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Role of β-arrestin in μ-opioid and TRPV1 receptor signalling

PhD. thesis

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Declaration

I, Vendula Nagy Marková, hereby declare that this PhD thesis is my original work and that it has not been previously submitted (or any of its part) for any academic degree earlier or at another institution. All sources of information used in this thesis have been acknowledged appropriately through citations and references.

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Mgr. Vendula Nagy Marková

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Abstract

β-Arrestin belongs to the protein family which has a huge impact not only on GPCR signaling, but its role exceeds the function of the membrane channel, its own signaling cascade, or as a scaffold protein, etc. Here we aimed to study β-arrestin roles on the MOR behaviour in the plasma membrane or μ-opioid receptor (MOR) signaling and effect on adenylyl cyclase (AC) function using the siRNA to decrease the expression of β-arrestin isoforms. Furthermore, we focused on investigating the role of β-arrestin on the crosstalk between MOR and TRPV1 channels, which are important parts of pain transduction. For this purpose, we used HEK293 cells that stably expressed MOR-YFP or transiently transfected with TRPV1-CFP.

We observed that both β-arrestin isoforms have an effect on the lateral mobility of MOR in the plasma membrane and the silencing of one or another β-arrestin isoforms abolishes the effect of MOR agonists to affect its diffusion in the plasma membrane. Interestingly, silencing of β-arrestin1 diminish the internalization of MOR induced by the endogenous agonist endomorphin-2. On the other hand, silencing of β-arrestin2 did not abolish the endomorphin-2 induced MOR internalization. Moreover, both isoforms exhibit a distinct impact on the inhibition of AC induced by the agonists of MOR. Forskolin-induced AC activity was enhanced in cells lacking β-arrestin2 and suppressed by silencing the β-arrestin1. Furthermore, we observed an important role of $G_{\alpha s}$ in forskolin-induced cAMP accumulation in cells lacking β-arrestins. For the first time, we showed a possible interaction of β-arrestin1 with AC activated by isoprenaline.

The next part of our investigation was to focus on the role of β-arrestin2 in the MOR-TRPV1 crosstalk. We observed that the elimination of β-arrestin2 abolished the effect of MOR or TRPV1 agonists to induce changes in the lateral mobility of one receptor or the other. Furthermore, the level of β-arrestin2 within the plasma membrane was decreased after activation of MOR or TRPV1 in cells expressing both receptors and that β-arrestin2 plays an important role in MOR-induced ERK1/2 phosphorylation in cells expressing TRPV1.

In the last part of our study, we examined the possible cooperation between TRPV1 and TLR4 in the plasma membrane and observe potential crosstalk between TLR4 and TRPV1 after TRPV1 activation.

Together, our study demonstrates the differences between the β-arrestin isoforms in MOR signaling and in modulation of AC activity and that β-arrestin2 is an important mediator in the crosstalk between MOR and TRPV1.

Key words: β-arrestin, μ-opioid receptor, TRPV1 receptor, TLR4 receptor, signaling, adenylyl cyclase, receptor lateral mobility.

Abstrakt

β-Arrestin se řadí do rodiny proteinů, které mají velký vliv nejen na signalizaci GPCR, ale jeho role je i u membránových kanálů, dále má své vlastní signální kaskády nebo jako tzv. “scaffold“ protein atd. V této práci jsme si dali za cíl studovat roli β-arrestinu na chování MOR v plazmatické membráně, signalizaci MOR a vliv na funkci AC. Jeho roli jsme zkoumali za pomoci siRNA metody, díky které se sníží exprese obou izoforem β-arrestinu. Dále jsme se zaměřili na zkoumání role β-arrestinu2 v komunikaci mezi MOR a TRPV1 kanálem, kdy tyto dva receptory jsou důležitou součástí přenosu bolesti. K tomuto účelu jsme použili buňky HEK293 stabilně exprimující MOR-YFP nebo transientně transfektované TRPV1-CFP.

Zjistili jsme, že obě izoformy β-arrestinu mají vliv na laterální mobilitu MOR v plazmatické membráně a umlčení jedné nebo druhé izoformy β-arrestinu zabrání změně difúze MOR v plasmaticke membráně vyvolané agonisty MOR. Zajímavé je, že umlčení β-arestinu1 snižuje internalizaci MOR vyvolanou endogenním agonistou endomorfinem-2. Na druhou stranu umlčení β-arrestinu2 nemělo téměř žádný efekt na internalizaci MOR vyvolanou endomorfinem-2. Obě izoformy navíc vykazují odlišný vliv na inhibici AC vyvolanou agonisty MOR. Aktivita AC indukovaná forskolinem byla zvýšena v buňkách s nedostatkem β-arestinu2 a potlačena umlčením β-arestinu1. Kromě toho jsme pozorovali důležitou roli $G_{\alpha s}$ v produkci cAMP vyvolané forskolinem v buňkách, kterým chybí obě formy β-arrestinu. Poprvé jsme prokázali možnou interakci β-arrestinu1 s AC aktivovanou isoprenalinem.

