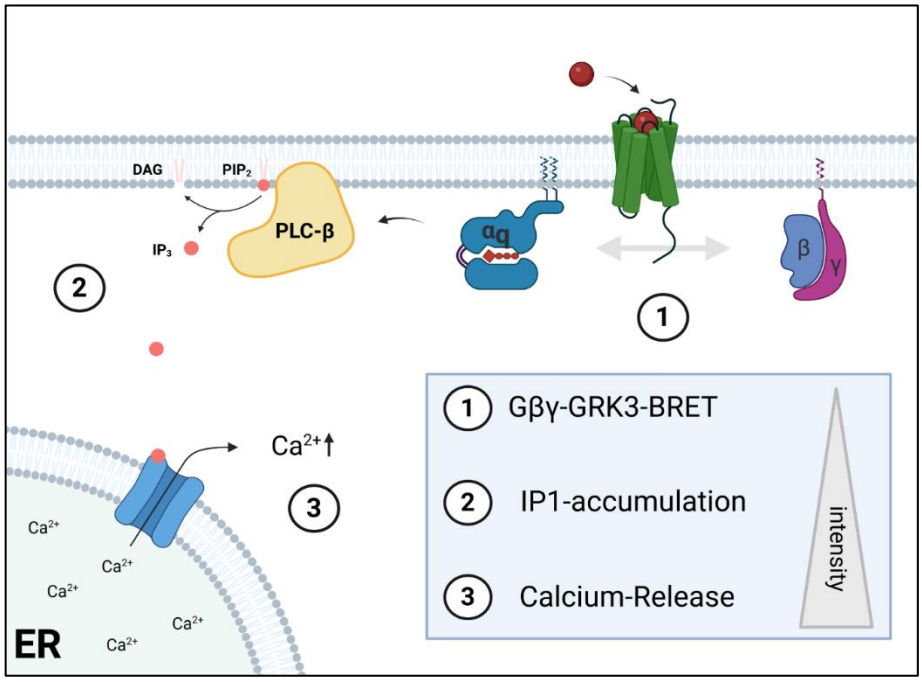


Figure 29 illustrates the classical AT1R-$G\alpha_q$ axis. As portrayed, activation of the AT1R leads to dissociation of the heterotrimeric $G\alpha_q\beta\gamma$ protein into the $G\alpha_q$- and $G\beta\gamma$-subunits. $G\alpha_q$ then activates phospholipase C$\beta$ which in turn cleaves the membrane phospholipid $PIP_2$ into DAG and $IP_3$. $IP_3$ diffuses through the cytoplasm to its receptor which is a calcium ion channel, localized to the endoplasmic reticulum (ER) membrane. Binding of $IP_3$ then leads to opening of the channel and the release of calcium ions from the ER into the cytosol, accompanied by further calcium- regulated responses (McCudden et al. 2005). In this context, dissociation of the heterotrimeric G protein is considered the upstream event of the cascade, $IP_3$ formation the midstream event, and calcium release the downstream event. Moreover, the signal

strength is amplified from upstream to downstream, which facilitates the detection of downstream events (Figure 29, blue box).

To study a potential Ang1-7-induced activation of classical readouts of the AT1R-G$\alpha_q$ axis, we used assay systems located at the three different levels of the cascade from upstream to downstream. Firstly, our upstream approach was the G$\beta\gamma$-GRK3-BRET assay (Figure 30), which provides a way to monitor the release of the G$\beta\gamma$-subunit from the G$\alpha_q\beta\gamma$ heterotrimer or, more precisely, its interaction with a GRK3 derivative, which indirectly indicates that dissociation of the heterotrimer must have occurred.

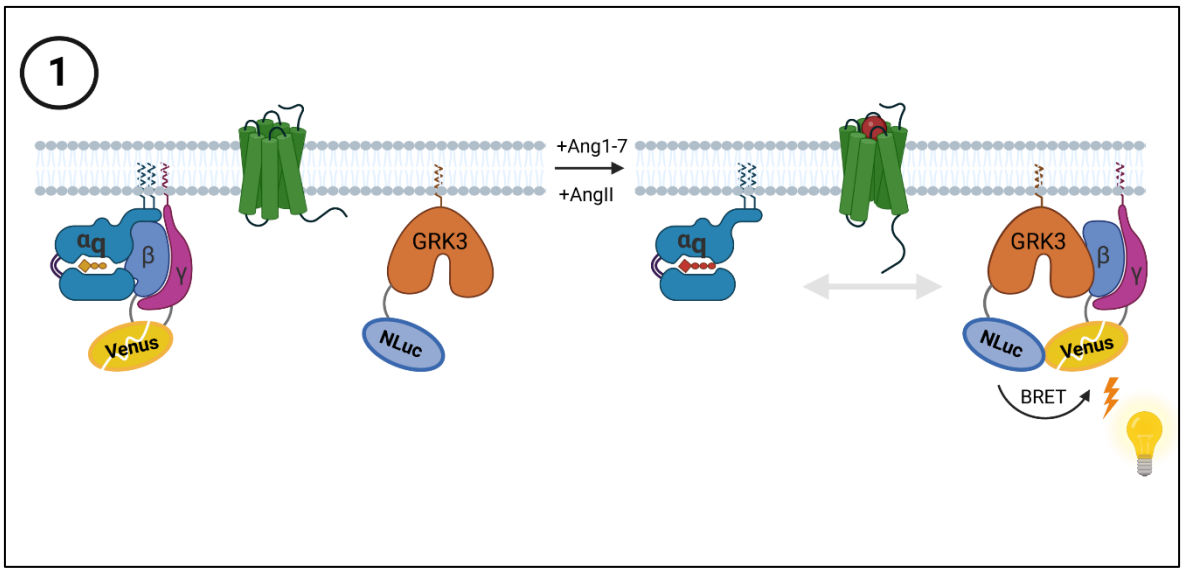


***Figure 30: Gβγ-GRK3-BRET assay principle:***

*Activated AT1R leads to the separation of the heterotrimeric G$\alpha_q\beta\gamma$ protein into G$\alpha_q$ and G$\beta\gamma$, of which the G$\beta\gamma$-subunit is fused to a Venus energy acceptor protein. G$\beta$ and G$\gamma$ are each associated with corresponding parts of the Venus protein (split Venus), thus only the G$\beta\gamma$-dimer forms a functional Venus protein. G$\beta\gamma$ can now bind to a modified GRK3 (see Methods), which is linked to a Nano-luciferase (Nluc) and anchored to the membrane. Nluc (energy donor) and Venus (energy acceptor) are now in close proximity resulting in an increase in the BRET signal. Figure was created with "BioRender.com".*

After $IP_3$ is generated by PLC-β activation, its degradation is initiated simultaneously with the onset of $IP_3$ dephosphorylation via $IP_2$ to $IP_1$. Further $IP_1$ degradation can be effectively blocked by LiCl (lithium chloride), making $IP_1$ accumulation under these conditions an ideal proxy for AT1R-induced $IP_3$ production (Figure 31). This was used as our midstream approach.

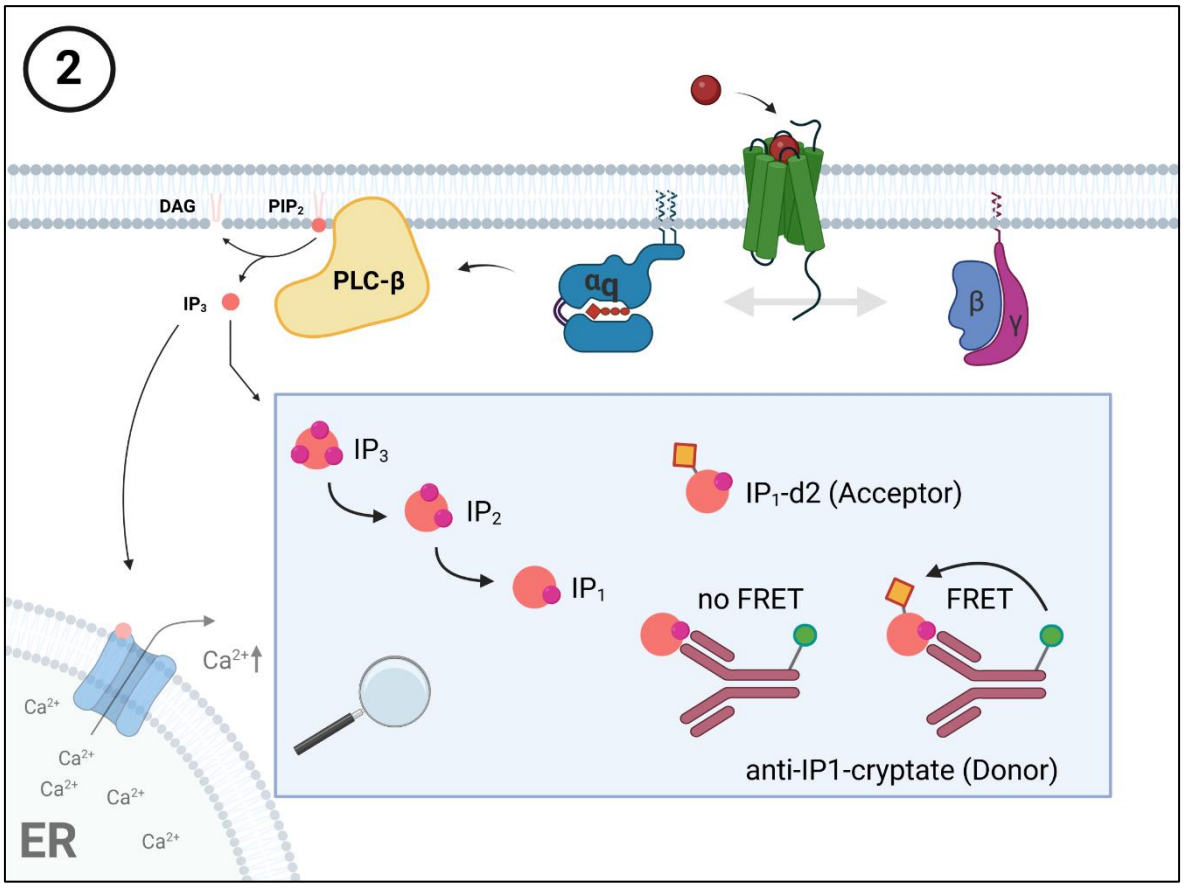


***Figure 31: (HTRF®)-IP$_1$-accumulation assay principle:***

*For the detection of inositol monophosphate (IP$_1$), an IP$_1$-directed antibody coupled to an energy donor (terbium-cryptate) is used. In addition, "artificial" IP$_1$ molecules (IP$_1$-d2) carrying an energy acceptor (d2) are added. The antibody binds the IP1-d2 and a FRET signal is generated when the donor is excited (blue box, right). Activation of the AT1R-G$\alpha_q$ pathway leads to the formation of natural IP$_1$, which now competes with the artificial IP$_1$-d2 for the antibody binding site. The more natural IP$_1$ is formed, the more IP$_1$-d2 is displaced by the antibody and the lower the measured FRET. Figure was created with "BioRender.com".*

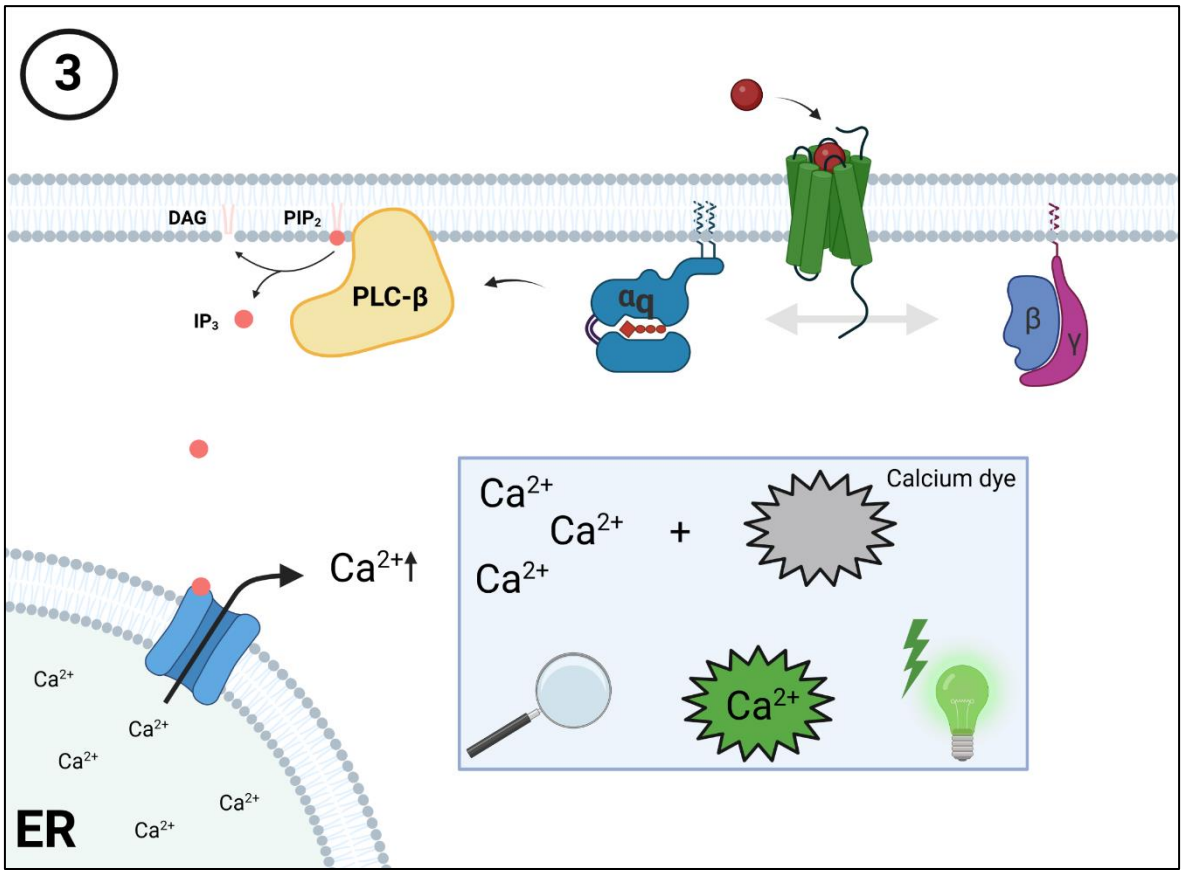


***Figure 32: Principle of the Calcium-mobilization assay:***

*Changes in intracellular calcium are detected with a fluorescence-based approach that uses a calcium-sensitive dye that penetrates the intracellular space. When calcium accumulates intracellularly (for example, after AT1R-G$\alpha_q$ activation), it binds to the dye, followed by an increase in the fluorescence intensity of the dye. Figure was created with "BioRender.com".*

Finally, we examined as downstream approach whether we could detect an AT1R-evoked increase in cytosolic calcium upon stimulation by Ang1-7 (Figure 32).

For our upstream Gβγ-GRK3-BRET approach, we utilized HEK293 cells that have been genome-edited using CRISPR-Cas9 technology. These cells are depleted of the seven Gα-protein subunits (G$\alpha_{q/11}$, G$\alpha_{s/olf}$, G$\alpha_{12/13}$ and G$\alpha_z$), hence the name Δseven cells, but still express the endogenous PTX-blockable G$\alpha_{i/o}$ protein members (Benkel et al. 2022). A cellular background devoid of all functional Gα proteins is accomplished by PTX pre-treatment of Δseven cells (Δseven+PTX = "zero functional G") (Grundmann 2018). Thus, re-expression of the G$\alpha_q$ subunit then allowed us to specifically study the activation of this subunit after transiently expressing the AT1R. As can be observed in Figure 33 (A), stimulation with the full agonist AngII led to a rapid increase in the monitored ΔBRET signal, with the highest responses at 100nM to 1µM. Ang1-7, in contrast, caused no

detectable change in the ΔBRET (Figure 33 (A and B)), consistent with the literature reporting $G\alpha_q$ not to be activated by Ang1-7 (Galandrin et al. 2016; Teixeira et al. 2017; Namkung et al. 2018). However, as we detected a clear $G\alpha_q$ involvement for Ang1-7-promoted pERK accumulation, we hypothesized that activation of $G\alpha_q$ -mediated by Ang1-7 may be problematic to detect using this upstream assay.

Consequently, for the following midstream approach, the $IP_1$ accumulation, we took advantage of a wild-type HEK293 cell line stably expressing the AT1R, thus providing a constant level of translated receptor. Here, $IP_1$ production was observed after treatment with high concentrations of Ang1-7 peptide, but very weak compared to AngII (Figure 33 (C-E)). Although we did observe a trend toward $IP_1$-accumulation with increasing concentrations of Ang1-7, it did not result in a statistically significant increase in the measured $IP_1$-production at 100µM (Figure 33 (E)).

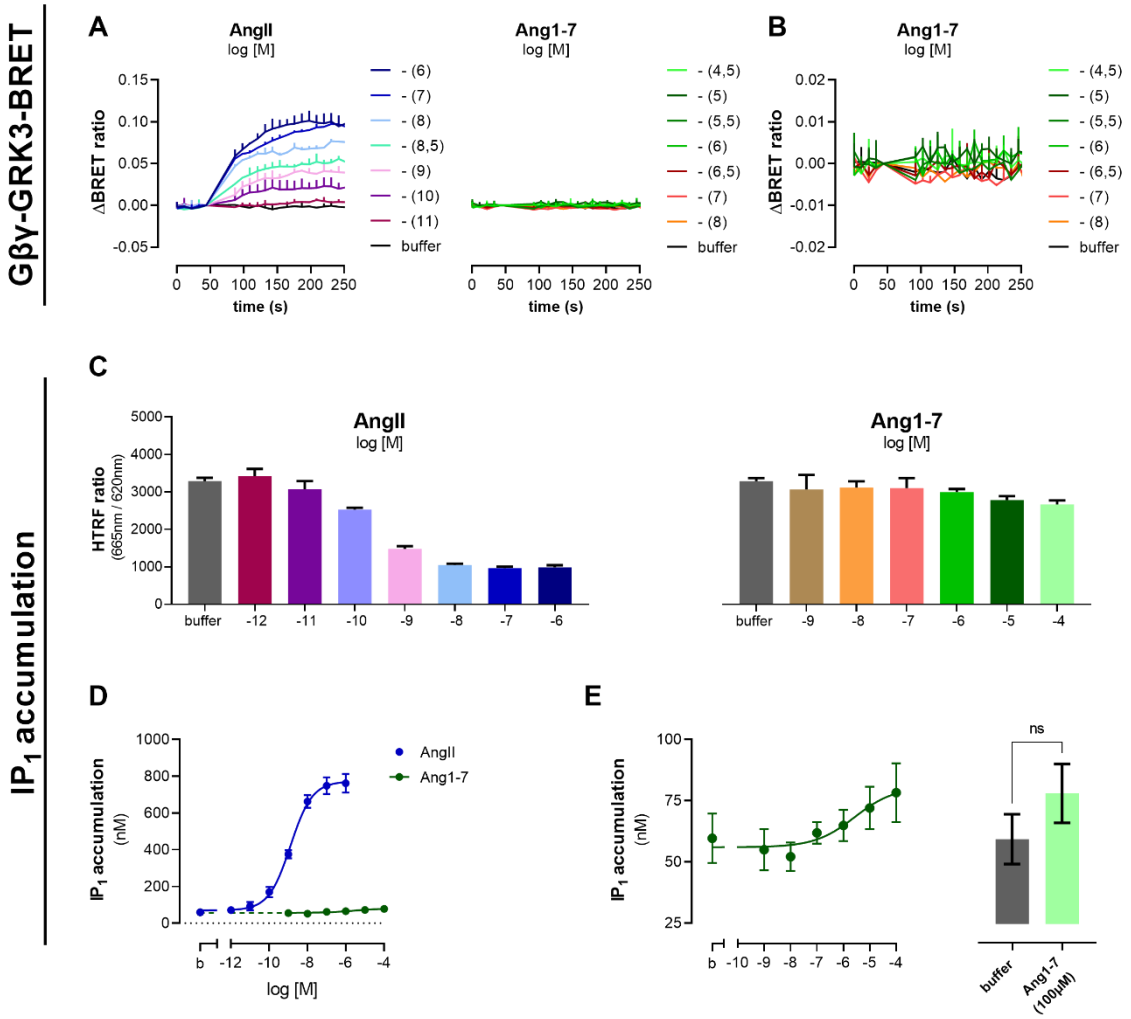

***Figure 33: Effects of Ang1-7 and AngII on the classical AT1R-Gα$_q$ axis using Gβγ-GRK3 interaction and IP$_1$ accumulation as readouts:***

*Representative traces of the Gβγ-GRK3-interaction upon Ang1-7 and AngII stimulation measured as changes in the BRET ratio. Δseven+PTX-cells stably expressing the AT1R were used and the Gα$_q$ subunit was reintroduced for analysis among the other constructs (see Materials and Methods) (Posokhova et al. 2013). (B) "Zoom in" on the protein interaction induced by Ang1-7 shown in (A). (C) Representative IP$_1$-accumulation-HTRF®- ratios in wt-HEK293 stably expressing the AT1R for AngII and Ang1-7 applied as indicated in the legend. High IP$_1$ accumulation results in a reduction of the HTRF®- ratio (see assay principle). (D) Summary of AngII- and Ang1-7- (or Ang1-7 alone (E)) provoked IP$_1$-accumulation, converted and shown as nanomolar IP$_1$ levels. Representative data represent a technical duplicate +SD for Gβγ-GRK-BRET and triplicate for IP1 +SD. Summary IP1 is n = 9 ±SEM. Statistical significance was assessed by a one-sample t-test. ns indicates non-significant.*

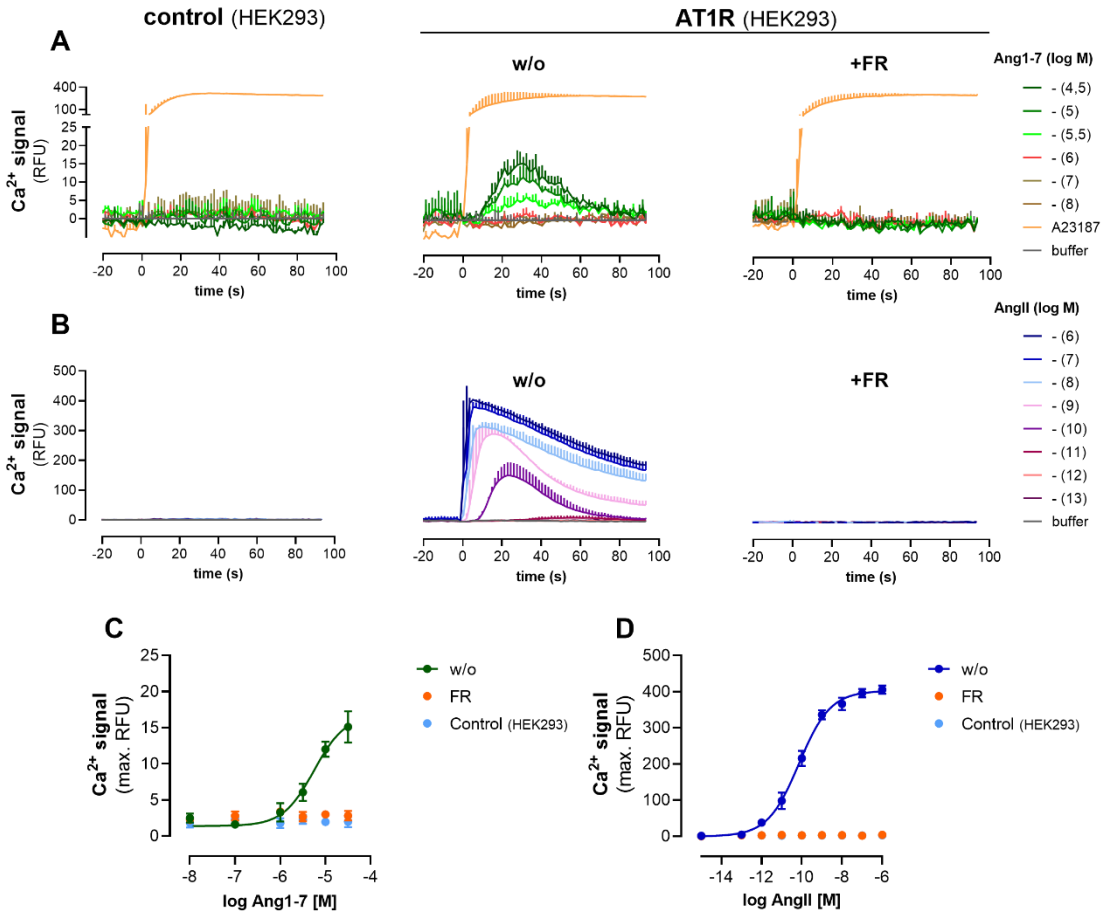


***Figure 34: Ang1-7- and AngII- mediated Ca$^{2+}$ responses at the AT1R:***

*Representative Ca$^{2+}$-responses within 90s monitored for wild-type HEK293 cells stably expressing the AT1R or not (control HEK293) (left) stimulated with Ang1-7 (A) or AngII (B) in the absence (w/o) or presence of the specific Gα$_q$ inhibitor FR (1µM) (+FR). Calcium ionophore A23187 (5µM) was applied as a viability control.*

*Data are summarized as concentration-effect curves (max. response) for Ang1-7 (C) and AngII (D). Representative traces are shown as means+SD, concentration-effect curves are means±SEM of at least three biological replicates performed as duplicates.*

Since we observed a tendency for Ang1-7 to induce $IP_1$-accumulation in our wild-type HEK293 cells stably expressing the AT1R, we next performed the $Ca^{2+}$ release experiments in these cells. Here, Ang1-7 treatment resulted in a concentration-dependent $Ca^{2+}$-mobilization (Figure 34 (A)). The increase in $Ca^{2+}$-mobilization was entirely AT1R specific, as control HEK293 did not elicit $Ca^{2+}$-release for either Ang1-7 (Figure 34 (A)) or the full agonist AngII (Figure 34 (B)). Ang1-7- mediated $Ca^{2+}$-responses were smaller in amplitude than those observed for AngII, demonstrating a very weak partial agonism for Ang1-7 at this pathway (Figure 34 (C and D)). Moreover, both Ang1-7- and AngII-provoked $Ca^{2+}$-responses were completely $G\alpha_q$-mediated, as can be seen from the loss of detected $Ca^{2+}$-mobilization when cells were pretreated with FR. Of note, a difference in the kinetic profile of the evoked signals was again found between the two peptides, with Ang1-7 evoking a short and transient pattern, whereas AngII produced a sustained increase in intracellular calcium at high concentrations.

Altogether, and in line with our findings in the previous pERK experiments (Figure 28), our data suggest that AT1R activation by Ang1-7 is followed by the activation and signal transduction through $G\alpha_q$-proteins. Both the Ang1-7-induced pERK- and $Ca^{2+}$-responses show a transient kinetic pattern (Figures 28 and 34), which may indicate a different mode of $G\alpha_q$-protein activation by Ang1-7 compared with AngII. Furthermore, our data show that Ang1-7 activation of the $G\alpha_q$ is difficult to detect depending on the chosen readout, which would explain its reputation as a non-$G\alpha_q$-protein activator (Paz Ocaranza et al. 2020; Silva et al. 2020; Teixeira et al. 2017; Galandrin et al. 2016).

## 4.4 Ang1-7 induces distal downstream signaling via Gα-proteins.

Next, we wanted to gain a deeper knowledge of Ang1-7 signaling at the AT1R, particularly with regard to its previous characterization as β-arrestin-biased agonist. To do this, we chose to employ the highly sensitive method of label-free dynamic mass redistribution (DMR) which allows to measure changes in cell morphology as a result of global cell activation in real time (Schröder et al. 2010; Schröder et al. 2011). The detailed assay principle is illustrated in Figure 35.

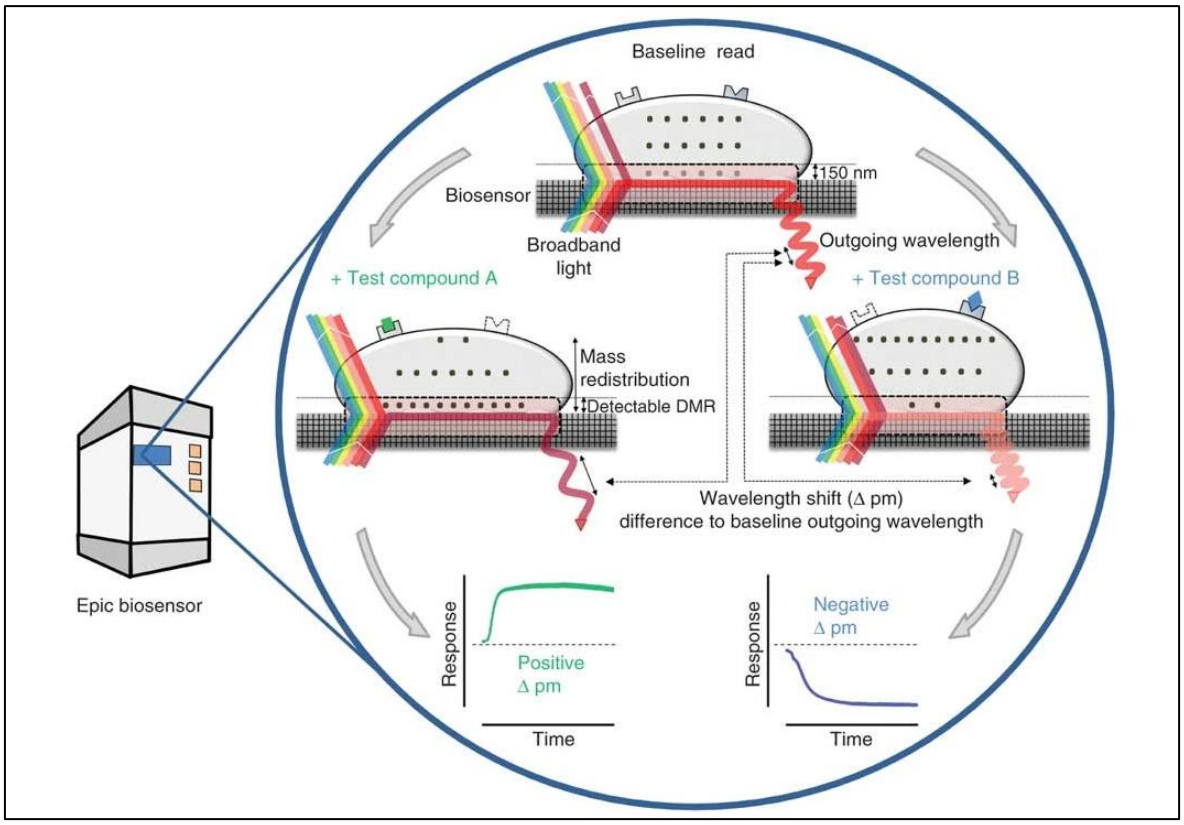


***Figure 35: Scheme for detection of dynamic mass redistribution (DMR):***

*To monitor global cell activation by dynamic mass redistribution, cells are seeded on an optical biosensor that is illuminated with broadband light, resulting in reflection of a specific wavelength ("baseline read"). Stimulation of a receptor leads to changes in cell morphology accompanied by mass redistribution in close proximity to the sensor. This in turn results in a shift of the reflected wavelength which is detected over time (adapted from Schröder et al. 2011).*

Ang1-7 treatment of wild-type HEK293 cells transiently expressing the AT1R caused concentration-dependent changes in cell morphology, as measured by substantial upward shifts in the detected reflected wavelengths (Figure 36 (A)). Ang1-7-induced global cell activation was of lower amplitude and potency compared to the full agonist AngII (Figure 36 (B)), again demonstrating that Ang1-7 acts as a partial agonist in terms of signaling at the AT1R. Concentration-dependent traces of Ang1-7 are shown with a greater magnification in Figure 37. In addition, in Ang1-7-induced DMR responses, a time-dependent transient component was observed that peaks after a short time but decays rapidly and remains constant (Figure 37). AngII, on the other hand, again produced a strong and sustained response over the entire measurement period.

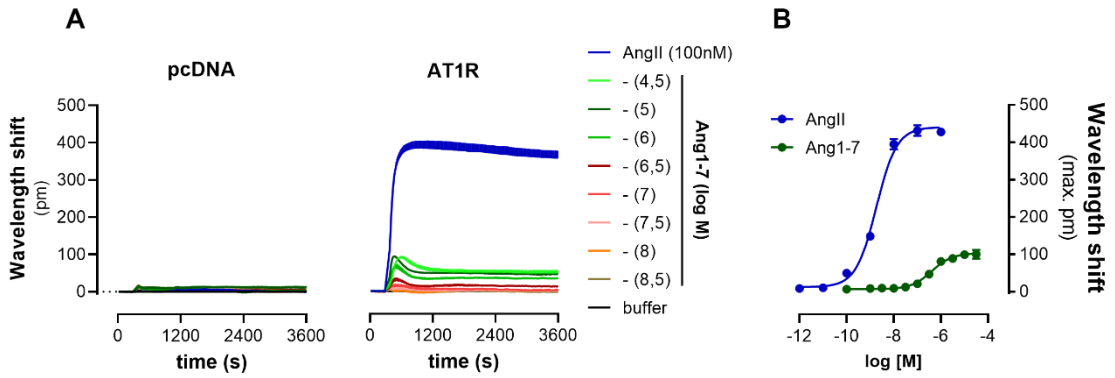


***Figure 36: Cell morphological changes measured by dynamic mass redistribution (DMR):***

*Wild-type HEK293 cells transiently transfected with plasmid-DNA encoding the AT1R or with empty vector (pcDNA) and stimulated with Ang1-7 and AngII as indicated. (A) Optical recordings are depicted as mean+SD of a representative experiment performed in technical triplicates. (B) Corresponding concentration effect curves to (A) with concentrations plotted against maximal provoked effects. Ang1-7 is n=7, AngII is n=3. Data is shown as mean±SEM.*

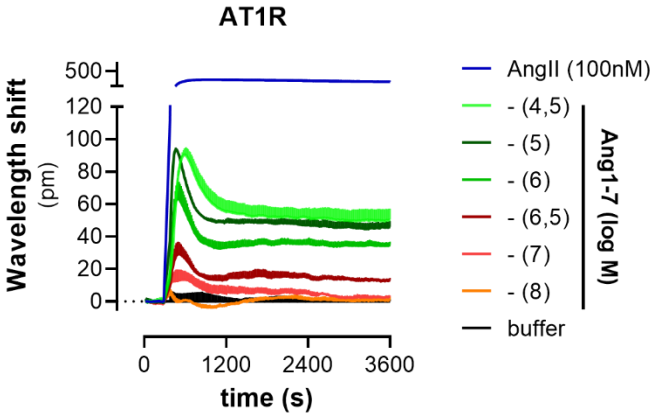


***Figure 37: Ang1-7-evoked cell morphological changes by AT1R stimulation:***

*"Zoom in" of the representative DMR recordings of the experiment shown in Figure 36 (A).*

Since we were able to measure appropriate Ang1-7 DMR traces, we wanted to further investigate these observed changes in cell morphology for the involvement of G proteins. Analogous to our ERK1/2-phosphorylation measurements, we therefore followed Ang1-7-mediated changes in cell morphology in the presence of the specific $G\alpha_q$- and $G\alpha_i$-inhibitors (FR and PTX). As shown in Figure 38, concentration-dependent responses elicited by Ang1-7 were substantially reduced by blocking $G\alpha_q$-proteins (+FR). These responses were further reduced by PTX treatment, demonstrating a contribution of $G\alpha_q$- and $G\alpha_i$-proteins in Ang1-7-promoted downstream signaling.

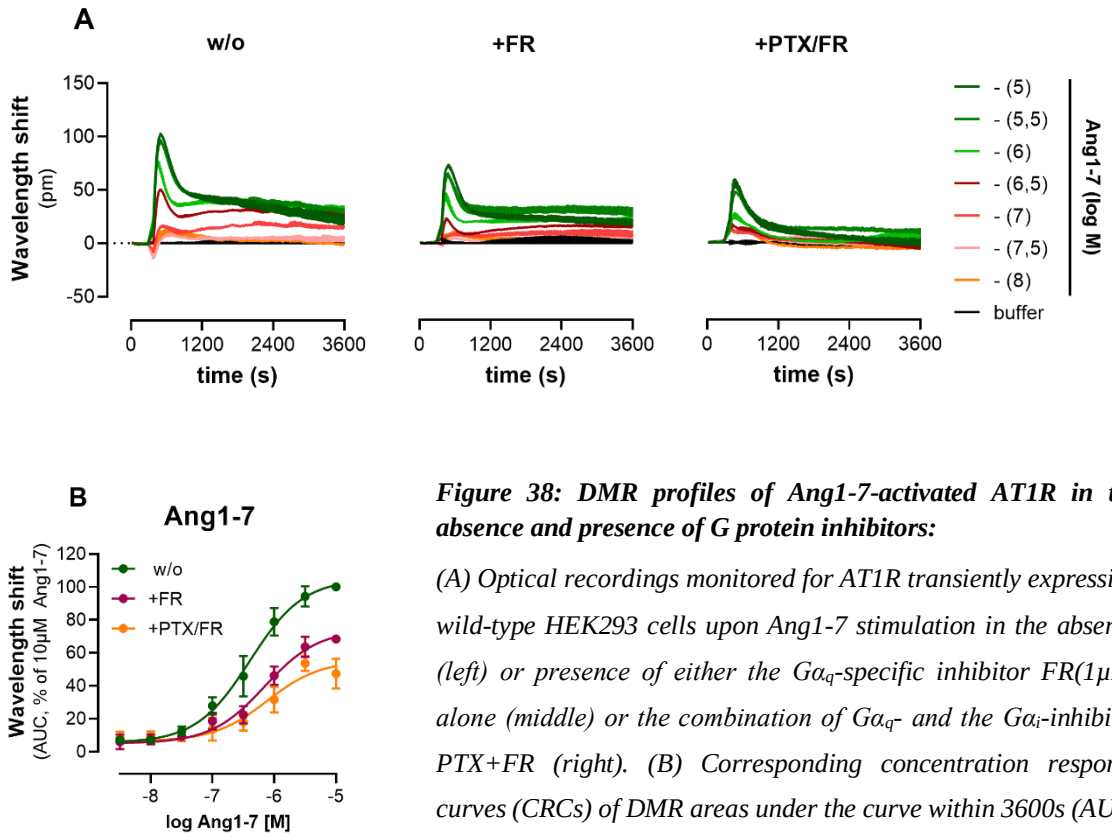


***Figure 38: DMR profiles of Ang1-7-activated AT1R in the absence and presence of G protein inhibitors:***

*(A) Optical recordings monitored for AT1R transiently expressing wild-type HEK293 cells upon Ang1-7 stimulation in the absence (left) or presence of either the $G\alpha_q$-specific inhibitor FR(1µM) alone (middle) or the combination of $G\alpha_q$- and the $G\alpha_i$-inhibitor PTX+FR (right). (B) Corresponding concentration response curves (CRCs) of DMR areas under the curve within 3600s (AUC) in relation to Ang1-7 (10µM) response in the absence of inhibitors (w/o). Optical recordings are shown as mean+SD and were performed in technical triplicate. CRCs represent four biologically independent experiments, presented as mean±SEM.*

The same signaling profile for Ang1-7 was also observed in HEK293 cells stably expressing the AT1R (Figure 39). In comparison to Ang1-7, responses of the full agonist AngII were stronger reduced in the presence of FR, underlining that AngII-promoted signaling at the AT1R predominantly relies on $G\alpha_q$ -activation (Tóth et al. 2018b). In addition to the strong contribution of $G\alpha_q$, a lower contribution of $G\alpha_i$-proteins could also be shown for AngII when an additional PTX pretreatment of the cells was conducted. This is fully consistent with the literature reporting that AngII stimulation of the AT1R leads to activation of Gα-protein members of the $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$ families, with $G\alpha_q$ being preferentially activated (Saulière et al. 2012; Ushio-Fukai et al. 1998; Kawai et al. 2017).

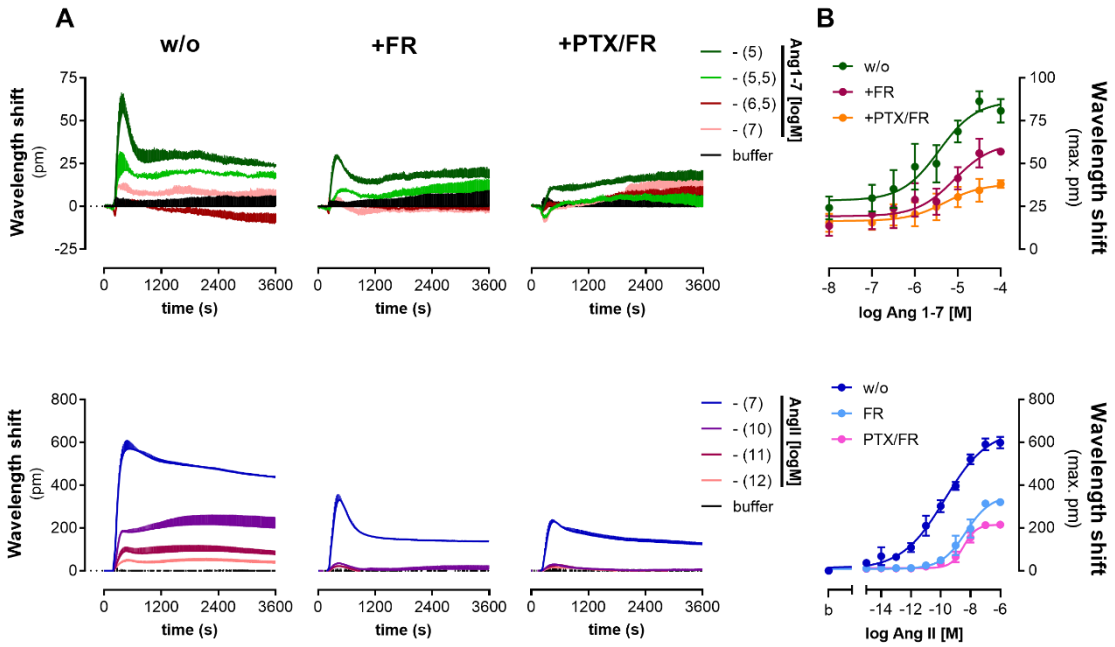


***Figure 39: Comparison of Ang1-7- and AngII- mediated whole cell activation through the AT1R in the absence and presence of G protein inhibitors:***

*(A) Representative DMR recordings of wild-type HEK293 cells stably expressing the AT1R upon Ang1-7 (top, panel) and AngII (bottom, panel) stimulation in the absence (w/o) or presence of the $G\alpha_q$ (+FR) (1µM) or $G\alpha_q$-plus $G\alpha_i$-inhibitor (+PTX/FR). (B) Summarized data of Ang1-7 (top) and AngII (bottom) presented as concentration response curves (maximum response) without (w/o) or with $G\alpha_q$ (+FR) or $G\alpha_i$-inhibition (+PTX/FR), respectively. DMR traces are mean+SD and represent one of four experiments performed independently in technical triplicate and summarized in the CRCs shown as mean±SEM.*

We also observed that a remaining DMR signal is detected after FR/PTX inhibition. Since endogenous $G\alpha_{12/13}$ -proteins are still present and activatable in the presence of both inhibitors, it could be assumed that the residual signal for AngII and Ang1-7 is due in whole or in part to this Gα-protein family.