V další části našeho zkoumání jsme se zaměřili na úlohu β-arrestinu2 v komunikaci mezi MOR-TRPV1. Pozorovali jsme, že knockdown β-arrestinu2 anuluje účinek agonistů MOR nebo TRPV1 na vyvolání změn laterální pohyblivosti jednoho nebo druhého receptoru. Kromě toho byla hladina β-arrestinu2 v plazmatické membráně snížena po aktivaci MOR nebo TRPV1 v buňkách exprimujících oba receptory. β-Arrestin2 hraje důležitou roli v MOR indukované fosforylaci ERK1/2 v buňkách exprimujících TRPV1.

V poslední části naší studie jsme se zabývali možnou spoluprací mezi TRPV1 a TLR4 v plazmatické membráně a pozorovali jsme potenciální vzájemné ovlivňování mezi TLR4 a TRPV1 po aktivaci TRPV1.

Celkově naše studie poukazuje na rozdíly mezi izoformami β-arrestinu v signalizaci MOR a modulaci aktivity AC a také že β-arrestin2 je důležitým mediátorem v komunikaci mezi MOR a TRPV1.

Klíčová slova: β-arrestin, μ-opioidní receptor, TRPV1 receptor, TLR4 receptor, signalizace, adenylát cykláza, laterální pohyblivost receptoru

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List of Abbreviations

A

AC	adenylyl cyclase
AP-1	activator protein 1
AP-2	adaptor protein 2
ASK	apoptosis signal-regulated kinase
ATP	adenosine triphosphate

B

β-arr1	β-arrestin1
β-arr2	β-arrestin2
β-AR	β-adrenergic receptor
β_2V_2R	β_2-adrenergic-vasopressin$_2$ receptor
BRET	bioluminescence resonance energy transfer

C

cAMP	cyclic adenosine monophosphate
CamKII	calmodulin kinase II
C5L2	complement fragment receptor
C5aR1	complement C5a receptor
CCR	chemokine receptor
CFP	cyan fluorescent protein
CGRP	calcitonin gene-related peptide
CXCR	C-X-C motif chemokine receptor

D

Dapp	apparent diffusion coeficient
DAG	diacyl glycerol
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DARC	duffy antigen receptor for chemokines
DMEM	Dulbecco´s modified Eagle´s medium

DOR	δ-opioid receptor
DRG	dorsal root ganglion

E

ECL	extracellular loop
End-2	endomoprhin-2
ERK1/2	extracellular signal-regulated kinase 1/2

F

FBS	fetal bovine serum
FCS	fluorescence correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer

G

GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GIRK	G protein-coupled inwardly rectifying potassium channel
GLP1	glucagon-like peptide-1 receptor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanine triphosphate

H

HEK293	human embryonic kidney cells 293

I

IBMX	3-Isobutyl-1-methylxanthine
ICL	intracellular loop
IP3	inositol triphosphate

J

JNK	c-Jun N-terminal kinase

K

KOR	κ-opioid receptor

L

LPS	lipopolysaccharide

M

M2R	muscarinic receptor 2
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MOR	μ-opioid receptor

N

NTSR1	neurotensin receptor 1
NPY1	neuropeptide Y receptor Y1

O

ORL-1	opioid receptor like 1

P

PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
PNS	post nuclear supernatant
PTH1	parathyroid hormone receptor 1
PTX	pertussis toxin

T

TLR	Toll-like receptor

TM	transmembrane
TRP	transient receptor potential
TRPV	transient receptor potential vanilloid

V

V_2R	vasopressin receptor 2
VGCC	voltage-gated calcium channel
VL-PAG	ventrolateral periaqueductal grey

Y

YFP	yellow fluorescent protein

1 Introduction

β-Arrestins play a crucial role in regulating the signaling of G protein-coupled receptors (GPCRs), which are a large family of membrane receptors that mediate various physiological processes in the body. Originally, β-arrestins were discovered as molecules that "arrest" the activation of GPCRs by preventing further G protein signaling after receptor activation. However, it is now known that β-arrestins also serve as key regulators of GPCR signaling, by serving as scaffolding proteins that recruit other signaling molecules to the receptor, leading to the activation of additional signaling pathways such as the mitogen-activated protein kinase (MAPK) pathways or internalization of receptors. B-Arrestins may affect not only GPCRs, but also channels or receptor tyrosine kinase (RTKs).

MOR and TRPV1 have been shown to interact and modulate each other's activity. MOR is involved in pain relief and addiction, and its activation by opioids leads to inhibition of neurotransmitter release, which reduces pain signaling. However, opioids are highly addictive due to their ability to produce a sense of euphoria and pain relief, which can lead to repeated use and eventually addiction. Furthermore, side effects caused by opioid use can have a significant impact on patient lives. β-Arrestins play a crucial role in regulating the signaling of MOR, and recent research has revealed that they can modulate the signaling of MOR in a biased manner. This means that different ligands can selectively activate different signaling pathways downstream of the receptor, leading to different physiological effects. TRPV1, on the other hand, is a thermosensitive ion channel involved in pain signaling and inflammation. As with MOR, β-arrestin can affect the TRPV1 function and is important in desensitization of TRPV1. Clearly, both receptors play an important role in pain sensation and relief, which makes them interesting targets for the research and development of new therapeutic approaches to pain management.

Unfortunately, the lack of convenient and accurate methods to study the crosstalk of both receptors and simultaneously the involvement of β-arrestins creates a poorly explored scientific area. It is even more important to understand the molecular mechanisms underlying the β-arrestin function in the signaling pathways of both receptors. Here, our objective was to investigate the involvement of β-arrestins in mu-opioid receptor (MOR) and TRPV1 signaling crosstalk and in addition the possible new partner for β-arrestin signaling such as adenylyl cyclase.

2 Literature overview

2.1 β-Arrestin

β-arrestins act as important signaling and scaffolding proteins that can interact and affect many non-receptor proteins in cells. Due to the ability of the β-arrestins to bind to a large number of different signaling proteins, β-arrestins may play a crucial role in several cell events such as cell survival, cell migration, apoptosis, cell proliferation, cytoskeletal organization, or development (Laporte and Scott 2019). We may classify 4 different arrestin isoforms as arrestin1, 2, 3, and 4. Arrestin1 and 4 are in the subclass of visual arrestins, and arrestin2 and 3 belong to the nonvisual class of arrestins. First, visual arrestins were discovered to be part of the retinal photoreceptor region (Dorey and Faure 1977). Lately, non-visual arrestins were identified as a component of β2-adrenergic receptor desensitization machinery and based on their discovery, arrestin2 and 3 are named β-arrestin1 and β-arrestin2, respectively (Attramadal et al. 1992; Parruti et al. 1993).

2.1.1 Structure

β-Arrestin1 and β-arrestin2 share more than 75% sequence identity. Their inactive or basal states are very similar. β-Arrestins are mostly folded from anti-parallel β-strands connected with small loop regions (Zhan et al. 2011). Two main domains (N and C domains) are linked with the middle loop and upon activation, the N and C domain undergo approximately 20° rotation with respect to each other. The C tail of the β-arrestins is folded back in the N domain, supporting the basal state of the β-arrestins (Figure 1). However, upon activation, the C tail is released from the N domain and can interact with proteins of the endocytic machinery such as adaptor protein 2 (AP2) and clathrin. However, a recent study observed that β-arrestin1 may undergo activation upon the binding of phosphatidylinositol 4,5-bisphosphate (PIP2) to its C domain without releasing the C tail of the β-arretsin1 (Zhai et al. 2023). Additionally, the basal state of the β-arrestins is supported by two interdomain interactions known as polar core and three-element interaction (Chen et al. 2018) (Figure 1).

We may describe three parts of β-arrestins as sensors that can recognize the activated GPCR. First, the phosphorylation sensor is located in the N domain of the β-arrestin together with the C tail. This sensor is capable of recognizing the phosphorylated receptor at its C terminus or intracellular loop 3 (ICL3) (Shukla et al. 2013). The activation

sensor consists of the finger loop and the C loop. The finger loop typically interacts with the transmembrane core of activated GPCR (Kang et al. 2015). The last sensor is called the membrane sensor located in the C domain. The C domain includes mainly hydrophobic and hydrophilic residues that can interact with the phosphoinositide groups in the inner layer of the plasma membrane and generate a tighter interaction between the β-arrestins and receptors (Lally et al. 2017) (Figure 1).

It is interesting to point out that there are only 5 published structures of activated β-arrestins together with the hormone-responsive GPCR. All structures were resolved within the last 4 years and were determined mostly together with conformation-selective antibodies such as Fab30 or ScFv16 which support and stabilize the structure of the complex. Individually, there are arrestin structures with M_2-muscarinic receptor (M_2R) (Staus et al. 2020), β_1-adrenergic receptor (β_1AR) (Lee et al. 2020), vasopressin receptor 2 (V_2R) (Bous et al. 2022) and two with neurotensin receptor 1 (NTSR1) (Yin et al. 2019; Huang et al. 2020).