In conclusion, our DMR data highlight a clear involvement of $G\alpha_q$- and $G\alpha_i$-proteins in the Ang1-7-promoted signaling through the AT1R, compared to the completely $G\alpha_q$-mediated $Ca^{2+}$-mobilization and ERK1/2-phosphorylation responses. Therefore, our findings show that Ang1-7, despite other reports (Teixeira et al. 2017; Galandrin et al. 2016), causes canonical Gα-protein signaling and cannot be considered Gα-protein independent. Most importantly, in contrast to the prevailing view of Ang1-7 as a non-$G\alpha_q$ activator (Paz Ocaranza et al. 2020), we identify a distinct, albeit weak compared to AngII, involvement of

$G\alpha_q$ for Ang1-7 using highly sensitive methods such as measurement of amplified effectors ($Ca^{2+}$ and pERK) or whole-cell responses (DMR).

## 4.5 β-arrestins are dispensable for Ang1-7-induced DMR and ERK1/2-phosphorylation.

In the concept of β-arrestin signaling, β-arrestins and G proteins are considered separate signal transducers (see also Introduction). Thus, β-arrestin-biased agonism is defined by the fact that β-arrestins can mediate signaling independently of G proteins (G protein-independent vs. β-arrestin-dependent) (Dewire et al. 2007; Dewire et al. 2008; Wei et al. 2003; Rankovic et al. 2016; Smith and Rajagopal 2016). The characterization of Ang1-7 as an AT1R β-arrestin-biased agonist was based on its described ERK1/2-stimulation without $G\alpha_q$ activation (Silva et al. 2020). Furthermore, β-arrestin-biased signaling was thus regarded to contribute to Ang1-7's cardiac antihypertrophic effects (Teixeira et al. 2017; Paz Ocaranza et al. 2020).

According to our results, we could no longer assume $G\alpha_q$-protein independence for Ang1-7 signaling at the AT1R. On the other hand, a remaining DMR signal is detected after FR/PTX inhibition, which could be due to a potential β-arrestin-biased signaling. Therefore, we decided to investigate the presumed role of β-arrestins in Ang1-7 signaling more closely. For this purpose, we used knockout HEK293 cells in which both non-visual arrestins (β-arrestin1/2) were deleted by CRISPR/Cas9 genome editing, hereinafter referred to as Δarr cells. Δarr cells were then genetically modified to stably express the AT1R (see Methods).

Because our DMR approach allowed us to adequately detect Ang1-7 signaling in HEK293 cells stably expressing the AT1R, we chose this assay initially to assess the global cell activation triggered by this peptide. As can be seen from Figure 40, despite the lack of β-arrestins, Ang1-7-induced changes in cell morphology were unaffected and resembled closely in their signaling pattern those obtained in wild-type HEK293 cells (compare Figures 39 and 40). Therefore, the monitored responses elicited by Ang1-7 were not attributable to β-arrestins. This was also true for the non-biased full agonist AngII. Only inhibition of $G\alpha_q$ and $G\alpha_i$ markedly reduced the provoked responses, indicating that these Gα-proteins are strongly involved in signal transduction. Furthermore, as seen in wild-type HEK293 cells (Figure 39), weak responses for both peptides were also preserved in Δarr cells when $G\alpha_q$- and $G\alpha_i$-proteins were blocked, underscoring that these residual signals are not induced by β-arrestins.

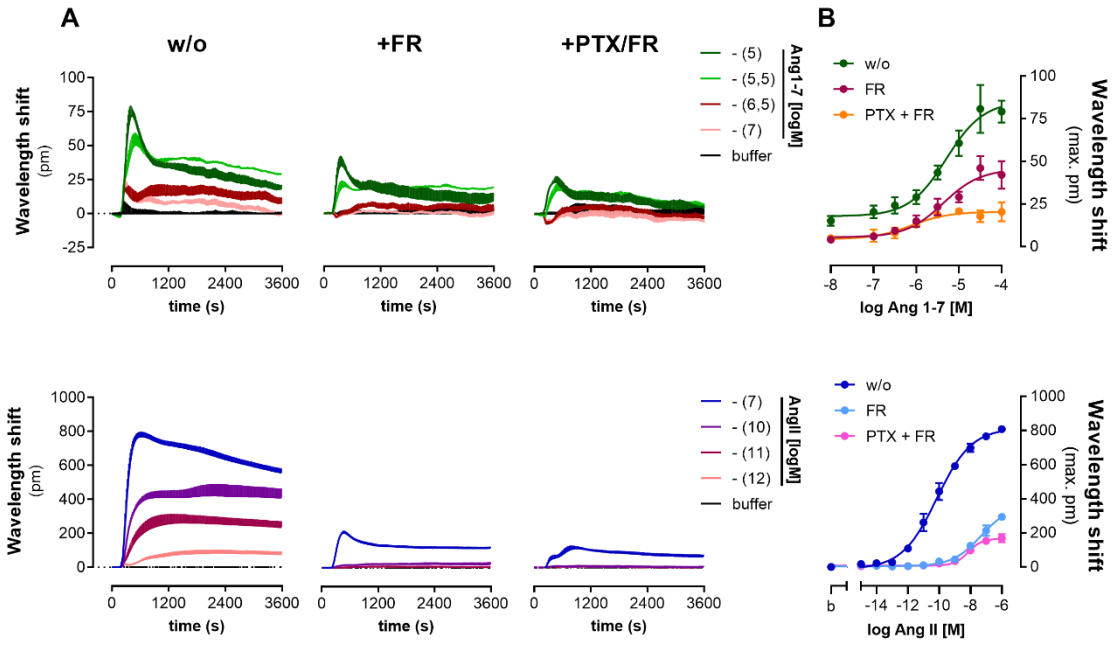


***Figure 40: DMR biosensing of cell morphological changes in β-arrestin1/2-depleted (Δarr) HEK293 cells induced by Ang1-7 and AngII:***

*(A) DMR responses of Δarr cells stably expressing the AT1R treated with Ang1-7 (upper panel) or AngII (lower panel) without (w/o) or with $G\alpha_q$- (+FR) (1µM) and $G\alpha_i$ -blockade. (B) Data summarized as concentration versus DMR peak response (max. response) curves presented as mean±SEM of four biologically independent experiments. Representative DMR traces are depicted as mean+SD and were performed in triplicate.*

Because the phosphorylation of the extracellular signal-regulated kinases (ERK1/2) serves as the most important signaling readout for β-arrestin-dependent signaling (Gurevich and Gurevich 2018), we next analyzed ERK1/2-activation in our generated Δarr cells stably expressing the AT1R. Stimulation by Ang1-7 (30µM) led to accumulation of pERK in a partially agonistic manner compared to AngII (Figure 41). Both the time dependence and the transient kinetic pattern of ERK1/2-activation triggered by Ang1-7 were fully comparable to what we observed in wild-type HEK293 cells. ERK1/2-phosphorylation induced by the full agonist AngII also remained strong and long-lasting over time as in the wild-type HEK293 cells (compare Figures 28 and 41). Moreover, ERK1/2-phosphorylation induced by Ang1-7 was strongly reduced under $G\alpha_q$-inhibition (+FR) and undetectable under additional $G\alpha_i$-blockade (+PTX/FR). Therefore, the absence of β-arrestins had no effect on Ang1-7-promoted ERK1/2-activation, indicating that β-arrestins are dispensable for the evoked pERK accumulation. Furthermore, these results show again that pERK accumulation triggered by Ang1-7 is controlled by G proteins. The same holds true for the full agonist AngII.

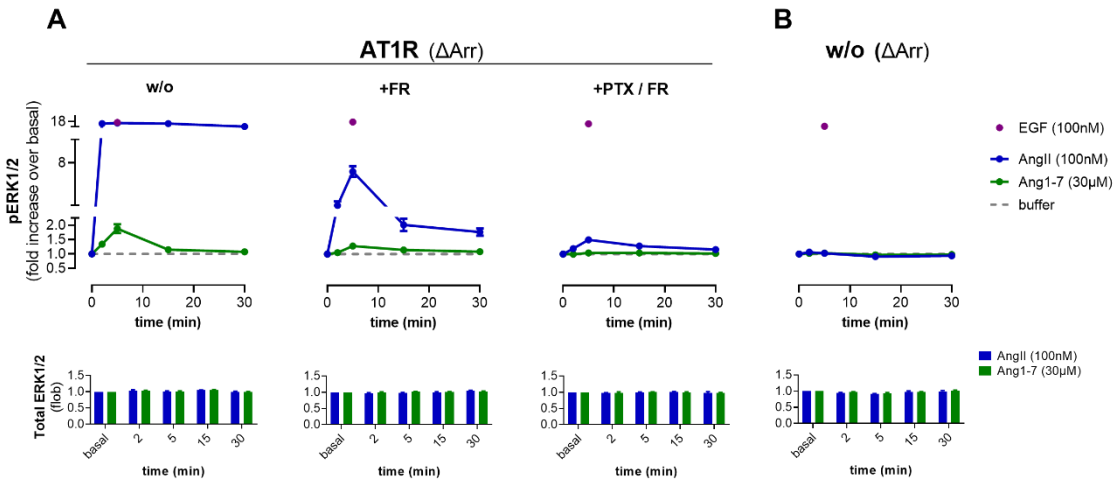


***Figure 41: AT1R-mediated ERK1/2-phosphorylation by angiotensin peptides in β-arrestin1/2-depleted HEK293 cells:***

*Temporal pattern of ERK1/2-phosphorylation and total-ERK1/2 induced by Ang1-7 (30µM) and AngII (100nM) stimulation of Δarr cells stably expressing the AT1R (A) or not (B). The contribution of $G\alpha_q$- and $G\alpha_i$-proteins to the detected responses was determined either by pretreatment with the $G\alpha_q$-specific inhibitor FR (1µM) alone (+FR) or in combination with the $G\alpha_i$-inhibitor PTX (+PTX/FR). EGF was used as a viability control. pERK1/2- and total ERK1/2-profiles are mean±SEM of four independent experiments each performed in triplicate.*

## 4.6 ERK1/2-phosphorylation triggered by AT1R requires active Gα-proteins, mainly of the $G\alpha_q$ family.

To corroborate the findings from our studies, showing that G proteins contribute to Ang1-7-promoted ERK1/2-phosphorylation via the AT1R, we next took advantage of our previously introduced HEK293 knockout cell line Δseven, which lacks all Gα-subunits except the $G\alpha_i$ family members. After pretreatment with PTX (Δseven+PTX) to generate a condition without activatable G proteins ("zero functional G"), neither Ang1-7 (30µM) nor AngII (100nM) stimulation resulted in detectable pERK accumulation over 30 minutes in cells transiently expressing the AT1R (Figure 42, middle), thus highlighting again the key role of G proteins in the ERK1/2-activation achieved for both peptides. Remarkably, by letting these cells re-express $G\alpha_q$, we were able to recover the Ang1-7-promoted pERK responses (Figure 42, right). Notably, the maximum pERK response achieved almost reached the amplitude detected in wild-type HEK293 cells transiently expressing the AT1R (see Figure 28). Furthermore, the transient signal profile for Ang1-7 also reappeared. Thus, our results suggest that $G\alpha_q$ is not only required but is also the main driver of Ang1-7-induced ERK1/2-activation.

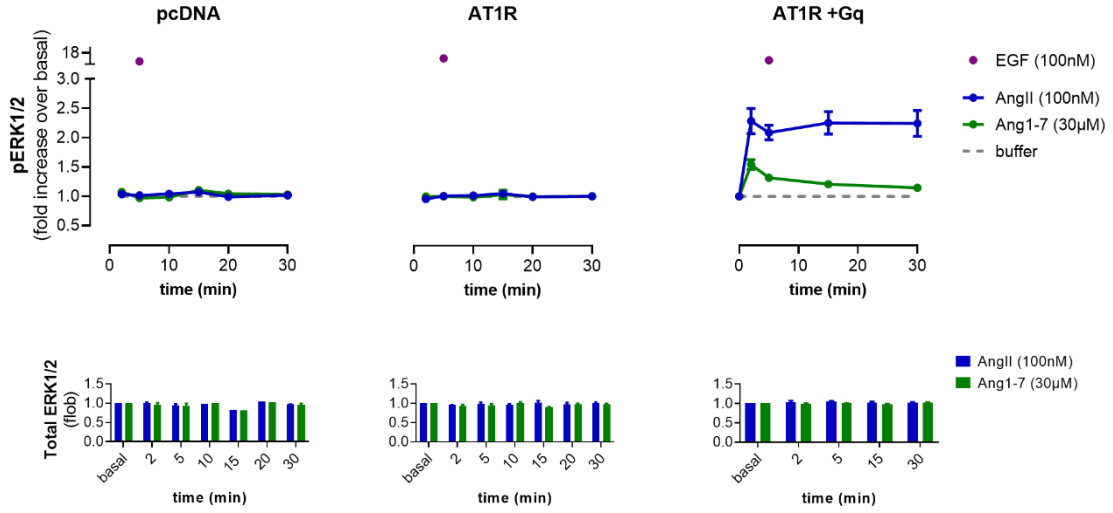


***Figure 42: The heterotrimeric G protein subunit Gα$_q$ is the main-driver of Ang1-7-mediated ERK1/2-phosphorylation through the AT1R:***

*Ang1-7- (30µM) and AngII- (100nM) induced kinetic pERK1/2- and total ERK1/2- profiles of HEK293 cells lacking functional Gα-protein subunits (Δseven+PTX) transiently expressing the AT1R (middle) or not (left). (Right) Re-expression of functional Gα$_q$-subunits in the AT1R-expressing Δseven+PTX cells followed by treatment with Ang1-7 (30µM) and AngII (100nM). Epidermal growth factor (EGF) (100nM) was applied for viability control. pERK1/2- and total-ERK1/2- profiles were performed in triplicate and are presented as mean±SEM, with pcDNA n=2, AT1R n= 4, AT1R+Gq n= 7.*

On the other hand, the accumulation of pERK evoked by the full agonist AngII was partially restored in comparison with the wild-type HEK293 cells transiently expressing the AT1R (Figure 28), which may reflect the involvement of other Gα-proteins in addition to Gα$_q$. Nevertheless, the time-dependent pattern of AngII-provoked pERK accumulation was again sustained and long-lasting, thus supporting the hypothesis that Gα$_q$ is responsible for the different kinetic pERK1/2-profiles of these angiotensin peptides (Figure 42, right).

We also examined the contribution of Gα$_i$-proteins on pERK accumulation. For this purpose, we first did not perform PTX inhibition in our Δseven cells, which means that endogenous Gα$_i$-proteins can still be activated (Figure 43 (A)). In this condition we then additionally overexpressed either the Gα$_{i2}$- or Gα$_o$-subunit to avoid overlooking of any potential Gα$_i$ -mediated effects (Figure 43 (B, C)). Consistent with our hypothesis that Gα$_q$ is the main driver of Ang1-7-provoked ERK1/2-phosphorylation, the presence of endogenous and even overexpressed Gα$_i$-proteins hardly resulted in measurable pERK for Ang1-7, indicating that Gα$_i$-proteins play a minor role. For AngII, on the contrary, ERK1/2-phosphorylation re-emerged, demonstrating not only a more pronounced Gα$_i$-contribution to AngII-evoked

pERK than for Ang1-7, but also that $G\alpha_i$-activation alone leads to a more transient kinetic pattern for the full agonist. This is fully consistent with the observations in our previous pERK experiments (Figure 28), where the AngII-induced pERK accumulation changed from a long-lasting to a transient time-dependent pattern when $G\alpha_q$ was blocked by FR treatment.

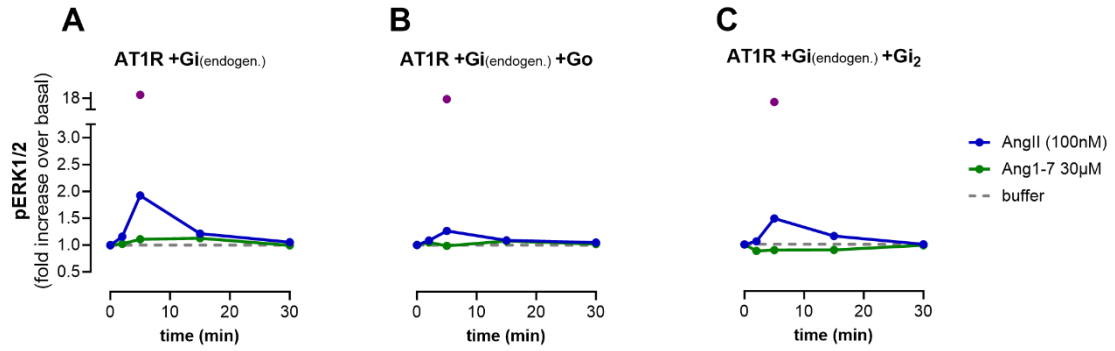


***Figure 43: $G\alpha_i$-protein influence on Ang1-7- and AngII- induced pERK accumulation downstream of the AT1R:***

*Time dependent pattern of ERK1/2-phosphorylation in Δseven cells transiently expressing the AT1R in which endogenous $G\alpha_i$ was not inhibited by PTX (A) and additionally either the $G\alpha_o$- (B) or $G\alpha_{i2}$-subunit was re-expressed (C). pERK1/2 profiles are mean±SD and represent one representative experiment (n=1) performed in triplicate.*

Finally, to rule out the possibility of overlooking β-arrestin-dependent AT1-ERK1/2 signaling for Ang1-7, we additionally overexpressed both β-arrestins. As Δseven+PTX cells endogenously express β-arrestins, measurement of β-arrestin-dependent ERK1/2-phosphorylation could be expected. However, in the absence of Gα-proteins, neither the abundance of endogenously nor overexpressed β-arrestins led to detectable pERK accumulation for both angiotensin peptides (Figure 44). This verifies our previous findings, showing β-arrestins not to be responsible for Ang1-7-promoted pERK responses.

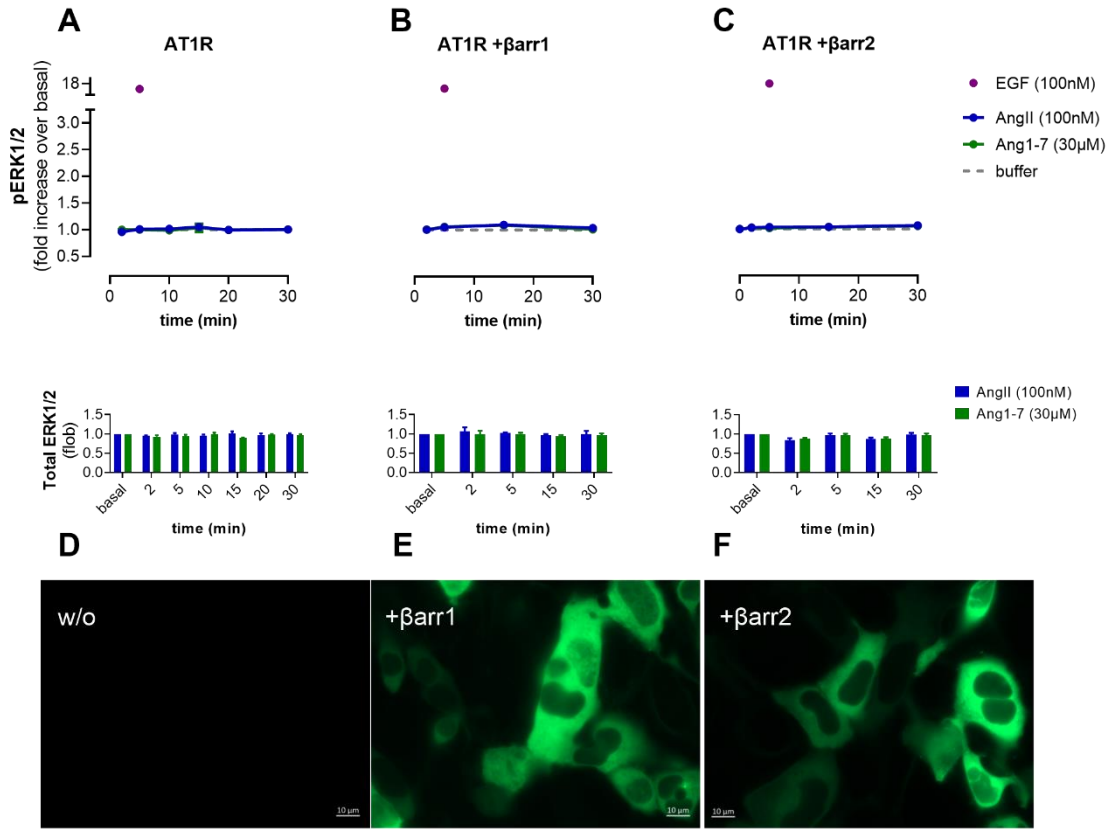


***Figure 44: β-arrestins do not initiate ERK1/2-phosphorylation through the AT1R upon Ang1-7 treatment in the absence of active Gα-proteins:***

*Ang1-7- (30µM) and AngII- (100nM) mediated ERK1/2-phosphorylation and total-ERK1/2 in G protein-deficient HEK293 cells (Δseven+PTX) transiently expressing the AT1R (A) and additionally β-arrestin1-GFP (B) or β-arrestin2-GFP (C). Cell viability was determined by EGF treatment (100nM). (D) Representative fluorescence microscopic image of cells shown in (A). (E+F) Demonstration of the overexpression of GFP-labeled β-arrestin1 or 2 in Δseven+PTX-cells by fluorescence microscopy, corresponding to (B+C). Images are representative of three independent experiments, scale bar=10µm. pERK1/2 and total ERK1/2 kinetic profiles are shown as mean±SEM of at least three biologically independent experiments, performed in triplicate.*

Altogether, our findings in Δseven+PTX cells confirm Gα-proteins as the true drivers of Ang1-7-induced pERK, in contrast to the prevailing view that Ang1-7 acts as a β-arrestin-biased agonist leading to β-arrestin-dependent ERK1/2-phosphorylation at the AT1R. To our surprise, we identified $G\alpha_q$, the Gα-protein subunit that is reported not to be activated by Ang1-7, to be the main-transducer of its ERK1/2-activation. Notably, whereas the full agonist AngII primarily promotes a long-lasting increase in pERK via $G\alpha_q$-activation, Ang1-7 shows a transient kinetic profile with lower efficacy via the same Gα-protein.