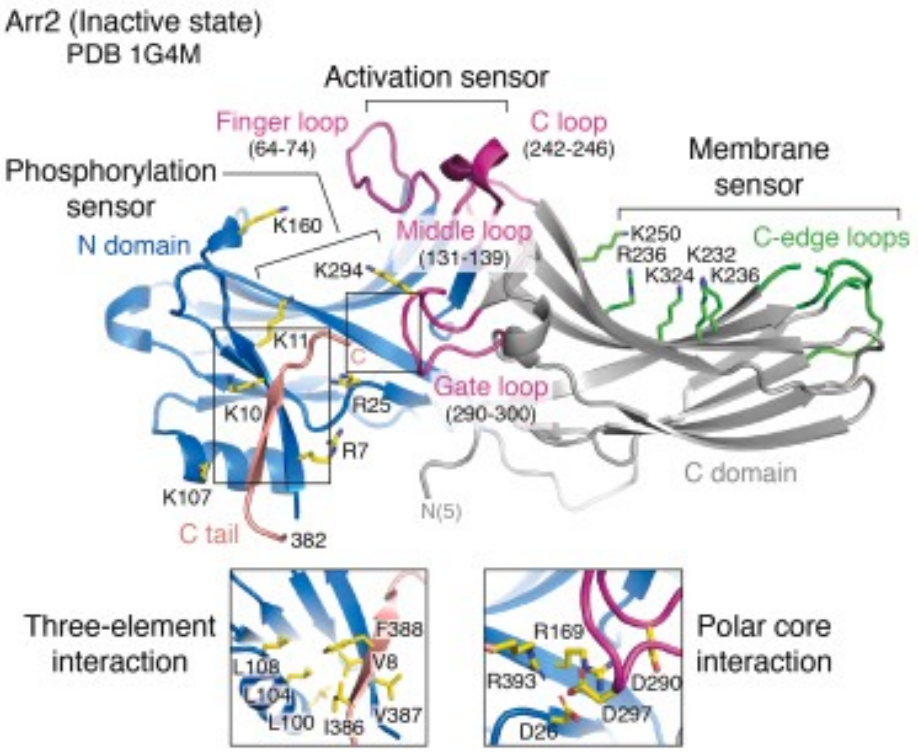

Figure 1 *The structure of β-arrestin1 in its inactive state. The inactive state is stabilized by two interdomains "polar core" and "three-element interaction" showed in a detail. N domain is presented in blue colour and C domain in grey colour. Amino acid residues for the phosphorylation sensor and membrane sensors are showed in yellow and green, respectively. Activation sensor is created by finger loop and C loop represented by purple colour (Chen and Tesmer 2022).*

2.1.2 β-Arrestins and GPCRs

Nevertheless, β-arrestins are mostly known for their regulation of seven transmembrane receptors recognized as GPCR. GPCRs are G protein-coupled receptors that are largely expressed among tissues and cells. GPCRs are widely studied due to their involvement in many diseases, sense of smell or taste, etc. as they respond to hormones, metabolites, neurotransmitters, or cytokines. We can divide GPCRs into six classes according to their functions, Class A - rhodopsin receptors (chemokine receptors, opioid receptors, angiotensin receptors, adrenergic receptors, etc.), Class B - secretin family (calcitonin receptors, glucagon receptors, etc.), Class C -metabotropic glutamate receptors, Class D - fungi mating pheromone receptors, Class E - cAMP receptors, Class F–frizzled and smoothened receptors (Ghosh et al. 2015).

The classic GPCR signaling pathway begins with the binding of the appropriate ligand to the GPCR. The binding of the ligand induces receptor conformational changes that allow the interaction between the activated receptor and the heterotrimeric G protein with bound GDP. The heterotrimeric G protein consists of 3 subunits α, β, and γ. Subsequently, the guanine nucleotide exchange factor (GEF) is responsible for replacing the GDP on the G_α subunit for GTP which leads to the dissociation of the G_α subunit from the $G_{\beta\gamma}$ subunit. Different subtypes of G_α subunits as $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ continue in the signaling through the distinct second messengers as cAMP, calcium, DAG, and IP3 (Gilman 1987). However, the signaling pathway of activated GPCR does not end with the activation of heterotrimeric G proteins. After the dissociation of the Gα and Gβγ subunit form the receptor, the subtype of the kinase family called G protein-coupled receptor kinases (GRKs) terminates the G protein signal and phosphorylates the C terminus or ICL of the receptor in a distinct phosphorylation pattern (Moore et al. 2007). The phosphorylation of the receptor is the signal for the binding of the β-arrestins which abolishes the further interaction between the activated receptor and the heterotrimeric G proteins (Lefkowitz and Shenoy 2005).

β-Arrestins serve as a scaffold for endocytic proteins such as clathrin and AP-2 (Goodman et al. 1996). The complex of GPCR and β-arrestin undergoes internalization in clathrin-coated pits. The decrease in the number of activated receptors in the plasma membranes prevents cell damage caused by sustained signaling. The clathrin-mediated

endocytosis is called the down-regulation of GPCRs. The receptor could be degraded in the lysosomes or recycled back to the plasma membrane (Lefkowitz and Shenoy, 2005).

2.1.2.1 Engagement of β-arrestin and GPCRs

There are only two isoforms of β-arrestin which raises the question: how do the two isoforms of β-arrestin distinguish between hundreds of GPCRs and their phosphorylation patterns? With more observed structures of activated β-arrestin with the activated GPCR, we may have the answer. It seems that β-arrestins are capable of engaging the receptor in several different ways. The first results of different engagement of β-arrestin and receptor were achieved with the β_2AR with replaced C terminus by V_2R (β_2V_2R). Surprisingly, two different structures of the interaction between the β-arrestin1 and β_2V_2R were observed using single-particle electron microscopy. One of them shown in Fig 2 is the interaction called "tail interaction" where the β-arrestin1 is hanging only on the C terminus of the β_2V_2R. The second structure revealed the 'core interaction' where β-arrestin1 forms a tight interface with the transmembrane (TM) core of the β_2V_2R. Presumably, the finger loop of the β-arrestin is responsible for the core interaction (Shukla et al. 2014). Lately, the functions of these distinct interactions were tested on the mutant of the β-arrestin lacking the finger loop part, which abolishes the core interaction together with β_2V_2R, β_2AR, and V_2R. The tail interaction does not prevent the receptor internalization and β-arrestin signaling however it suppresses the desensitization of G protein signaling (Cahill et al. 2017).

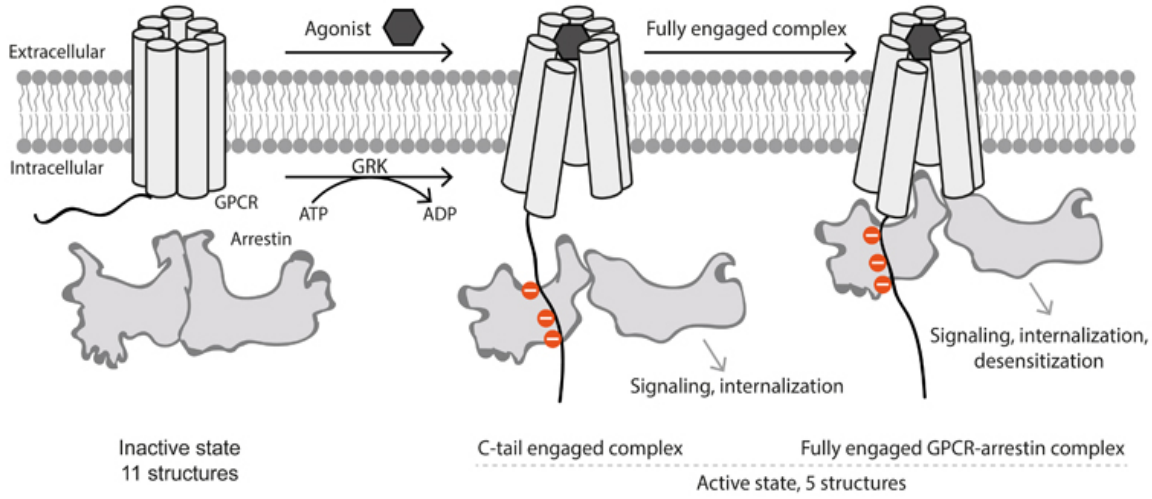

Figure 2 *Distinct engagement of β-arrestin with GPCR. On the left side, inactive state of β-arrestin expressed in the cytoplasm. Upon the activation of the GPCR with the agonist the phosphorylation of the GPCR C terminus is provided by GRKs and the C tail (center) or the core engagement complex of the β-arrestin and GPCR (right) appears (Sente et al. 2018).*

2.1.3 Isoforms of β-arrestins

As mentioned above, β-arrestin1 and 2 share a 75 % sequence identity, and their structure is similar. Studies of the roles of β-arrestin isoforms in vivo showed developmental defects such as abnormal lung and liver development or even embryonically lethal for mice lacking both β-arrestin isoforms. On the contrary, mice lacking only one of the β-arrestin isoforms did not show differences in phenotype compared to the wild type, but they differ in responding to the distinct agents (Zhang et al. 2011). These results may suggest that the two isoforms can substitute each other; however, we may find many differences in signaling, cell distribution, protein partners, involvement in diseases, etc.