## 4.7 Excursus: $G\alpha_q$ is involved in the Ang1-7-induced β-arrestin recruitment to the AT1R.

β-arrestin binding to an active GPCR is preceded by its phosphorylation by protein kinases of the G protein-coupled receptor kinases (GRK)-family and, depending on the pathway, also by members of the PKA/PKC-family. The latter and especially GRKs of the GRK2/3 subfamily are dependent on prior G protein activation, in which a released Gβγ-subunit from the heterotrimeric G protein is required for GRK2/3 recruitment to the receptor. Conversely, GRK5/6 subfamily is not activated by G protein βγ-subunits (Gurevich and Gurevich 2019; Gurevich and Gurevich 2020).

For peptides previously characterized as β-arrestin-biased at the AT1R, it has been reported that they lead mainly to GRK5/6- and not to GRK2/3-dependent phosphorylation of the AT1R (Kim et al. 2005; Kawakami et al. 2022). Nevertheless, our data showed that $G\alpha_q$ is implicated in Ang1-7 signaling at the AT1R, hence $G\alpha_q$-proteins could be also involved in the Ang1-7-mediated β-arrestin recruitment to the AT1R, presumably by engaging GRK2/3.

To investigate this hypothesis, we decided to repeat our previous experiments on β-arrestin recruitment (see Figure 22), except that we also applied the specific $G\alpha_q$ inhibitor FR, which blocks the dissociation of the $G\alpha_q$ heterotrimers. Notably, Ang1-7-induced β-arrestin recruitment to the AT1R was affected when $G\alpha_q$-proteins were blocked (Figure 45 (A+B)), which suggests that $G\alpha_q$ is also involved here. This was particularly evident for β-arrestin1, as reflected by a significant decrease in its engagement with the AT1R under FR treatment (Figure 45 (C)). In addition, β-arrestin1 coupling to the AT1R was also slowed by $G\alpha_q$-blockade. However, the recruitment of β-arrestin2 was only slightly and not significantly reduced, suggesting that the effect of $G\alpha_q$ on the recruitment of the two β-arrestins seems to differ in its magnitude (Figure 45 (C+D)).

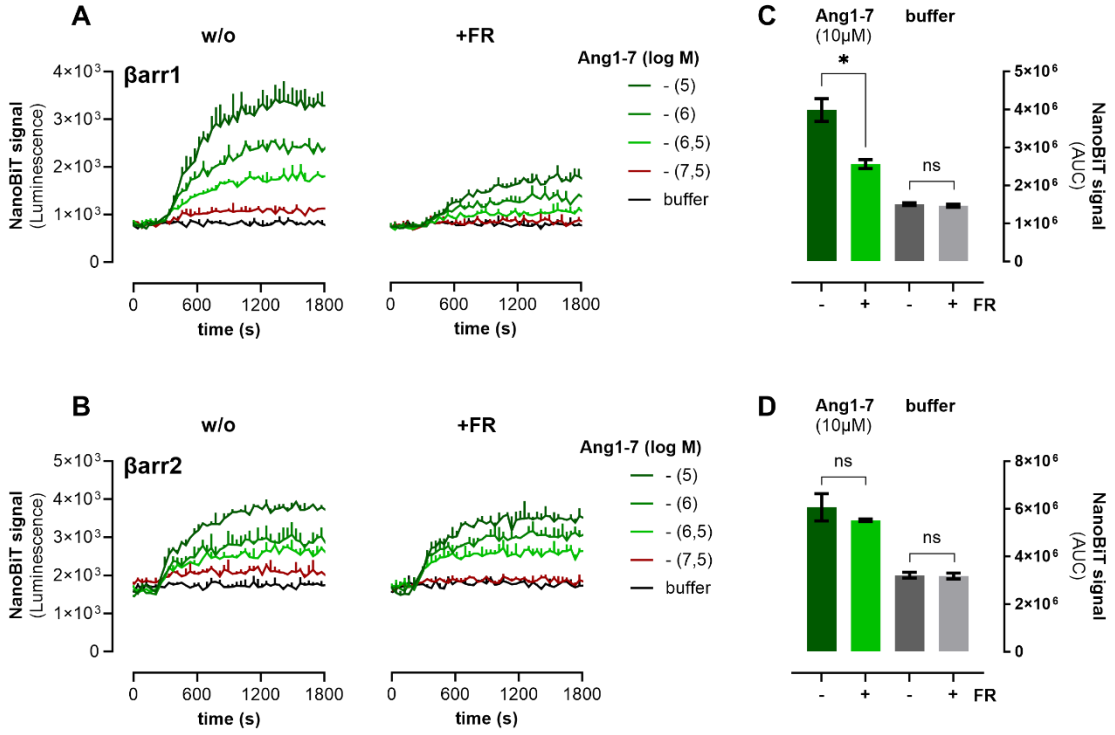


***Figure 45: Ang1-7-mediated β-arrestin recruitment to the AT1R in the absence and presence of specific $G\alpha_q$-inhibitor FR:***

*Representative real-time NanoBiT® luminescence traces of β-arrestin1-lgBiT (A) or β-arrestin2-lgBiT (B) recruitment to the AT1R-SmBiT upon Ang1-7 stimulation (concentrations as indicated) in wild-type HEK293 cells pretreated (right) or not (left) with the $G\alpha_q$-inhibitor FR (1μM). Comparison of the β-arrestin recruitment induced by Ang1-7 (10μM) or assay buffer (control) with or without pretreatment with FR (1μM) (C = βarr1, D = βarr2), shown as area under the curve (AUC) of measured luminescence within 30 minutes. Data represent the mean±SEM of three independent experiments. Statistical analysis was calculated using a two-tailed, unpaired t-test with *P<0.05, **P<0.01, ns= non-significant.*

This is consistent with the observations for the full agonist AngII (Figure 46), where $G\alpha_q$ inhibition resulted in a greater decrease in the maximal engagement of β-arrestin1 with the receptor than β-arrestin2 (Figure 46 (A+B)). Moreover, FR-inhibitory effects were only visible after ligand-induced receptor activation, as indicated by the fact that basal luminescence was not affected by FR (Figures 45 and 46 (C+D), grey). This leads to an interesting side note: the pre-recruitment of β-arrestin2 to the AT1R that we observed (see also Figure 23), recognizable by its higher basal luminescence compared to β-arrestin1, does not appear to be $G\alpha_q$ dependent, as it was unaffected by $G\alpha_q$-inhibition.

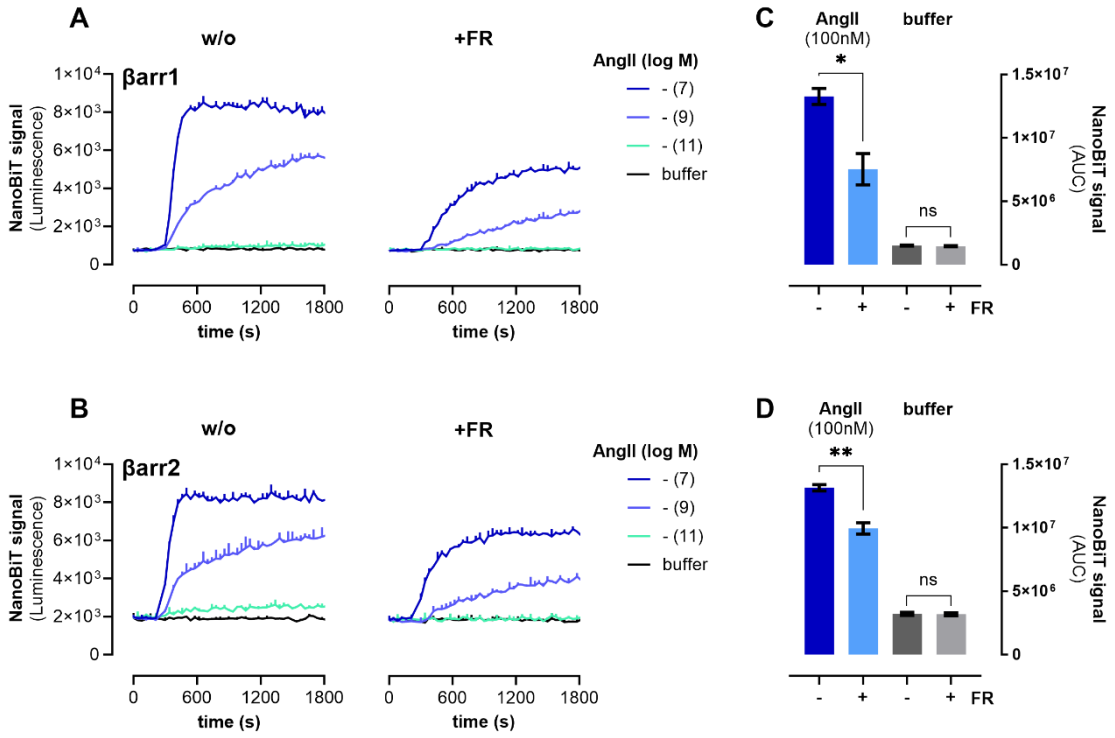


***Figure 46: AngII-induced β-arrestin recruitment to the AT1R in the absence and presence of specific $G\alpha_q$-inhibitor FR:***

*Representative AngII-provoked real-time monitoring of β-arrestin1-lgBiT (A) or β-arrestin2-lgBiT (B) engagement with the AT1R-SmBiT in wt-HEK293 cells in the absence (left) or presence (right) of the specific $G\alpha_q$-inhibitor FR (1µM). Comparison of the β-arrestin recruitment induced by AngII (100nM) or assay buffer (control) with or without pretreatment with FR (1µM) (C = βarr1, D = βarr2). Data are mean±SEM of at least three independent assays and are presented as area under the curve (AUC) within 30 minutes of detection of luminescence. Statistical analysis was calculated using a two-tailed, unpaired t-test with *P<0.05, **P<0.01, ns= non-significant.*

Because FR could reduce but not abolish the Ang1-7 mediated recruitment of β-arrestin1 to the AT1R, we asked whether the engagement of this β-arrestin to the receptor could also occur independently of prior G protein activation. For this purpose, we again employed the HEK293 knockout cells Δseven pretreated with PTX to reach the "zero functional G" stage. In Δseven+PTX cells, both Ang1-7 and AngII provoked robust β-arrestin1 engagement with the AT1R (Figure 47, w/o-condition), demonstrating in agreement with the literature that β-arrestins are recruited to ligand-activated GPCRs independent of the presence of active G proteins (Grundmann 2018; Hunyady et al. 1994a). Moreover, in the absence of functional G proteins, Ang1-7 induced only a partial maximal recruitment of β-arrestins compared to the full agonist AngII.

Since we observed a reduced recruitment of β-arrestins to the AT1R in our wild-type HEK293 cells when the endogenous $G\alpha_q$-proteins were blocked by FR (Figure 45, 46), we next reintroduced the $G\alpha_q$-subunit into our "zero functional G" cells by transient transfection to look for possible modulatory effects of this subunit. The presence of activatable $G\alpha_q$-proteins resulted in an enrichment in the rate and maximal interaction of β-arrestin1 with the AT1R for both angiotensin peptides (Figure 47, +Gq-condition). This was more prominent for Ang1-7, albeit maximal detected luminescence obtained with AngII was never reached.

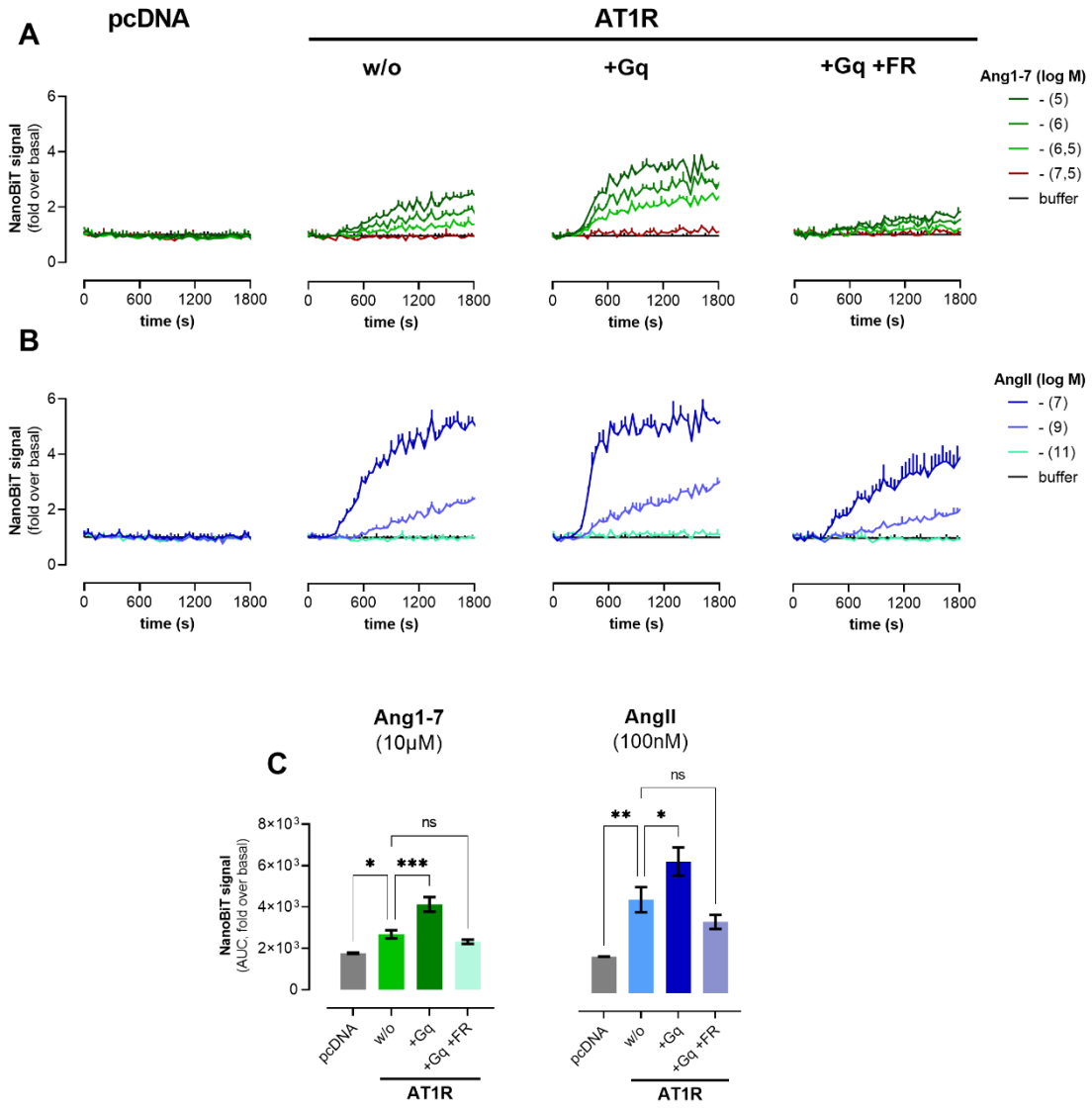


***Figure 47: Heterotrimeric $G\alpha_q$-protein activation is required for the detected enhancement of β-arrestin1 engagement with the AT1R by Ang1-7 and AngII:***

*Representative Ang1-7- (A) and AngII-induced (B) NanoBiT® signals in Δseven+PTX cells ("zero functional G") transiently expressing the AT1RSmBiT (AT1R) or not (pcDNA) and β-arrestin1-lgBiT. Recruitment of β-arrestin1 to the AT1R was determined without (w/o) or with re-expression of $G\alpha_q$-proteins either blocked or*

*not blocked by prior treatment with the specific inhibitor FR (10µM) (+Gq and +Gq+FR). Traces are mean+SD and representative of one out of at least four independent experiments. (C) Quantification of Ang1-7- (10µM) and AngII- (100nM) mediated β-arrestin1 recruitment to the AT1R calculated as area under the curve (AUC) of the measured fold over basal ratios within 30 minutes. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test with *P<0.05, **P<0.01,***P<0.001, ns= non-significant.*

The observed enrichment in β-arrestin1 recruitment was a consequence of activation of $G\alpha_q$ heterotrimers, as shown by the removal of $G\alpha_q$-induced effects by FR inhibition (Figure 47, +Gq+FR-condition). Interestingly, when $G\alpha_q$ was blocked by FR, we saw a trend, although not statistically significant, to even lower promoted β-arrestin1 recruitment than in the w/o-condition. This can be explained by the excess of $G\alpha_q$. Although blocked by FR, $G\alpha_q$ heterotrimers compete with β-arrestin1 proteins for the active AT1R and thereby interfering with β-arrestin1 coupling.

Encouraged by our finding that $G\alpha_q$ is also involved in Ang1-7-mediated β-arrestin recruitment to the AT1R, we wondered whether its extent could be modulated by the cellular level of $G\alpha_q$. Therefore, we switched back to our wild-type HEK293 cells, but instead of inhibiting endogenous $G\alpha_q$ by FR (cf. Figure 45, 46), we additionally overexpressed $G\alpha_q$-proteins. Remarkably, in the presence of excess $G\alpha_q$, the recruitment of β-arrestin1 to the AT1R upon Ang1-7 stimulation was greatly enhanced (Figure 48). Additionally, $G\alpha_q$-overexpression altered the kinetics and amplitude of the traces obtained for Ang1-7 towards faster and higher luminescence signals.

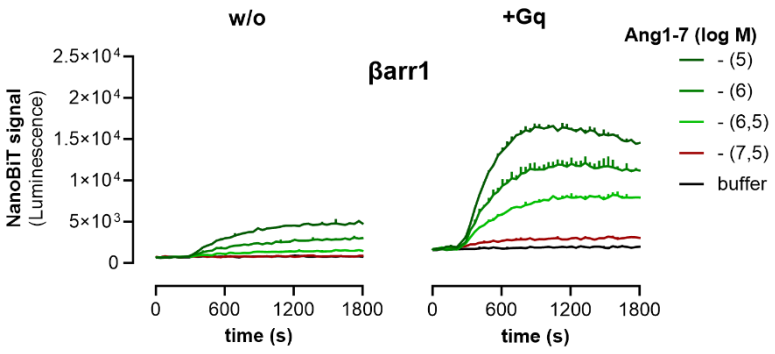


***Figure 48: $G\alpha_q$ influence on the Ang1-7-promoted β-arrestin1 recruitment:***

*Representative real-time traces of Ang1-7-activated wild-type HEK293 cells transiently expressing the AT1R-SmBiT and β-arrestin1-lgBiT with or without co-expressing heterotrimeric Gα-protein subunit $G\alpha_q$. Data are mean+SD.*

A comparison of Ang1-7- and AngII-mediated β-arrestin recruitment in $G\alpha_q$-overexpressing HEK293 cells is shown in Figure 49 for β-arrestin1 and in Figure 50 for β-arrestin2. In the

presence of overexpressed $G\alpha_q$-proteins, Ang1-7 triggered a stronger enhancement for β-arrestin1 recruitment than for β-arrestin2, which is in line with our previous observations (Figure 45) where inhibition of $G\alpha_q$-proteins impaired β-arrestin recruitment to the AT1R. This discrepancy could be explained by the higher propensity of β-arrestin2 to interact with the AT1R that we observed. Therefore, β-arrestin2 may be less subject to $G\alpha_q$-initiated modifications at the receptor, such as recruitment and subsequent phosphorylation by G protein-dependent protein kinases such as GRK2. In addition, treatment with the full agonist AngII also yielded in an increased recruitment of both β-arrestins. However, the detected increase in luminescence was less pronounced than for Ang1-7. As summarized in Figure 49/50 (C+D), this results in a higher calculated efficacy for Ang1-7 relative to AngII in $G\alpha_q$-overexpressing cells (+Gq condition) than when $G\alpha_q$ is "only" endogenously present (w/o condition).

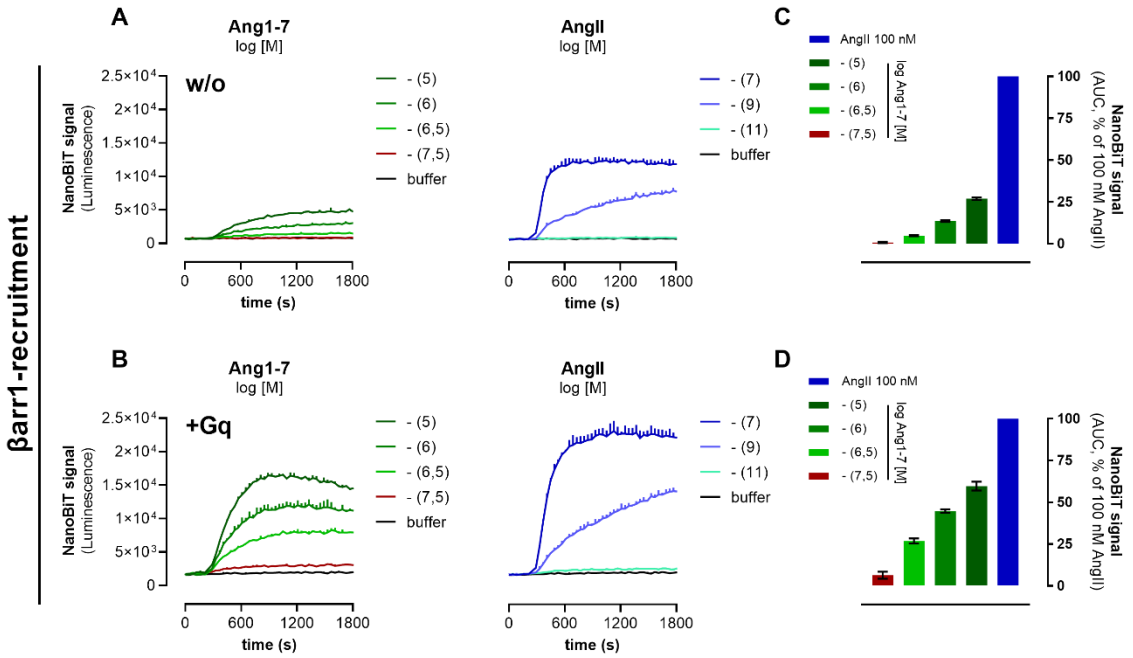


***Figure 49: Comparison of Ang1-7- and AngII- mediated β-arrestin1 engagement with the AT1R in the absence and presence of $G\alpha_q$ excess:***

*Real-time measurements of Ang1-7- and AngII- mediated increase in detected luminescence in wt-HEK293 cells transiently expressing the AT1R-SmBiT and β-arrestin1-lgBiT without (A) or with additional $G\alpha_q$-overexpression (B). Traces are mean+SD and represent one of at least three biologically independent experiments, that are quantified in (C+D). Quantification is expressed as area under the curve within 30 minutes, normalized to AngII (100nM) response and shown as mean±SEM.*

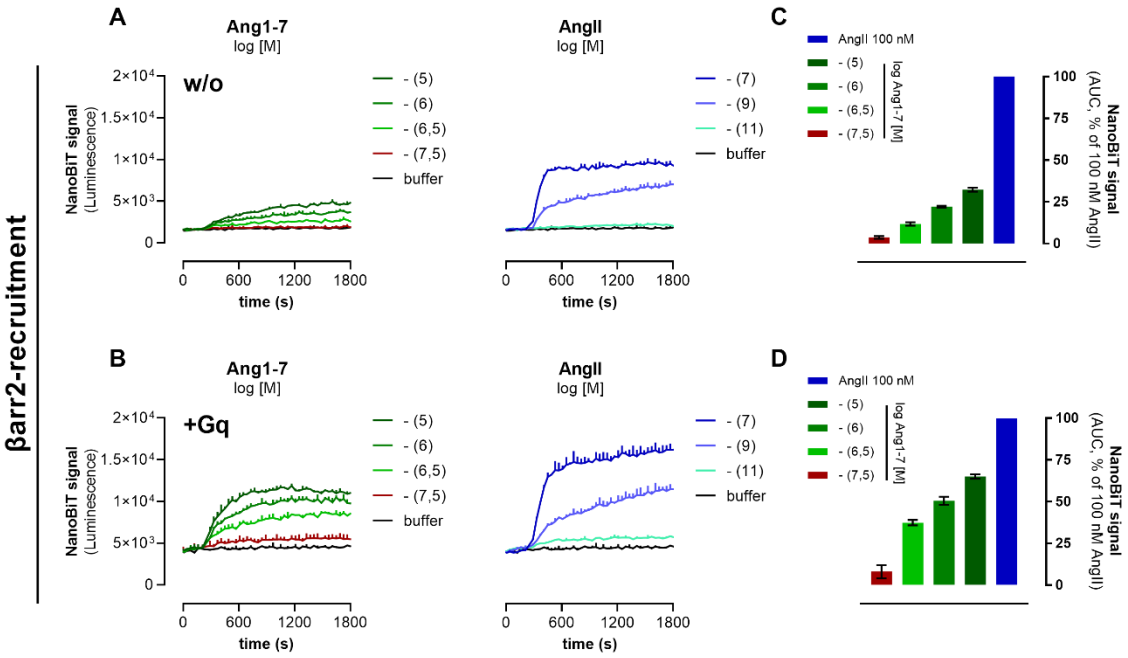


***Figure 50: Recruitment of β-arrestin2 to the AT1R upon Ang1-7 and AngII treatment in the presence or absence of excess $G\alpha_q$:***

*Real-time measurements of β-arrestin2 recruitment to the AT1R after treatment with Ang1-7 and AngII (as indicated) in the presence or not of additional overexpressed $G\alpha_q$-proteins (A, B). Graphs are mean+SD and are representative of one out of three biologically independent experiments. For quantification, the (AUCs) areas under the curve within 30 minutes for Ang1-7 were calculated and then normalized to AngII (100nM). Data are mean±SEM.*

Hence, our data show not only that $G\alpha_q$ can play a modulatory role in the Ang1-7-promoted β-arrestin recruitment, but also that the expression level of $G\alpha_q$ in the cell may control the extent of β-arrestin recruitment to the AT1R achieved by this peptide. This further underscores that for Ang1-7 even β-arrestin recruitment and G protein activation cannot be considered separately but influence each other.

## 4.8 β-arrestins and GRK2 do not drive but modulate the G protein driven accumulation of pERK downstream of the AT1R.

As mentioned above, a common feature of β-arrestins is that they can interact with a wide range of proteins, including members of all three tiers of the classical MAPK cascade (Luttrell et al. 2001; McDonald et al. 2000; Song et al. 2009). This property enables them to bring the members of a signaling pathway closer together in space and time, which is also termed the scaffold function of β-arrestins. In this way, for example, they can facilitate the execution of the signaling pathway and exert a modulating effect on the signaling response.

Scaffold function for β-arrestins in the downstream signaling from the AT1R to ERK1/2 is well described (Tohgo et al. 2002; Gurevich and Gurevich 2008; Dewire et al. 2007). Having demonstrated that β-arrestins do not initiate the pERK accumulation upon Ang1-7 stimulation at the AT1R, we now wanted to test whether the recruited β-arrestins have a modulatory effect on the generated pERK response and act as scaffolds.

For this purpose, we considered our Δseven+PTX cells ("zero functional G") as an optimal background to distinguish between signal transduction and scaffold functions of β-arrestins. Since neither endogenous nor overexpressed β-arrestin1/2 transduced an increase in pERK (see Figure 44) but only G protein re-expression resulted in re-emergent pERK accumulation (Figure 42), in a preliminary approach Δseven+PTX cells expressing the AT1R and $G\alpha_q$ by transient transfection were simultaneously transfected with increasing amounts of plasmid DNA encoding β-arrestin2.

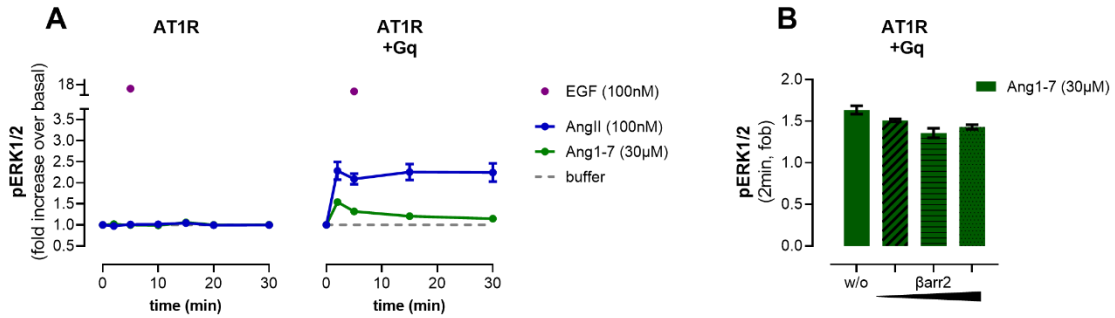


***Figure 51: Effect of β-arrestin2 on the AT1R-Gα$_q$-mediated accumulation of pERK1/2 by Ang1-7 stimulation:***

*(A) pERK1/2 profiles (from Figure 42) of angiotensin peptide stimulated Δseven+PTX cells transiently expressing the AT1R without (left) or with (right) re-expression of Gα$_q$. (B) Ang1-7- (30µM) provoked pERK accumulation in Δseven+PTX cells transiently expressing AT1R and Gα$_q$ (as fold increase over basal levels)*

*after 2 minutes in the presence of endogenously expressed β-arrestins (w/o condition) or by overexpression of β-arrestin2, achieved by transfection of increasing amounts of β-arrestin2 plasmid-DNA (0.125µg/0.5µg/1.5µg).*

We started measuring Ang1-7-provoked pERK accumulation at 2 minutes because this was the peak of the Ang1-7-induced pERK responses. Since no protein expression level is measured, a quantitative comparison of the signals is precluded, only allowing us a qualitative assessment. As shown (Figure 51 (B)), overexpression of β-arrestin2 resulted in a slight decrease of the detected pERK in comparison to the w/o condition, which only contains endogenously expressed β-arrestins.

Figure 52 plots the increase of Ang1-7- and AngII-evoked ERK1/2-phosphorylation in the presence of overexpressed β-arrestin1 or 2. There was a trend towards a reduction in the evoked pERK responses for both peptides. This was apparent at 2 minutes for Ang1-7 and at later time points for AngII, although it was not statistically significant (Figure 52 (B+C)). The transient Ang1-7 and the long-lasting AngII kinetic profiles were maintained when β-arrestins were overexpressed, re-emphasizing that these characteristic temporal patterns are mediated by $G\alpha_q$ alone. Therefore, the presence of overexpressed β-arrestin1 or 2 did not have a different but rather a comparable effect on the pERK responses elicited by the two angiotensin peptides through the AT1R.

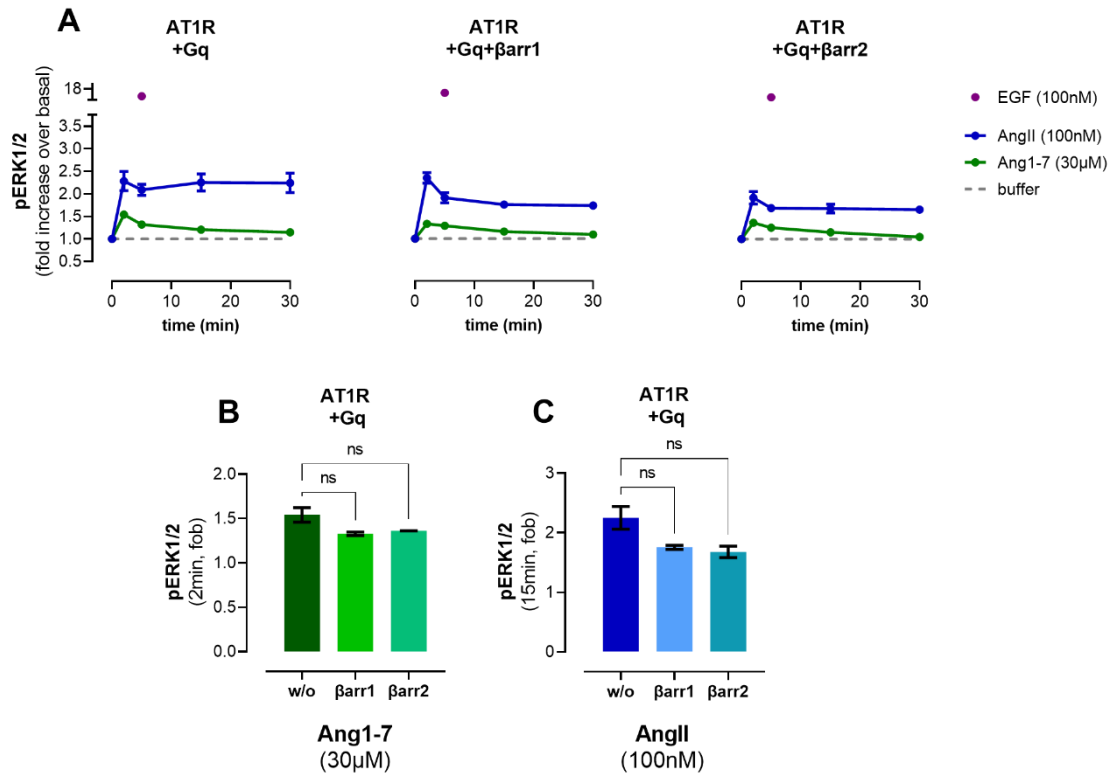


***Figure 52: Effect of β-arrestin1 and -2 on the kinetic profile of AT1R-Gα$_q$-promoted ERK1/2-phosphorylation by Ang1-7 and AngII:***

*(A) Temporal pattern of ERK1/2-phosphorylation induced by Ang1-7 (30µM) or AngII (100nM) in Δseven+PTX cells transiently (re)-expressing the AT1R and Gα$_q$ either endogenously expressing β-arrestins (left) or with additional overexpression of β-arrestin1 (middle) or -2 (right). (B) Comparison of Ang1-7-(30µM) promoted pERK accumulation after 2 minutes with or without overexpression of β-arrestin1 or -2 and (C) corresponding AngII- (100nM) mediated ERK1/2-phosphorylation after 15 minutes. One-way ANOVA with Dunnett's multiple comparison was used for statistical analysis. Data shown in kinetic profiles and column plots represent at least three independent experiments (each performed in triplicate) and are expressed as mean±SEM.*

Since the negative regulatory effect of β-arrestin on the AT1R-ERK1/2 pathway was only tendential and not significant, we asked ourselves whether GRKs might also play a regulatory role here. GRKs phosphorylate the activated GPCR, thus representing the de facto first step in β-arrestin recruitment to the receptor (Gurevich et al. 2012; Drube et al. 2022). We decided to overexpress GRK2 in our experimental approach because of its reported role in interacting with components of the MAP kinase cascade (Penela et al. 2010; Jiménez-Sainz et al. 2006).

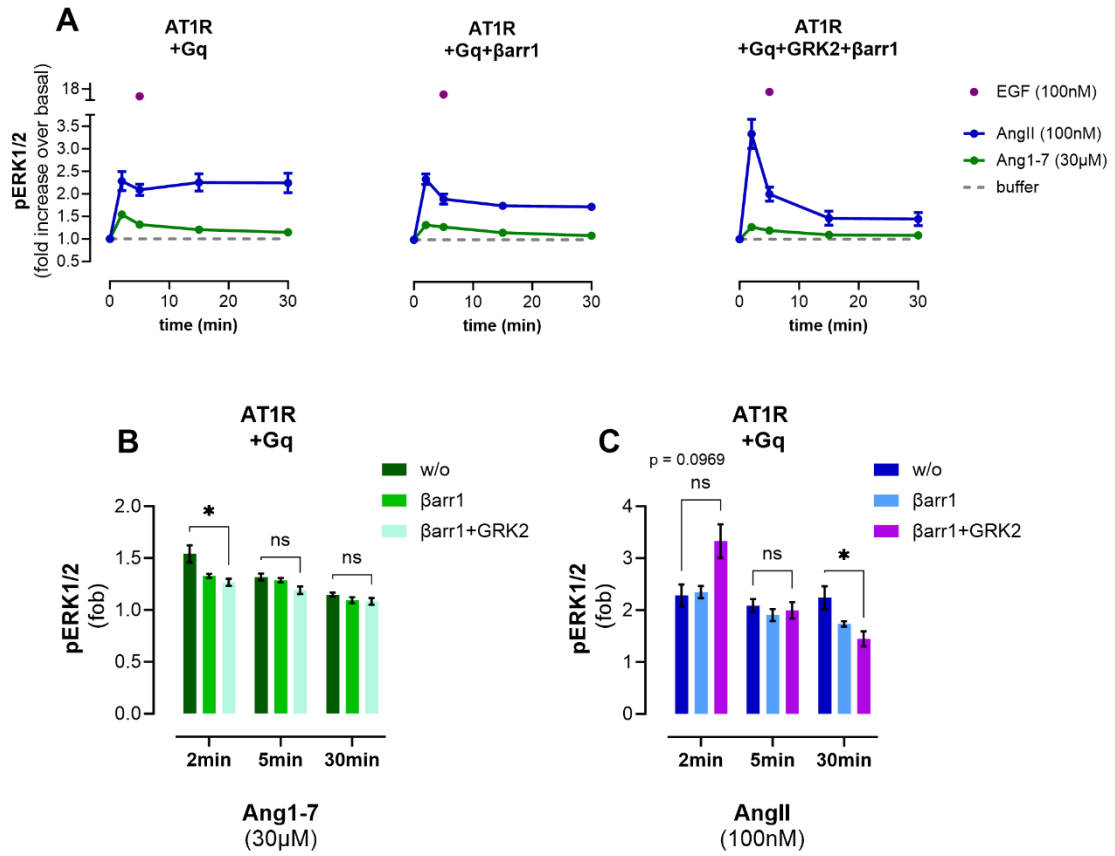


***Figure 53: Effect of GRK2 on the kinetic profile of AT1R-Gα$_q$-mediated ERK1/2-phosphorylation by Ang1-7 and AngII:***

*(A) Temporal pattern of ERK1/2-phosphorylation upon Ang1-7 (30µM) or AngII (100nM) stimulation of Δseven+PTX cells (re)-expressing AT1R and Gα$_q$ (left) in the presence of transiently overexpressed β-arrestin1 (middle) or the combination of overexpressed GRK2 with β-arrestin1 (right). Kinetic profiles are mean±SEM of at least three independent experiments carried out in triplicate. Comparison of Ang1-7- (30µM) promoted pERK accumulation after 2, 5 and 30 minutes with or without overexpression of β-arrestin-1 and GRK2. Corresponding AngII- (100nM) mediated ERK1/2-phosphorylation is shown in (C). Two-way ANOVA with Dunnett's multiple comparison was used for statistical analysis. Data shown in kinetic profiles and column plots represent at least three independent experiments (each performed in triplicate) and are expressed as mean±SEM.*

As shown in Figure 53, co-expression of GRK2 and β-arrestin1 resulted in a reduced Ang1-7-promoted ERK1/2- activation, which is consistent with the prominent desensitization function of GRKs and β-arrestins (Hunyady et al. 2000). Strikingly, ERK1/2-phosphorylation upon AngII stimulation was strongly affected. Interestingly, pERK responses were modulated in two directions (Figure 53 (A), right): While the detected phosphorylated ERK1/2 was strongly elevated within 2 minutes, indicating a positive modulation, it declined after 5 minutes to levels comparable to those in the Gα$_q$- and Gα$_q$+β-

arrestin1- conditions, and it decreased further at later time points, suggesting a negative modulation (Figure 53 (C)).

In summary, working in the "zero functional G" background (Δseven+PTX) allowed us to verify that the observed GRK2-modulating effects on Ang1-7 and AngII-induced pERK accumulation were completely G protein dependent (Figure 54). For both angiotensin peptides, neither endogenously expressed GRKs and β-arrestins (upper panel, left) nor their overexpression alone or in combination transduced ERK1/2-activation through the AT1R (upper panel). On the contrary, as demonstrated before, only re-expression of active G proteins (here $G\alpha_q$) led to the phosphorylation of ERK1/2, highlighting G proteins to be the sole driver of the transduced ERK1/2-activation. This $G\alpha_q$-protein-mediated pERK accumulation can be modulated and fine-tuned depending on the presence of β-arrestins and GRK proteins, emphasizing their role as signaling scaffolds. In our case, we saw a trend for both β-arrestins in a slight reduction on $G\alpha_q$-mediated ERK1/2-phosphorylation, but when additionally overexpressing GRK2, we were able to delineate a decrease in Ang1-7-provoked pERK accumulation and strikingly positive and negative modulation on $G\alpha_q$-driven pERK responses for full agonist AngII.