It is necessary to mention that both isoforms have the nuclear localization sequence but only β-arrestin2 carries the nuclear export sequence on its C-terminal (Ma and Pei 2007). Looking at the signaling functionality of both isoforms, it is necessary to look first for their interaction partners and their redistribution. β-Arrestin1 binding partners are more associated with the plasma membrane compared to β-arrestin2 interaction partners. This fact suggests that the binding partners of β-arrestin1 are more associated with the GPCR and the cell cycle. However, overlapping of the possible signaling pathways showed more functional activity of β-arrestin2 which may point out β-arrestin2 affects the signaling pathway away from the receptors (van Gastel et al. 2018).

With a novel BRET methodology, we can define even more detailed differences between the β-arrestin isoforms. A recent study focused on differences in β-arrestin1 or 2 binding conformations to the parathyroid hormone receptor 1 (PTH1). Interestingly, β-arrestin1 is more able to form the functional “hanging complex” with PTH1 compared to β-arrestin2 which requires the ‘core complex’ for its proper function. Furthermore, the translocation of β-arrestin2 to the plasma membrane does not require the presence of GRK compared to β-arrestin1 (Haider et al. 2022).

Differences between β-arrestin isoforms do not include just molecular function but we can find a distinct role of the isoforms in diseases. For example, one study described the opposite effect of β-arrestin1 and 2 on microglia-mediated inflammation and pathogenesis in Parkinson's disease (Fang et al. 2021). Furthermore, β-arrestin isoforms may play a role in various types of cancer. In practise, overexpression of β-arrestin1 in transgenic mice resulted in rapid initiation and growth of the xenograft tumor than in wild-type or overexpressed β-arrestin2 transgenic mice (Zou et al. 2008). On the contrary, the expression of β-arrestin2 in a murine model of lung cancer prevents tumor growth (Raghuwanshi et al. 2008). It is obvious that there are clear differences between the two isoforms of β-arrestin however the deeper investigation is still very important.

2.1.4 Signaling pathways of β-arrestin

The role of β-arrestins is not coupled only with GPCRs. Now we know, β-arrestins may have several other functions. Mainly they serve as a scaffold with hundreds of signaling proteins including ion channels, tyrosine kinase receptors, transporters, endocytic machinery, protein kinases, etc. (Ma et al. 2021).

2.1.4.1 MAPK

One of the well-studied signaling pathways where β-arrestin is involved is MAPK (mitogen-activated protein kinase) cascade. MAPKs are an important part of the signaling pathways and they respond to various stimuli like cytokines, growth factors, mitogens, etc. MAPKs are associated with several cell events like apoptosis, cell differentiation, and proliferation. MAPKs cascade consists of three kinases from the upstream: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK. There are three distinct groups of MAPKs: ERK1/2, JNK (JNK1, JNK2, JNK3 isoforms and p38 (α, β, γ, δ). All of them react to their upstream kinases (DeWire et al. 2007).

Activation of MAPK could be G protein-dependent or independent. We know that MAPK activation might be caused by the Gi or Go protein (Ahn et al. 2004). The first evidence of β-arrestin involvement in GPCR signaling was reported by the β-arrestin-mediated recruitment of nonreceptor tyrosine kinase c-Src to GPCR (Luttrell et al. 1999). Lately, it was shown that β-arrestin plays an important role in the activation of ERK1/2. In this case, β-arrestin acts as a scaffold protein for the kinases involved in the MAPK cascade. Upon activation of $AT1_AR$, β-arrestin gets activated and forms a complex that includes MAPKs such as Raf-1, MEK1, and ERK1/2 which leads to the activation of ERK1/2 (Luttrell et al. 2001). On the other hand, we can find GPCRs where β-arrestin-mediated ERK1/2 activation depends on the G protein activity. Concretely, the use of pertussis toxin (blocks the $G_{\alpha i}$ activity) completely abolished the activation by β-arrestin upon the CCR7 chemokine receptor CCR7 (Kohout et al. 2004). To point out the differences between β-arrestin isoforms even in their scaffolding role, a recent study of purified β-arrestin1 and 2 discovered that β-arrestin2 binds to ERK1 / 2 with higher affinity than β-arrestin1 (Perry-Hauser et al. 2022).

The scaffolding role of β-arrestin for another MAPK such as JNK was confirmed shortly after the observation of ERK1 / 2. Using the co-immunoprecipitation assay the complex of β-arrestin2 together with ASK1 (MAP3K), MKK4/7 (MAP2K), and JNK3 was observed (Song et al. 2009; Zhan et al. 2013).