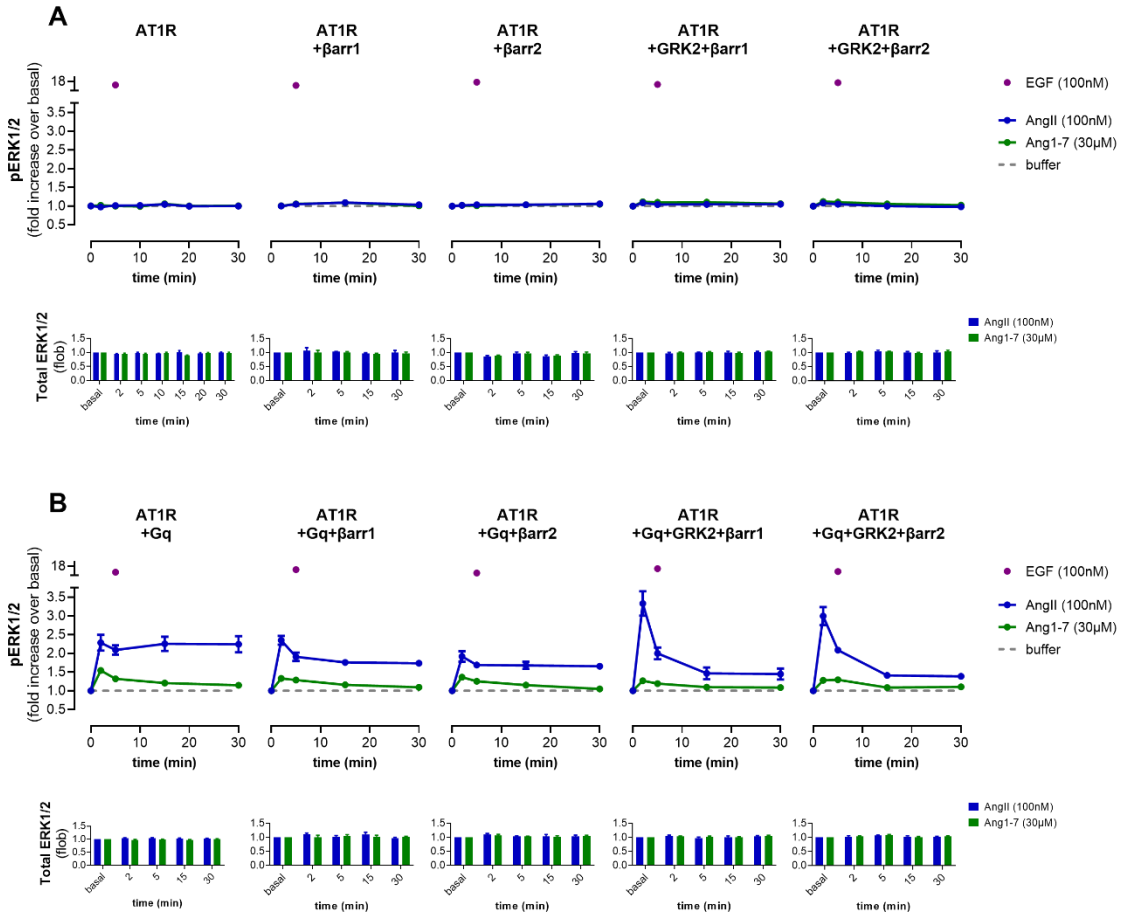


***Figure 54: AT1R-mediated ERK1/2-phosphorylation induced by angiotensin peptides is not transduced by, but modulated by the presence of GRKs and β-arrestins:***

*Ang1-7- (30µM) and AngII- (100nM) evoked pERK1/2- and total ERK1/2 kinetic profiles of "zero functional G" (Δseven+PTX) HEK293 cells transiently expressing the AT1R without (A, upper panel) or with re-expression of the heterotrimeric G protein subunit $G\alpha_q$ (B, lower panel) in the absence (left) or presence of either both β-arrestins alone or in combination with GRK2. Epidermal growth factor (100nM) was used as viability control. Data are mean±SEM of at least three biologically independent experiments performed in technical triplicate.*

## 4.9 Ang1-7 acts as "AT1R-internalizer" promoting strong angiotensin II type 1 receptor endocytosis.

Given the central role of β-arrestins in terminating GPCR signaling by mediating the endocytosis of the receptor (Turu et al. 2019) and the substantial Ang1-7-mediated recruitment of β-arrestins to AT1R, we next decided to investigate how Ang1-7 affects AT1R internalization. For this purpose, we fused the receptor N-terminally to a so-called SNAP-tag®. The SNAP-tag® is a peptide with enzymatic activity ($O^6$-guanine nucleotide alkyl transferase) that can be exploited to covalently attach small molecules to the protein

and thus to the SNAP-AT1R (Keppler et al. 2003; Maurel et al. 2008). This allowed us to perform diffusion-enhanced resonance energy transfer (DERET) experiments in which we coupled a luminescent terbium cryptate derivative (SNAP-Lumi4®-Tb) to the SNAP-AT1R (Levoye et al. 2015). The exact principle of the assay is shown in Figure 55. With DERET, we were able to directly monitor the internalization of the AT1R in real-time.

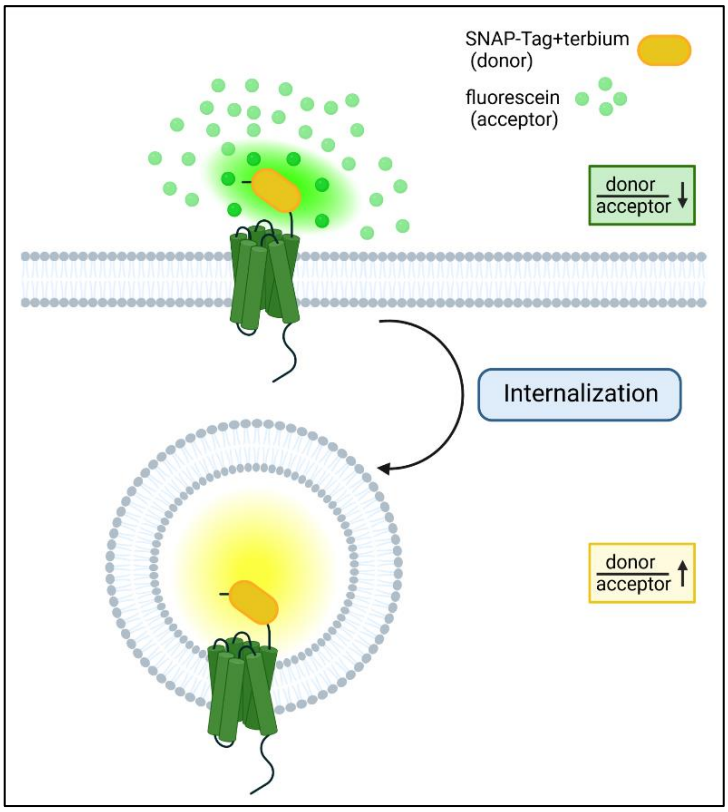


***Figure 55: Illustration of the DERET assay principle:***

*To achieve diffusion-enhanced resonance energy transfer (DERET), a terbium cryptate energy donor (SNAP-Lumi4®-Tb) is attached to the SNAP-tag of cells expressing the SNAP-AT1R on their surface. Subsequent addition of the cell-impermeable fluorescein energy acceptor then results in DERET from donor to acceptor when the donor is excited by a light source. Because the energy acceptor, fluorescein, is used in excess, the emission of the donor is quenched, resulting in a low donor/acceptor ratio (top). Ligand activation of SNAP-AT1R induces receptor internalization, preventing cell-impermeable fluorescein from following. This results in increased donor emission from internalized receptors, which is reflected in a higher donor/acceptor ratio (bottom) and is monitored in real time. Modified from S.N Roed et al. 2014 (Roed et al. 2014). Figure was created with "BioRender.com".*

Remarkably, Ang1-7 (30µM) stimulation of HEK293 cells transiently expressing the SNAP-AT1R resulted in reproducible high level of receptor internalization, which was reflected by a marked increase in the detected DERET signal over time (Figure 56 (A)). The kinetic profiles of the observed increase in DERET signals were similar for Ang1-7 and AngII. It is important to note that, for the first time, the effects mediated by Ang1-7 were superior to those of the full agonist AngII. This can be seen from the lower maximal DERET signals obtained by AngII (1µM) treatment. Moreover, monitored internalization was entirely AT1R-driven and was not the result of unspecific endocytosis related to the SNAP protein, as control HEK293 cells transiently expressing the SNAP-tagged CD86 transmembrane

protein did not show changes in DERET over 1 hour when challenged with Ang1-7 or AngII (Figure 57).

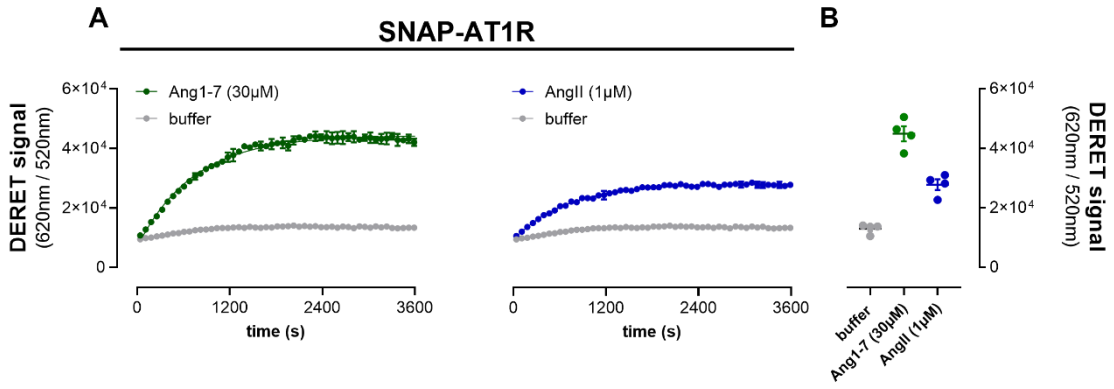


***Figure 56: Real time recordings of SNAP-AT1R internalization measured by DERET:***

*(A) Representative recordings of wild-type HEK293 cells transiently expressing the SNAP-AT1R stimulated with Ang1-7 (30µM), AngII (1µM) or assay buffer as control. Internalization was monitored for 1 hour. Detected changes in the DERET were fitted by a one-phase association kinetic model and thereby calculated plateaus are summarized in (B) for four independent experiments shown as mean±SEM. Representative data are mean±SD.*

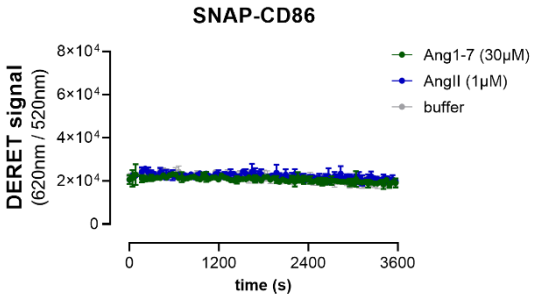


***Figure 57: DERET of SNAP-CD86 expressing HEK293 cells upon Ang1-7 and AngII treatment:***

*Representative changes in DERET of wild-type HEK293 cells transiently expressing the transmembrane protein SNAP-CD86 followed by stimulation with assay buffer with or without angiotensin peptide Ang1-7 (30µM) or AngII (1µM). Data shown are n=1.*

Intrigued by our DERET results, we next set out to verify our observations using a different approach. Since SNAP-tag® technology provides a means of attaching small molecules of choice to the receptor, we took advantage of this by labeling SNAP-AT1R with a red fluorescent substrate (SNAP-Surface® 649) prior to the addition of angiotensin peptides. As a result, we were now able to follow the localization of SNAP-AT1R expressed by HEK293 cells using fluorescence microscopy. For comparability with DERET, HEK293 cells were transfected and treated using the same transfection paradigm and assay conditions (see Methods). As can be seen from Figure 58, SNAP-AT1R expression was initially (1 minute) localized to the plasma membrane, as we observed the highest fluorescence predominantly

at the cell surface. While cells treated with assay buffer maintained the surface localization of the receptor after 60 minutes, indicating that no internalization occurred (bottom right), Ang1-7 (30µM) instead promoted substantial internalization of the SNAP-AT1R, analogous to our DERET assays (top right). This has led us to identify Ang1-7 once again as a potent "AT1R internalizing" peptide.

The endocytosis induced by Ang1-7 was particularly evident by the now low fluorescence in the membrane and the strong accumulation in the cell interior. In addition, Ang1-7 internalization was characterized by a central accumulation of vesicles, which was visible under the microscope by a punctate strong fluorescence intensity. On the other hand, in agreement with our DERET measurements, we detected a less pronounced internalization for AngII (1µM) after 60 minutes, recognizable by the fact that SNAP-AT1Rs were still present in greater numbers in the membrane (middle right). Notably, AngII-induced endocytosis resulted in the accumulation of dispersed vesicles near the plasma membrane rather than in a central cluster. This suggests that the two peptides may differ in the route of endocytosis that they mediate for the AT1R.

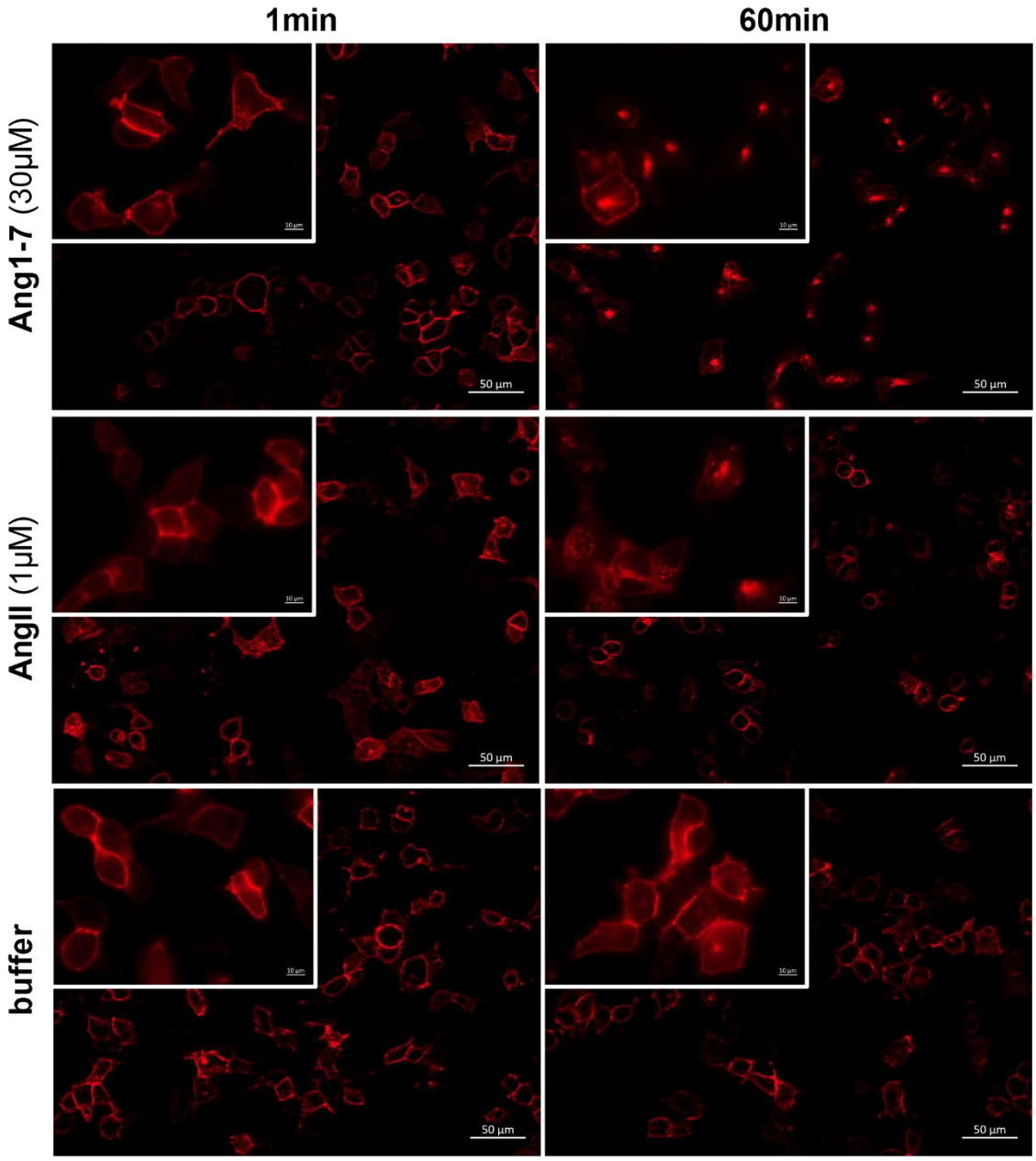


***Figure 58: Internalization of SNAP-AT1R followed by fluorescence microscopy:***

*Structured illumination micrographs of wild-type HEK293 cells transiently expressing SNAP-AT1R with SNAP-tag covalently linked to a red fluorophore (SNAP-Surface® 649) followed by treatment with Ang1-7 (30µM) (top row), AngII (1µM) (middle row) or assay buffer (bottom row). Cells were stimulated at the indicated times, fixed with PFA, and then examined by fluorescence microscopy (see Methods for details). Structural images were acquired at 20x magnification (50µm, scale bar) using Apotome2.0, while corresponding inserts were acquired at 63x magnification (10µm, scale bar) using conventional fluorescence microscopy. Images are representative of four independent experiments.*

Having also demonstrated by microscopy the extraordinary ability of Ang1-7 to mediate AT1R internalization, which was even superior to that of the full agonist AngII, we consequently asked how AT1R signaling for the two peptides behaved in parallel. For this purpose, HEK293 cells transiently expressing SNAP-AT1R derived from the same transfection pool were used to perform a comprehensive analysis in which internalization and signaling experiments were conducted in parallel. DERET was hence chosen to detect internalization. Simultaneously, we used $Ca^{2+}$ mobilization, dynamic mass redistribution (DMR) and ERK1/2-phosphorylation assays to assess AT1R signaling according to our previously performed measurements (Figure 59).

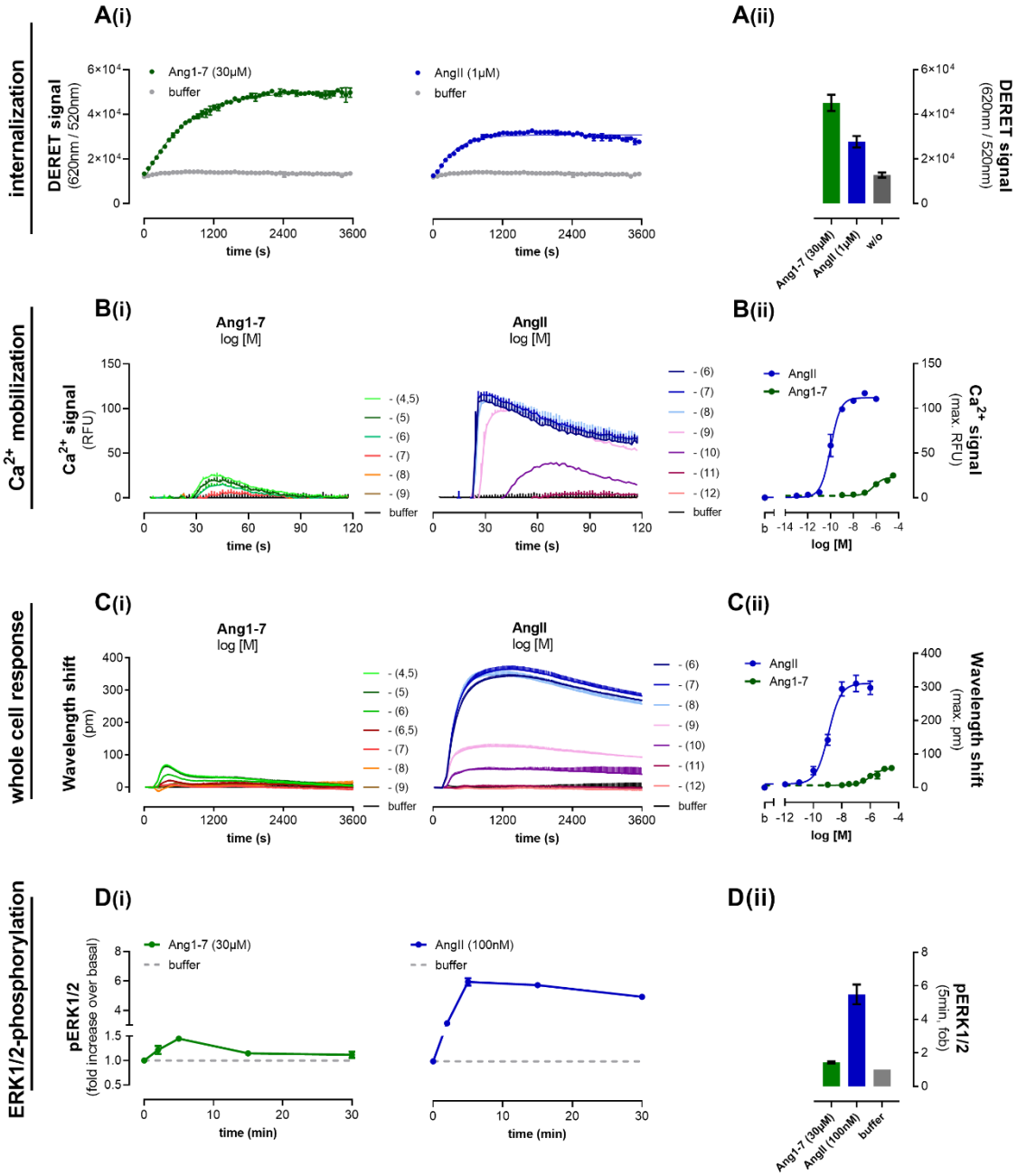


***Figure 59: Comparison of SNAP-AT1R-mediated internalization and signal transduction by Ang1-7 and AngII:***

*Representative kinetics (A(i)-D(i)) and quantification (A(ii)-D(ii)) of HEK293 cells transiently expressing the SNAP-AT1R in response to Ang1-7 and AngII (at concentrations as indicated) of (A) DERET internalization, (B) $Ca^{2+}$ mobilization, (C) whole-cell response (DMR), and (D) ERK1/2-phosphorylation. Representative traces are shown as mean±SD. Quantifications are presented as concentration versus maximal response curves for $Ca^{2+}$-mobilization (max. RFU) (B(ii)) and whole-cell response (max. wavelength shift) (C(ii)). ERK1/2-phosphorylation is summarized by the mean of the phospho-ERK1/2 fold increase over basal detected after 5 minutes (D(ii)), whereas DERET signals are quantified by the plateau value reached, calculated by a one-phase association model (A(ii)). Quantified data consist of four biologically independent experiments (or two*

*for the $Ca^{2+}$ -assay) and are presented as mean±SEM. Assays were performed in technical triplicate for DERET, pERK and DMR and in duplicate for $Ca^{2+}$-mobilization.*

Again, a reproducible, robust increase in measured DERET-signal, which reached a maximum at approximately 30 minutes and remained constant thereafter, was observed upon activation of AT1R by Ang1-7. Underscoring our observations of Ang1-7 as a potent "AT1R-internalizer", the maximal responses obtained for the full agonist AngII, however, did not reach the DERET signals elicited by Ang1-7 (Figure 59 (A(i)+A(ii)).

In contrast, Ang1-7 continued to act as a weak partial agonist at the SNAP-AT1R, as evidenced by the low efficacies or maximal effects obtained for Ang1-7 in $Ca^{2+}$-mobilization-, whole cell response-, and pERK-accumulation-assays compared to the full agonist AngII (Figure 59 (B-D)). The detected SNAP-AT1R signaling induced by the angiotensin peptides was in complete line with our previous results. Thus, we can rule out any SNAP-tag effect on AT1R signaling behavior. In addition, Ang1-7 responses not only differ in amplitude from those evoked by AngII, but also exhibit their characteristic transient profile in comparison to the long-lasting and sustained AngII responses.

The demonstration that Ang1-7 acts as a weak partial agonist at the AT1R in terms of signal transduction, but as a strong "AT1R-internalizer", brings us back to our original question: What is the role of β-arrestins if it is not β-arrestin-signaling for β-arrestin-biased Ang1-7? Is it internalization of the AT1R? To address this, we finally performed a DERET internalization experiment in our previously presented β-arrestin-depleted HEK293 cell line (Δarr), which we have manipulated to stably express the SNAP-AT1R. As shown in Figure 60, the surface levels of SNAP-AT1R were comparable to the transiently expressing HEK293 control cells, as indicated by the similar DERET signals obtained when stimulated with assay buffer (Figure 60, grey). The absence of β-arrestins resulted in an almost complete loss of Ang1-7-provoked AT1R endocytosis, highlighting the critical role of β-arrestins in Ang1-7-induced AT1R internalization. The same was true for the full agonist AngII, for which no internalization was detectable in Δarr cells.

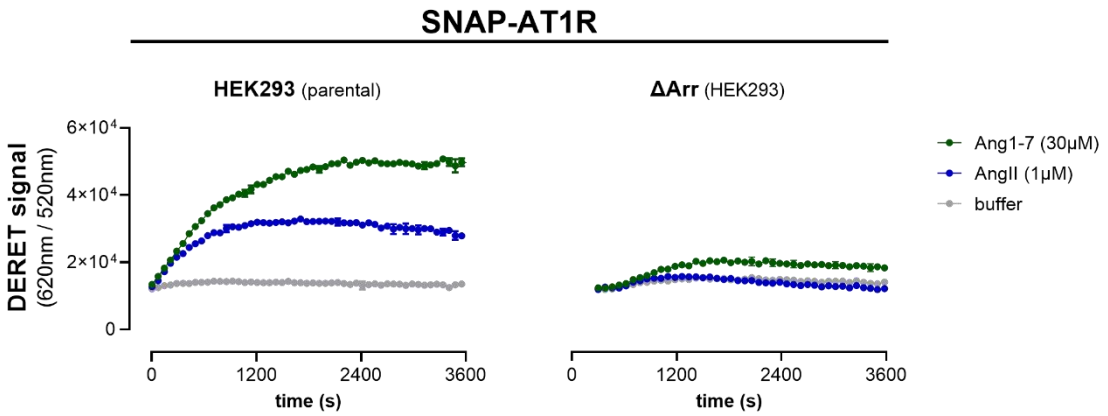


***Figure 60: Effect of β-arrestins on angiotensin peptide-induced SNAP-AT1R internalization:***

*Comparison of Ang1-7- (30µM) and AngII- (1µM) induced SNAP-AT1R internalization in wild-type (left) to β-arrestin1/2-depleted (Δarr) HEK293 cells, monitored as change in detected DERET signals for 1 hour each of a single representative experiment (n=1).*

# 5 Discussion

The paradigm of functional selectivity, or biased signaling, has created a whole new pharmaceutical concept that has shifted drug screening and development to the search for novel biased compounds (Urban et al. 2007; Whalen et al. 2011). However, the strict dichotomy of β-arrestins as independent signal transducers in addition to G proteins has been challenged in recent years and is a source of controversy in the field.

In this work, we tested whether the endogenous Ang1-7 peptide is a true $G\alpha_q$-protein-independent, β-arrestin-biased agonist at the AT1 receptor and how this might contribute to its widely reported beneficial effects in the body.

## 5.1 Ang1-7 signaling at the AT1R is not $G\alpha_q$-independent.

Based on our main quest of this work, we initially focused our investigations on the first aspect, the reported G protein independence of Ang1-7 signaling at the AT1R. We found that Ang1-7 signaling distal to the AT1R cannot be described as G protein independent but is de facto G protein dictated. To reach this conclusion, we applied several assays that detect signaling events from upstream (Gβγ-GRK3-BRET) to downstream ($Ca^{2+}$ release, DMR, pERK accumulation), with a special focus on the involvement of $G\alpha_q$-proteins.

Consistent with the literature (Galandrin et al. 2016; Teixeira et al. 2017), G protein activation for Ang1-7 was barely detectable at the level of G protein dissociation (Figure 33). However, it was evident in our downstream approaches, which allowed us to identify Ang1-7 as a weak partial agonist at the AT1R. In particular, using highly specific pharmacological inhibition (FR and PTX), we were able to clearly delineate the involvement of $G\alpha_q$- and $G\alpha_i$-proteins in Ang1-7-induced cell morphological changes as measured by optical DMR sensing (Figure 38). Notably, $G\alpha_q$-protein activation was shown to be responsible for the small $Ca^{2+}$ release induced by Ang1-7 stimulation at the AT1R (Figure 34) in contrast to *Galandrin et al.* and *Teixeira et al.* who questionably assumed that Ang1-7 was completely independent of $G\alpha_q$-proteins in terms of its $Ca^{2+}$ release at the AT1R, although they observed a slight increase at higher concentrations (Galandrin et al. 2016; Teixeira et al. 2017). Furthermore, using FR, we found that $G\alpha_q$-proteins, which are reported to be least activated by Ang1-7 (Namkung et al. 2018), play a central role in the Ang1-7-promoted pERK accumulation by the AT1R (Figure 28).

Our data illustrate how technical advances in the sensitivity of assays, as well as the discovery of highly specific inhibitors (PTX/FR) of particular signal transducers ($G\alpha_i$, $G\alpha_q$), not only allow studying signaling pathways in greater detail, but also provide a more accurate picture of the signaling cascades that are taking place. Moreover, our observations are fully consistent with recent findings in the field showing that mutants of the AT1 receptor, such as the AT1-DRY mutant, which had been reported to be G protein independent (see Introduction), promote weak G protein activation (Pietraszewska-Bogiel et al. 2020). The same is true for SII, the prototype of all β-arrestin-biased peptides at the AT1R (see Introduction). SII is a vivid example of the changes taking place in the field. Originally classified as G protein independent based on radiolabeling studies such as [$^{35}$S]GTPγS binding (for G protein activation) or myo-[$^{3}$H]-inositol (for IP-accumulation) (Wei et al. 2003; Holloway et al. 2002; Hansen et al. 2008), novel BRET-based biosensors and high sensitive downstream signaling assays have shown that SII induces G protein-dependent signaling at the AT1R through more than one G protein subfamily (Namkung et al. 2018; Saulière et al. 2012; Grundmann 2018).

## 5.2 $G\alpha_q$-dependent Ang1-7 signaling at the AT1R exhibits a characteristic transient signaling profile.

Our results revealed that not only the sensitivity of the chosen method, but also the time dependence of the measurement (kinetic aspects) is crucial for the assessment of the signaling properties of the Ang1-7 peptide. Thus, time-dependent downstream measurements (real-time ($Ca^{2+}$ release) or point kinetics (pERK)) allowed us not only to identify Ang1-7 as a weak partial agonist at the AT1R, but also to discern a characteristic transient action profile compared to the full agonist AngII (Figure 27, 34). Importantly, we have demonstrated that this unique transient signaling profile was driven by G proteins of the $G\alpha_q$-subfamily as there was a loss of the obtained responses under $G\alpha_q$-inhibition with FR (Figure 28, 34).

Regarding $Ca^{2+}$ release, we detected a $G\alpha_q$-dependent weak and short response after Ang1-7 stimulation of the AT1R (Figure 34). We consider that, beside the assay sensitivity, the short duration of the Ang1-7-induced $Ca^{2+}$ mobilization may provide an explanation for the difficulties in the detection of its promoted $IP_1$-accumulation compared to AngII (Figure 33 (C)). This is because while we observed a long-lasting $Ca^{2+}$ mobilization for the full agonist AngII (Figure 33, 34), its $IP_1$ accumulation becomes more pronounced the longer the incubation period. Ang1-7, on the other hand, loses out to the

full agonist due to its transient $Ca^{2+}$ mobilization and thus does not profit in this type of $IP_1$-accumulation assay (Figure 33 (D)).

Likewise, a transient signaling profile for Ang1-7 was also particularly apparent when we measured phosphorylation of ERK1/2 (Figure 27). This characteristic pattern is consistent with other groups showing shorter and lower pERK accumulation for reported β-arrestin-biased ligands such as Ang1-7 and SII compared to the full agonist AngII (Teixeira et al. 2017; Zimmerman et al. 2012).

From a physiological perspective, sustained full agonist AngII activation, as seen in an overactive RAS with persistently elevated calcium levels, can lead to adverse outcomes, such as a prolonged contractile tone of vascular smooth muscle cells (Ohtsu et al. 2008; Higuchi et al. 2007), which, over time, can be a factor in the development of arterial hypertension (Ohtsu et al. 2008; Rhian M. Touyz and Ernesto L. Schiffrin 2000; Delaitre et al. 2021). Therefore, we propose that in the context of an uncontrolled RAS, transient calcium activation, as we see with Ang1-7, is a desirable property and may provide a first explanation for the protective effects of Ang1-7 on the vasculature. Furthermore, given the prominent role of ERK1/2 in cell proliferation and cell growth (Chang and Karin 2001), it is especially noteworthy that prolonged pERK accumulation may be associated with more detrimental effects on the cell (Cagnol et al. 2006; Gallo et al. 2019), whereas transient ERK1/2-activation could favor a beneficial cell state. This may further explain how Ang1-7 signaling through the AT1R exerts protective effects on the heart and other organs.

$G\alpha_q$-proteins were accountable for the prolonged and high pERK- and $Ca^{2+}$-responses to the full agonist AngII, and the same G protein family was responsible for the corresponding transient signals detected after Ang1-7 treatment of the AT1 receptor. We can only speculate about the reasons for the differences in $G\alpha_q$-activation for Ang1-7 vs. AngII. First, β-arrestin-biased ligands are known to stabilize a different conformation of the AT1 receptor than the balanced agonist AngII, which may directly influence the propensity of the receptor to couple to β-arrestins or G proteins (Nivedha et al. 2023; Devost et al. 2017; Saulière et al. 2012). Second, a different conformation or shorter activation of the $G\alpha_q$-protein itself is conceivable, which would then affect the subsequent activation of distal effector proteins (Draper-Joyce and Furness 2019; Klein Herenbrink et al. 2016). Finally, transient signals might be indicative of a preferential stimulation of proteins that terminate signaling pathways, such as ERK1/2 phosphatases (Chang and Karin 2001).

## 5.3 β-arrestins do not mediate the Ang1-7-promoted pERK accumulation at the AT1R.

β-arrestin-biased signaling for Ang1-7 at the AT1R has originally been inferred from its ability to not promote G protein activation but still lead to recruitment of β-arrestins and the activation of ERK1/2 kinases (Teixeira et al. 2017; Paz Ocaranza et al. 2020). Our data uncovered the clear involvement of Gα-proteins in Ang1-7-induced ERK1/2-phosphorylation, ruling out G protein independence. Therefore, we were prompted to further investigate the reported β-arrestin-dependence here.

ERK1/2-phosphorylation is a downstream signaling event, which has the technical advantage of being easier to measure, but the disadvantage that multiple transducers can act on this effector simultaneously (e.g., all four G protein classes and β-arrestins) (Goldsmith and Dhanasekaran 2007; Luttrell 2002). Therefore, to distinguish between G protein- vs. β-arrestin-dependent signaling, methods are needed that definitively eliminate one or the other to unambiguously dissect for the initial signal transducer. To achieve this, we took advantage of CRISPR-Cas9 genome edited HEK293 cells lines in combination with pharmacological inhibition (PTX), resulting in either complete knockout of Gα-proteins ("zero functional G", Δseven+PTX) or β-arrestin1/2 ("zero β-arrestin", Δarr).

We found that there was no detectable pERK accumulation in the absence of functional Gα-proteins after AT1 receptor stimulation with Ang1-7 (Figure 42). Furthermore, neither endogenous abundance of β-arrestin1/2 nor overexpression of a specific β-arrestin-isoform resulted in pERK accumulation in the absence of functional Gα-proteins (Figure 44). Only re-expression of $G\alpha_q$-proteins by transient transfection in our "zero functional G" HEK293 cells could restore the characteristic transient pERK signal profile of Ang1-7 (Figure 42, right). Conversely, ablation of β-arrestins (Δarr cells) had no effect on Ang1-7-induced pERK responses and only FR-treatment led to the abolishment of the signal in these cells (Figure 41).

Altogether, these findings are in agreement with our previous observations of $G\alpha_q$-protein involvement in Ang1-7-ERK1/2 signaling: Ang1-7-induced ERK1/2-phosphorylation through the AT1R is not a result of G protein-independent, β-arrestin-biased signaling. Instead, its ERK1/2-activation is entirely dependent on the presence of active G proteins, predominantly $G\alpha_q$-proteins, which account for the characteristic transient pERK response and which have been reported not to be activated by Ang1-7 stimulation (Namkung et al.

2018; Silva et al. 2020). In line with our conclusion, it is worth noting that a trend has emerged in recent publications addressing originally β-arrestin-dependent signaling outcomes at the AT1R that are now increasingly associated with G proteins (Smith and Pack 2021). A good example for this is the reported signaling mode for AT1 receptors induced by mechanical stress (Tóth et al. 2018b; Zou et al. 2004; Ramkhelawon et al. 2013). Mechano-activation of the AT1R was originally described to promote G protein-independent, β-arrestin-biased signaling. In particular, this was also determined by the detection of ERK1/2-phosphorylation (Rakesh et al. 2010; Wang et al. 2014). Furthermore, this β-arrestin-mediated signaling was reported to be responsible for the Frank-Starling mechanism in the heart (Abraham et al. 2016). However, it was later shown that the originally observed β-arrestin-biased AT1R signaling by mechanical stretch requires the presence of Gα-proteins (Dwivedi et al. 2018; Wang et al. 2018).

Furthermore, our data are fully consistent with novel publications showing that β-arrestins are not only dispensable for initiating pERK responses distal to a GPCR (O'Hayre et al. 2017), but de facto require the presence of functional Gα-proteins, even for described β-arrestin-dependent agonists (Grundmann 2018; Benkel et al. 2022). Notably, all of these novel publications took advantage of complete protein knockout via CRISPR-Cas9 genome editing (Doudna and Charpentier 2014), while older works used RNA interference (siRNA, etc.) (Wei et al. 2003; Ahn et al. 2004b), a method that can specifically silence the expression of proteins, achieving knockdown but not complete elimination of the protein of interest (Dana et al. 2017). For this reason, protein knockdown approaches (siRNA) may not be an ideal tool for discriminating unambiguously between signal transducers within the same pathway (Boettcher and McManus 2015). However, the differentiation of G protein- from β-arrestin-dependent signaling using the CRISPR-Cas9 knockout approach has also been controversial, as questions have been raised about the extent to which knockout may lead to the overrepresentation of other signaling pathways that compensate for the knockout (Luttrell et al. 2018). With respect to our data, there was no reason to conclude that Ang1-7 signaling at the AT1 receptor might be different due to alterations by CRISPR-Cas9 knockout. In fact, we saw that our studies in wild-type and CRISPR-Cas9-edited HEK293 cells were highly complementary. While we observed a loss of the transient pERK signal by $G\alpha_q$ blockade (FR-treatment) in our wild-type HEK cells (Figure 28), this very signal re-emerged in our “zero functional G” cells upon $G\alpha_q$ re-expression (Figure 42).

In summary, in our quest to determine whether Ang1-7 is a true $G\alpha_q$-protein independent but β-arrestin-biased agonist at the AT1R, we can now state that we have found the opposite to be true. CRISPR-Cas9 technology let us identify Ang1-7-promoted ERK1/2-activation at the AT1R as entirely G protein-dependent, not β-arrestins but rather $G\alpha_q$-proteins being the predominant signal-transducer of Ang1-7's pERK responses. Consequently, we suggest that the further classification of Ang1-7 as a β-arrestin-biased agonist at the AT1R is no longer valid.

## 5.4 β-arrestins and GRK2 do not drive but can modulate the G protein driven accumulation of pERK at the AT1R.

Since β-arrestins did not act as transducers for the phosphorylation of ERK1/2 distal the AT1R upon Ang1-7 stimulation, we next investigated the possible modulatory role of these proteins on its $G\alpha_q$-protein mediated pERK response. We included both, β-arrestin1 and β-arrestin2, in our pERK measurements because both β-arrestin isoforms have been described to have reciprocal regulatory effects on ERK1/2-activation at the AT1R (Ahn et al. 2004b). Additionally, β-arrestin-dependent pERK was reported to be associated with a more sustained increase in pERK by the AT1R (Ahn et al. 2004a). For this reason, we measured at different time points to avoid missing any potential β-arrestin effects.

We were able to see a trend toward reduced pERK responses for Ang1-7 and AngII when either β-arrestin1 or β-arrestin2 was overexpressed (Figure 52), pointing to the known prominent downregulatory function of β-arrestins (Turu et al. 2019). This was consistent with our findings in β-arrestin-depleted HEK293 cells (Δarr). Here, pERK responses appeared to be enhanced in the absence of β-arrestins (Figure 41). Interestingly, this was more evident for the full agonist AngII at later time points, suggesting a more modulatory role of β-arrestins on prolonged AngII-induced pERK accumulation. In contrast, the short duration of Ang1-7-induced pERK responses may be less susceptible to modulation by β-arrestins.

Our measurements did not indicate any differences of the β-arrestin isoforms in Ang1-7-ERK1/2 signaling (Figure 44, 52). Of note, the function of the individual β-arrestin isoforms is still controversial, as in some cases completely opposite roles of the isoforms have been described, depending on the tissue (Delaitre et al. 2021). For example, in the heart, the β-arrestin2 isoform is thought to have cardioprotective properties (Rakib et al. 2021), whereas it is associated with detrimental effects on smooth muscle cells (Rakib et al. 2021; Watari et

al. 2013; Kim et al. 2008). Overall for Ang1-7 we only saw a tendency but not a signifcant influence of β-arrestins on the provoked pERK responses at the AT1R (Figure 52).

Analogous to the β-arrestin isoforms, also reciprocal effects of GRK2/3- and GRK5/6-family members on the ERK1/2-phosphorylation after AT1 receptor stimulation has been reported (Kim et al. 2005). Interestingly, β-arrestin-biased peptides at the AT1R have been attributed to GRK5/6 recruitment because of their reported inability to activate Gα-proteins, thus not leading to Gα-protein dissociation with subsequent GRK2/3 activation (Kawakami et al. 2022). Since we have already proven $G\alpha_q$-proteins to be responsible for Ang1-7-mediated pERK responses, we concentrated our efforts on a potential GRK2 modulation here.

Co-expression of GRK2 with β-arrestins resulted in a slight reduction in the obtained pERK responses to Ang1-7 (Figure 53), underlining again the prominent role of β-arrestins and GRKs in downregulating AT1 receptor responses (Tóth et al. 2018b). In contrast, we could demonstrate a distinct regulatory function of GRK2/β-arrestins on the $G\alpha_q$-mediated ERK1/2-phosphorylation for the full agonist AngII (Figure 53, 54): On the one hand, they have a critical function in terminating AT1R signaling as shown by the negative modulation of pERK responses, especially at late time points, which may point to receptor desensitization/internalization (Figure 53 (C)). On the other hand, they may also act as regulatory scaffolds that can help facilitate the phosphorylation of ERK1/2. This may be seen by the potentiation of pERK at 2 minutes (Figure 53 (C)). These up- and down-regulatory effects of GRK2 on MAP kinase signaling have previously been reported in the literature (Robinson and Pitcher 2013; Elorza et al. 2000).

A possible explanation for the distinct influence of GRK2 on AngII- versus Ang1-7-promoted pERK could again be the differential $G\alpha_q$-protein activation. AngII-mediated $G\alpha_q$-activation could lead to a more extensive GRK2 activation and thus may promote the modulatory function of GRK2. Ang1-7, on the other hand, exhibits less or different $G\alpha_q$-activation, which is either unlikely to lead to sufficient GRK2 activation or results in a distinct activation, explaining the different modulatory effects on ERK1/2-phosphorylation for Ang1-7 and AngII.

## 5.5 Excursus: The pitfalls of defining and determining “ligand bias”.

As outlined in the introductory section, a biased ligand can be defined by its ability to stabilize a distinct conformation of a GPCR relative to the balanced ligand, which is then translated into the selective engagement of a different set of effector proteins (Wisler et al. 2018). Thus, a β-arrestin-biased ligand confers a receptor conformation that preferentially recruits β-arrestins over G proteins. One difficulty with this definition is what exactly the term “preferentially” describes. Here, there are three possible interpretations: i) Does it mean that a β-arrestin-biased ligand activates β-arrestins so favorably that it represents a kind of super-agonist on the β-arrestin pathway compared to the balanced ligand? ii) The reverse example is also possible. Losartan, an AT1 receptor blocker, is described as having a predominantly inverse agonistic effect on G proteins rather than on β-arrestin at the AT1R (Ferraino et al. 2021). So, in simple terms, losartan blocks G proteins rather than β-arrestins at the AT1R. Is losartan therefore indirectly β-arrestin-biased? Or is losartan a G protein-biased inverse agonist? iii) The most common variant, which also applies to Ang1-7, is that the β-arrestin-biased ligand acts as a partial agonist on both G proteins and β-arrestins compared to the balanced ligand, but with higher potency in favor of the β-arrestin pathway (Smith et al. 2018; Nivedha et al. 2023). All three examples illustrate the importance of clear terminology definitions. A recent comprehensive review in the field has addressed this issue and all critical aspects of ligand bias characterization in detail and is referenced here for further information (Kolb et al. 2022). In our case, we will only discuss one point that came to light directly in this work.