2.1.5 Biased agonism

GPCRs were classified as a switcher between the ‘on’ and ‘off’ states or the two states mode depending on the ligand binding. Drugs called agonists activate the signaling pathway, and in contrast, there are antagonists which block the downstream signaling. However, it was found that receptors might be stabilized in many conformations upon ligand binding that corresponds to the ligand affinity and efficacy for the receptor. Furthermore, some agonists were defined as biased agonists which means they have the ability to direct the signal through the G protein or β-arrestin as opposed to endogenous ligands that activate both signaling pathways at a similar level.

What is the molecular mechanism of biased agonism? The answer might be in a comparison of the β2AR-$G_{\alpha s}$ crystal structure with the rhodopsin-arrestin complex. In the inactive state of GPCRs, there is no space to bind to the G protein or β-arrestin. Interestingly, the active state of the receptor bound to G protein or arrestin is not as distinct

as expected. There is a small exception where the receptor bound to the G protein creates a larger pocket by extending the C terminal side of TM6. This movement opens the pocket in the activated receptor for the Ras-like domain of the G_{α} subunit (Zhou et al. 2017). The study of the GLP1 receptor where mutation at the beginning of TM6 to positively charged residues changes the receptor from unbiased to G protein biased supports the importance of TM6 in G protein binding (Yin et al. 2016). On the other hand, arrestin is mainly associated with the C-terminus of TM7 and the N-terminus of helix 8. More important for the arrestin association with the receptor is the C tail of the receptor and the phosphorylation pattern which is necessary for arrestin binding (Zhou et al. 2017).

Recently, biased agonism is a well-studied phenomenon that has implications in physiology and related pharmacology and drug discovery. In some cases, G protein or β-arrestin signaling pathways were related to beneficial effects in diseases or with side effects. For example, the Gi/o signaling pathway triggered by cannabinoid receptor 1 lead to improved neuronal cell viability in the Huntington disease model (Laprairie et al. 2016). The idea of a drug that could decrease the side effect of the drug only with beneficial effects was tested on the angiotensin receptor. The biased agonist called TRV120027 signals toward β-arrestin which causes better heart contractility and lower blood pressure (Violin et al. 2010). This drug is in clinical testing (Pang et al. 2017).

2.1.6 β-Arrestin coupled receptors

The basic characterization of biased agonism is the ability of the agonist to induce conformational changes of the receptor that led to the activation more towards G protein or β-arrestin. Since we know some agonists have this ability, it could raise the question of whether there is a ligand or even receptor which binds and signal through only one of the transducers.

The studies discovered some of the GPCRs that do not couple with G protein but exhibit the β-arrestin recruitment upon receptor activation. Among these receptors are the decoy D6 receptor, the complement C5a receptor, and the chemokine receptor (CXCR7) (Weber et al. 2004; Kalant et al. 2005; Rajagopal et al. 2010). In terms of concreteness, the complement C5a receptor (C5L2) did not show inhibition of the forskolin-stimulated cAMP response after using two different agonists such as C5a and C5a-desArg. On the other hand, recruitment of β-arrestin2 to the C5L2 receptor was much more effective in CHO cells treated with both agonists (Van Lith et al. 2009).

Recently, Pandey et al. conducted a study that focused on a more detailed description of the possible existence of β-arrestin coupled receptors. They compared two pairs of GPCRs, CCR2 with D6 and C5aR1 and C5aR2, in the context of G protein activation, β-arrestin recruitment, trafficking and GRK preferences, ERK1/2 activation, and the conformational changes of activated β-arrestin. D6-CCR2 were activated with the common agonist CCL7 (chemokine ligand) and C5aR1-C5aR2 share the native agonist C5a. Briefly, the study compared all subtypes of G_α proteins and observed the lack of G_α protein activity between the D6 and C5aR2 receptors using a new NanoBit assay. Furthermore. Both receptors robustly recruited both isoforms of β-arrestins upon activation. Furthermore, the activated D6 receptor does not require the presence of GRKs for β-arrestin recruitment as a result of constitutive phosphorylation in its basal state. Taken together, for the first time this study confirmed the existence of β-arrestin coupled receptors (Pandey et al. 2021).

Note of interest: there is an atypical chemokine receptor called Duffy antigen receptor for chemokines (DARC). DARC does not exhibit coupling either with G proteins or β-arrestins (Chakera et al. 2008). However, the receptor phenomenon needs to be further investigated.

2.1.7 Supercomplexes

We know that β-arrestin may create two different binding states with the activated GPCR described as core engagement or hanging state (Shukla et al. 2014). Particularly, the hanging state of β-arrestin bound to GPCR gives us thoughts about possible space for G protein binding. First, studies on parathyroid hormone receptor or β_2-adrenergic receptor revealed that G protein signaling was not attenuated by binding of β-arrestin to the receptor; however, they observed sustained G protein signaling even from endosomes (Ferrandon et al. 2009; Irannejad et al. 2013).