The extent to which a ligand is able to promote the recruitment of β-arrestins to its receptor is used as a measure of its β-arrestin-bias quality (see above). However, an aspect that is often overlooked in the context of G protein- versus β-arrestin-bias is that the recruitment of β-arrestins to the GPCR may itself be partially G protein-dependent (Drube et al. 2022). In fact, GRKs and protein kinase C, which phosphorylate the AT1R-C-terminus and thus critically influence β-arrestin recruitment to the receptor, are not considered in this context, but are themselves in part highly dependent on prior G protein activation/dissociation (Matthees et al. 2021). This makes the dichotomous view (G protein vs. β-arrestin) even more problematic because players occupying the critical interface between G proteins and β-arrestins cannot simply be neglected.

This is particularly evident in our β-arrestin recruitment data with Ang1-7, in which the extent of β-arrestin engagement with the AT1R can be modulated by the $G\alpha_q$-protein level in the cell (see 4.7). We hypothesize that increased $G\alpha_q$-protein presence and its subsequent activation may lead to increased GRK2 recruitment to the AT1R, explaining the enhanced β-arrestin recruitment under this condition (Figure 48-50). Thus, the presence and the level of expression of G proteins, GRKs and β-arrestins critically control the extent of β-arrestin recruitment to the AT1R. The differences in the efficacies obtained for Ang1-7 in the HEK-T cells compared to the parental HEK293 cells might be attributed to this (Figure 22, 24). Our data show that the recruitment of β-arrestins to the AT1R involves a complex interplay of G proteins and β-arrestins.

## 5.6 β-arrestin-bias as unique switch-off mechanism at the AT1R.

Our study sheds new light on the signaling mechanism of Ang1-7 at the AT1R by identifying G proteins, especially $G\alpha_q$-family members rather than β-arrestins, as the original transducers of its unique signaling responses. Nevertheless, we wondered what would be the consequences of its β-arrestin-bias if a β-arrestin-dependent signaling does not exist. Because we visualized the excellent ability of Ang1-7 to initiate β-arrestin recruitment to the AT1R, consistent with the literature (Galandrin et al. 2016; Teixeira et al. 2017), we therefore focused on the role of β-arrestins in mediating AT1 receptor endocytosis. This led to another key finding of this work: Ang1-7 exerts exceptional AT1R internalization properties via β-arrestins.

We found that Ang1-7 stimulation resulted in a high level of AT1 receptor internalization, which was reflected by a marked increase in the detected DERET signals over time (Figure 56). Downregulation of the AT1R by Ang1-7 was already reported in the early 2000s using indirect measures (Clark et al. 2001a; Clark et al. 2001b). However, our highly sensitive approach enabled us to measure directly signals resulting from internalized receptors in real time (Roed et al. 2014), revealing the strong internalization properties of Ang1-7, even surpassing the ones of AngII in this approach (Figure 56). In addition, Ang1-7 internalization was characterized by a central accumulation of vesicles, which was visible under the microscope by a punctate strong fluorescence intensity after 60 minutes (Figure 58, top). Instead, AngII-induced endocytosis of the AT1R resulted in the accumulation of dispersed vesicles near the plasma membrane rather than in a central cluster (Figure 58, center). Finally, we demonstrated β-arrestins to be the main mediator of AT1R internalization upon

Ang1-7 and AngII treatment, indicated by an almost complete loss of AT1R endocytosis in the absence of β-arrestins (Figure 60).

Consistent with our observations, AT1R internalization capacity has previously been reported for the synthetic β-arrestin-biased SII (Holloway et al. 2002) and for the β-arrestin-biased TRV027 and TRV023 (Kawakami et al. 2022; Szakadáti et al. 2015). *Szakadáti and colleagues* also found a different mode of induced AT1R internalization by these β-arrestin-biased peptides compared to the full agonist AngII (Szakadáti et al. 2015). Interestingly, a pattern of more centrally located endocytic vesicles, similar to that observed with Ang1-7, was shown microscopically after stimulation of AT1 receptors with SII and TRV023 (Szakadáti et al. 2015).

Different reasons can explain the distinct patterns of internalization caused by these AT1R ligands. The AT1 receptor is defined as class B receptor and thus internalizes with the bound β-arrestin (Oakley et al. 2000), the stability of this binding being determinant for the subsequent fate of the internalized receptor (Tóth et al. 2018b). Thus, β-arrestin-biased peptides led to weaker coupling of the β-arrestin to the AT1R and consequently to distinct trafficking of the AT1R in comparison to AngII (Szakadáti et al. 2015). The different stability of the β-arrestin coupling to the AT1R is thought to be due to the distinct AT1R conformations induced by the biased ligands or AngII (Devost et al. 2017; Saulière et al. 2012) and/or the corresponding activation of different GRKs (Kim et al. 2005), which could lead to distinct phosphorylation of the AT1R-C-tail and thus binding of β-arrestins (Kawakami et al. 2022). Both could ultimately lead to different β-arrestin conformations (Shukla et al. 2008; Lee et al. 2016) contributing to the differing stability of the AT1R-β-arrestin complex (Zimmerman et al. 2012; Kawakami et al. 2022; Cahill et al. 2017).

Furthermore, the evolving understanding of the role and importance of membrane phosphoinositides (PIPs) in GPCR internalization added another layer of complexity to the system (Moo et al. 2021; Kelly et al. 2014). For example, the regulatory role of PIPs in the assembly and dynamics of GPCR-β-arrestin complexes has been uncovered for several GPCRs in a recent study (Janetzko et al. 2022). In addition, *Tóth et al.* found that the internalization of the AT1R is critically controlled by the level of $PIP_2$ -lipids (phosphatidylinositol 4,5-bisphosphate) (Tóth et al. 2018b; Tóth et al. 2012). $PIP_2$ plays a crucial role in the cleavage of the vesicles from the membrane and acute depletion of $PIP_2$ has been shown to result in delayed or impaired AT1R internalization, with AT1 receptors

remaining on the cell surface (Tóth et al. 2012). This is particularly important because $PIP_2$ reduction can be achieved by AT1R-$G\alpha_q$-protein activation with concomitant PLCβ-dependent hydrolysis of $PIP_2$ to $IP_3$ and DAG (Figure 12). Thus, the extent of $G\alpha_q$-activation with subsequent $PIP_2$ hydrolysis has been shown to be directly related to AT1R internalization rates (Balla et al. 2012; Szakadáti et al. 2015). β-arrestin-biased peptides at the AT1R, which induce only weak $G\alpha_q$-protein activation, were found to promote enhanced AT1R trafficking from the membrane into early endosomes compared to the strong $G\alpha_q$-protein activator AngII (Szakadáti et al. 2015).

Ang1-7 would combine all the features described here that may explain its strong ability to promote internalization of AT1 receptors: First, it is a weaker $G\alpha_q$-activator through the AT1R than the synthetic β-arrestin-biased peptides SII or TRV023 or TRV027 (Namkung et al. 2018; Lavenus et al. 2018; Kawakami et al. 2022), and second, it also has the lowest affinity for the AT1R of those (Violin et al. 2010; Bosnyak et al. 2011; Miura et al. 2000). Both of these features may represent "ideal" requirements for a strong internalizing property at the AT1R, even superior to AngII. On the one hand, very weak $G\alpha_q$-activation may lead to less $PIP_2$ depletion and thus to high internalization rates of the AT1R. It would be very interesting to investigate whether Ang1-7 shows even better internalization properties than the synthetic β-arrestin-biased peptides SII and TRV023/TRV027 in our DERET approach, which could be an idea for further studies. On the other hand, its low affinity for the AT1R could lead to a distinct AT1R conformation, which in turn could produce a weaker AT1R-β-arrestin complex, yielding in a distinct internalization of the AT1R (Figure 58) (Tóth et al. 2018b). Although Galandrin and colleagues suggested a different β-arrestin-AT1R coupling for Ang1-7 versus AngII (Galandrin et al. 2016), further studies are needed to obtain a comprehensive picture of the exact trafficking routes taken by Ang1-7-internalized AT1 receptors.

From a physiological point of view, reduced AT1R internalization has been described as expression of an overactivated RAS that contributes to the development of several cardiovascular diseases (Bian et al. 2018). Furthermore, abnormal activation of the AT1R by autoantibodies was also shown to result in limited receptor internalization associated with sustained vasoconstriction (Bian et al. 2019). Therefore, Ang1-7 may play a key role as an intriguing endogenous counter regulator of pathologically elevated AT1R surface levels.

From the findings and notions presented in this study, further investigations can be envisaged in more physiological cellular settings ranging from primary cells, tissues, organs to animal models. For example, it is interesting to note that reduced internalization has also been shown for M2 muscarinic receptors stimulated by agonistic autoantibodies (Wallukat et al. 1999). It would therefore be exciting to know in this case whether the targeted development of an agent that could specifically internalize M2 receptors could counteract the negative effects of non-desensitization here. Moreover, based on the concept of how our body, within a peptide cascade such as the RAS, manages to convert the full agonist (AngII) into its "counter regulator" (Ang1-7) by simple proteolytic cleavage, it would be curious to investigate whether a similar concept can be found for other peptide cascades (hormone cascades, for instance) in the body. Finally, focusing on AT1R internalization by Ang1-7 and given the described imbalance of elevated AngII and depressed Ang1-7 levels in Covid-19 (see 1.6), it would be interesting to further investigate whether the deficiency of Ang1-7 and thus the absence of the important "counter-AT1R-internalizer" also can contribute to the pathogenesis in this manner.

In summary, our work has provided new insight into the enigmatic signaling mechanism of the Ang1-7 peptide at the AT1 receptor. Based on the reports of inducing true β-arrestin-bias signaling independent of $G\alpha_q$-proteins, we instead identified Ang1-7 as a $G\alpha_q$-dependent partial agonist with a unique transient signaling profile (pERK, calcium) and an intriguing β-arrestin-bias internalizer of the AT1 receptor. Both of these major findings may help explain the reported beneficial effects of Ang1-7 in a variety of diseases and pave the way for a new approach in drug screening: the search for β-arrestin-biased receptor internalizing compounds.

## 5.7 Summary

Endogenous angiotensin 1-7 is not simply a degradation product of AngII, but rather marks a biologically active peptide at the center of the alternative RAS-axis, a natural counterbalancing branch of the classical RAS.

Based on its property not to elicit canonical G protein signaling at the AT1R, specifically $G\alpha_q$ signaling, but instead to preferentially recruit β-arrestins to the receptor, Ang1-7 was designated as β-arrestin-biased ligand. Furthermore, its ability to nonetheless provoke ERK1/2-phosphorylation distal the AT1R was attributed to β-arrestin-dependent signaling, leading to the classification as a β-arrestin-biased agonist. Thus, $G\alpha_q$-protein-independent, β-arrestin-dependent signaling was suggested to further contribute to the cardioprotective and beneficial physiological effects of the Ang1-7 peptide.

While Ang1-7 leads to a substantial recruitment of β-arrestins to the AT1R, in full agreement with the literature, we were able to show that, instead of being G protein-independent, G proteins direct its signaling downstream of the AT1R. Moreover, we found that $G\alpha_q$, which is reported not to be activated, contributes to the signaling and, intriguingly, can also modify the magnitude of recruited β-arrestins. Strikingly, we show that Ang1-7-promoted ERK1/2-activation can no longer be considered as β-arrestin-dependent signaling. It is exclusively driven by G proteins. In particular, we identified $G\alpha_q$ as the main driver of Ang1-7-promoted pERK accumulation distal the AT1R. We next asked what role, if not signal transduction, the recruited β-arrestins could play upon Ang1-7 treatment of the AT1R. We therefore investigated the possible modulating influence as scaffolds in the provoked ERK1/2-activation. While we observed up- and down-regulatory modulation of AngII-induced pERK accumulation as a consequence of GRK2/β-arrestin scaffolding, we detected a slight reduction on the provoked pERK responses of Ang1-7, suggesting a distinct regulatory role of GRK2 here.

We then addressed the remaining question: If it is not β-arrestin-dependent signaling, what else could be the reason for the preferential recruitment of β-arrestins by Ang1-7? Given the prominent role of β-arrestins in the endocytosis of GPCRs, we investigated Ang1-7 for its ability to induce internalization of the AT1R. Remarkably, Ang1-7 stimulation resulted in strong AT1R endocytosis, allowing us to identify the peptide as a potent "AT1R internalizer".

Therefore, we believe that Ang1-7 may exert its beneficial effects by acting as an endogenous “switch-off” that effectively reduces the number of AT1 receptors on the cell surface, rather than by inducing β-arrestin-biased signaling.

This mechanism of action could serve as a natural model to open up a whole new field of drug development by searching for compounds that not only inhibit GPCRs by blockade, but also act more naturally and sustainably by downregulating the receptor.

# 6 Appendix

## 6.1 Infobox - ACE2 (angiotensin converting enzyme 2)

**ACE2** (angiotensin converting enzyme 2)

The transmembrane protein ACE2 is a homolog of the ACE (Donoghue et al. 2000). It converts circulating peptide substrates with its extracellularly extending catalytic domain and was discovered in 2000 (Patel et al. 2016). As a monocarboxypeptidase, it removes a single amino acid at the C-terminal end of its substrates, unlike the dipeptidyl peptidase ACE (which removes two amino acids) (Tipnis et al. 2000).

ACE2 forms the peptides Ang1-9 and Ang1-7 from the two RAS peptides AngI (Ang1-10) and AngII (Ang1-8), each truncated by one amino acid (see Figure 16). The conversion of AngII to Ang1-7 is 400-fold more efficient (Vickers et al. 2002; Rice et al. 2004), making ACE2 the key enzyme in the generation of Ang1-7.

Through its functions such as i) degradation of the two classical RAS peptides (AngI and AngII), and ii) major producer of the Ang1-7 heptapeptide, it becomes the critical interface between the classical and alternative RAS (Patel et al. 2016).

The enzyme is widely expressed in different organs (lung, kidney, cardiovascular system, brain) (Hamming et al. 2004; Gembardt et al. 2005).

In addition to its prominent role in the RAS, other roles of the enzyme have been described in the literature (Hashimoto et al. 2012; Clarke and Turner 2012). However, in light of the recent Covid-19 pandemic, the function of ACE2 as a portal of entry for coronaviruses (such as SARS-CoV1 and SARS-CoV2) is playing an important role in current research (Li et al. 2003; Turner et al. 2004; Jackson et al. 2022; Jamison et al. 2022).

## 6.2 Infobox - MAS receptor

### MAS receptor

The MAS receptor (also known as MAS1) is a GPCR whose coding gene was originally identified as a protooncogene in the context of a study of tumorigenicity (Young et al. 1986; Rabin et al. 1987). Its name is derived from the first three letters of the last name ("Massey") of the tissue donor from whose sample the gene for the study was derived (Bader et al. 2018).
The GPCR is ubiquitously expressed with highest receptor densities in brain and testis, followed by vasculature, heart, and kidney (Santos et al. 2018).
MAS is involved in important cardiovascular functions. MAS-ablated mice have been shown to develop endothelial dysfunction, profibrotic changes, and impaired cardiac function (Rabelo et al. 2008; Santos et al. 2006).
In 2003, **Ang1-7** was proposed as the endogenous ligand for the MAS receptor (Santos et al. 2003). Both, MAS and Ang1-7, form the alternative Ang1-7/MAS axis of the RAS, which with its described cardioprotective effects, represents a natural counterregulator to the classical RAS (Santos 2019).
However, the extent to which Ang1-7 mediates its beneficial cardiovascular effects via the MAS receptor remains controversial. Although tissues/organs from MAS-deficient mice show a partial loss of Ang1-7-inducible effects (Fraga-Silva et al. 2008; Mario et al. 2012; Santos et al. 2003; Tallant et al. 2005), recent data have challenged this concept by showing that Ang1-7 does not promote G protein signaling or desensitization in cells overexpressing the MAS (Tirupula et al. 2014; Zhang et al. 2012). Of note, a novel comprehensive study focusing on MAS signaling failed to detect any Ang1-7-induced signaling down the MAS. The group was also unable to measure binding of the peptide to the receptor, ruling out a direct interaction between Ang1-7 and the MAS receptor (Gaidarov et al. 2018). Due to the controversial and non-evidence-based data, the MAS receptor is still listed as an orphan GPCR and is therefore not classified as an endogenous Ang1-7 receptor (Karnik et al. 2017; Gaidarov et al. 2018).
Ang1-7 and the MAS receptor have similar protective effects, which may explain their initial correlation as ligand and receptor. MAS itself is a strongly constitutively active $G\alpha_q$-coupled receptor whose presence in the cell influences signaling cascades even in the absence of ligands (Karnik et al. 2017). Thus, MAS may have a direct effect on the RAS by reducing the signaling capacity of the AT1R, both through its constitutive activity and through possible dimerization with the AT1R (Canals et al. 2006; Kostenis et al. 2005).

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# 8 List of figures

# 9 List of tables

# 10 Abbreviations

| *Abbreviation* | *Full name* |
|---|---|
| µM | micromolar |
| 7TMR | seven-transmembrane receptor |
| A23187 | Calcimycin, calcium ionophore |
| Aa | amino acid(s) |
| Ang1-7 | angiotensin 1-7 |
| AngI | angiotensin I |
| AngII | angiotensin II |
| AP | adaptor protein |
| Arg | arginine |
| arr | arrestin |
| Asp | aspartic acid |
| AT1aR | angiotensin II type 1a receptor |
| ATP | adenosine-5’ triphosphate |
| BSA | bovine serum albumin |
| cAMP | 3’, 5’-cyclic adenosine monophosphate |
| Cas9 | CRISPR-associated endonuclease 9 |
| cDNA | complementary DNA |
| $CH_3COOK$ | potassium acetate |
| CNS | central nervous system |
| CRC | concentration response curve |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| DAG | diacylglycerol |
| DERET | diffusion enhanced resonance energy transfer |
| dH2O | demineralized water |
| DMEM (1X) | Dulbecco’s modified Eagle Medium |
| DMR | dynamic mass redistribution |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| E. coli | Escherichia coli |
| EC50 | concentration of half maximum effect |
| ECL | extracellular loop |
| EDTA | ethylenediaminetetraacetic acid |

| | |
|---|---|
| EGF | epidermal growth factor |
| $E_{max}$ | efficacy |
| ERK1/2 | extracellular signal regulated kinase |
| FBS/FCS | fetal bovine/calf serum |
| FR | FR900359 |
| FRET | fluorescence resonance energy transfer |
| G418 | Geneticin |
| GDP | guanosine-5’ diphosphate |
| GFP | green fluorescent protein |
| GPCR | G protein-coupled receptor |
| GRK | G protein coupled receptor kinase |
| GTP | guanosine-5’-triphosphate |
| h | hour(s) |
| HA | hemagglutinin |
| HBSS | Hank’s balanced salt solution |
| HCl | hydrochloric acid |
| HEK | human embryonic kidney cells, HEK293 cells |
| HEPES | *N*-2-hydroxyethylpiperazine-*N’*-2-ethane sulphonic acid |
| His | histidine |
| HTRF | homogenous time-resolved fluorescence resonance energy transfer |
| ICL | intracellular loop |
| Ile | isoleucine |
| $IP_1$ | inositol monophosphate |
| $IP_3$ | inositol 1,4,5-triphosphate |
| KCl | potassium chloride |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| LB | lysogeny broth |
| lgBiT | large BiT |
| LiCl | lithium chloride |
| MAPK | mitogen-activated protein kinase |
| $MgCl_2$ | magnesium chloride |
| min | minute(s) |
| $MnCl_2$ | manganese (II) chloride |

| | |
|---|---|
| MOPS | 3-(N-morpholino) propane sulfonic acid |
| $Na_2HPO_4$ | disodium hydrogen phosphate |
| NaCl | sodium chloride |
| NanoLuc® /Nluc® | Nano-luciferase |
| NaOH | sodium hydroxide |
| NGS | Next Generation Sequencing |
| nm | nanometer |
| nM | nanomolar |
| OD | optical density |
| OptiMEM™ | Opti-Minimal-Essential-Medium (MEM) |
| PBS | phosphate-buffered saline |
| PDL | poly-D-lysine |
| PEI | poly-ethylenimine |
| Pen/Strep | penicillin/streptomycin |
| pERK1/2 | phosphorylated ERK1/2 |
| PFA | paraformaldehyde |
| PH domain | pleckstrin homology domain |
| Phe | phenylalanine |
| $PIP_2$ | phosphatidylinositol 4,5-bisphosphate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLCβ | phospholipase C β |
| pm | picometer |
| PPI | protein-protein interaction |
| Pro | proline |
| PTX | pertussis toxin |
| RbCl | rubidium chloride |
| RFU | relative fluorescence units |
| RH domain | RGS-homology domain |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| RT | room temperature |
| s | second(s) |
| S.O.C. Medium | Super optimal broth with catabolite repression medium |
| siRNA | small interfering RNA |

| | |
|---|---|
| smBiT | small BiT |
| Tyr | tyrosine |
| UV-light | ultraviolet light |
| Val | valine |
| w/o | without |
| wt | wild-type |
| βarr1 | β-arrestin1 |
| βarr2 | β-arrestin2 |

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