With increasing information about β-arrestin structure in its activated state bound to the GPCR together with studies describing sustained G protein signaling even from the endosomes, researchers came up with a hypothesis of supercomplexes. This hypothesis was first confirmed in 2016 using functional assays such as the real-time cAMP assay or BRET assay for three different GPCRs: β_2AR, V_2R, β_2V_2R. The results demonstrated the possible formation of G protein, β-arrestin, and receptor supercomplexes in endosomes especially for the typical class B receptor (Thomsen et al. 2016). Later, structural insight in the

supercomplex formation of isolated β_2V_2R, β-arrestin, $G_{\alpha s}$, G_{β}, and G_{γ} was confirmed using the cryo-EM method. Here, it is necessary to mention that the supercomplex structure is made under artificial conditions together with nanobodies which are important to stabilize the active conformation of β-arrestin or $G_{\alpha s}$ (Nguyen et al. 2019).

2.2 μ-Opioid receptor

Opioid receptors belong to the family of GPCRs. We distinguish four different subtypes of opioid receptors, such as δ-opioid receptor (DOR), κ-opioid receptor (KOR), μ-opioid receptor (MOR), and the opioid receptor like-1 (ORL-1) (Mollereau et al. 1994). The distribution of all four classes is through the nervous system like the cortex, limbic system, spinal cord, midbrain, etc. (Volkow and McLellan 2016). Natural agonists of ORs are endogenous opioids such as endorphins, enkephalins, and dynorphins. ORs are involved in many physiological processes including analgesia, euphoria, stress, reward system, etc. (Inturrisi, 2002). However, MOR is one of the most involved receptors in analgesia and pain relief (Kieffer, 1999).

2.2.1 Pain

μ-Opioid receptors are located on the presynaptic and postsynaptic neuron membranes. MOR is coupled to the Gi/o protein family, and its activation leads to the inhibition of voltage-gated calcium channels in presynaptic neurons, which results in the inhibition of neurotransmitter release. Activation of MOR is also responsible for the activation of inward rectifying potassium channels that caused the hyperpolarization of the postsynaptic neuron. Inhibition of neuronal transmission of pain is the effect of opioid use and the cause of analgesia (Inturrisi, 2002). In addition to that, activation of MOR also reduces the production of cAMP and decreases the activity of protein kinase A (PKA). Accordingly, changes in PKA activity and production were connected to the long administration of opioids, where cAMP production increased and resulted in higher activity of PKA. Therefore, PKA activity might be involved in reduced analgesia and caused tolerance (Duman et al. 1988).

More than 20 % of adults suffer from chronic pain in the US. Combating chronic pain becomes a problem since the opioid crisis in the US. The use of opioids is the first choice for treating chronic pain. Although opioids provide fast pain relief, they have several side effects as respiratory depression, diarrhoea, nausea, vomiting, analgesic tolerance, and

abuse liability. All these side effects are responsible for the lower quality of life of patients and lead to overdose deaths. The opioid crisis in the United States is one of the most severe public health crises. The first wave of the crisis related to the nonmedical use of prescribed opioids was followed by the use of heroin as the second wave. The misuse of illegal synthetic opioids led to the crisis as the third wave and the most recent is the combination of psychedelic drugs and opioids. Fencing the crisis is even more difficult when opioids have beneficial therapeutic effects and cannot be forbidden as some other illegal drugs (Volkow and Blanco, 2021).

2.2.2 Opioids

Opium is the extract of the poppy plant *Papaver somniferum*. Opium contains morphine and codeine which have been used for pain relief for thousands of years (Pathan a Williams 2012). Interestingly, to find an even better drug to treat pain, heroin was synthetized from opium. However, heroin turned out to be a very addictive drug (Brownstein, 1993). We can distinguish three groups of opioids: endogenous, synthetic, and semi-synthetic. Some other semisynthetic opioids are oxymorphone or hydromorphone. Fully synthetic opioids are fentanyl, methadone, or meperidine. For example, fentanyl is multiple times more potent than morphine and, in combination with other drugs, becomes even more addictive (Ling and Wesson 1990).

Endogenous opioids are produced in the brain as neuropeptides and are derived from three precursors named prodynorphin, pro-enkephalin A, and pro-opiomelanocortin which enable the formation of three different endogenous opioids such as dynorphins, enkephalins, and endorphins, respectively (Shenoy and Lui 2023). All endogenous opioids are able to bind to MOR, however, endorphins have a higher affinity for MOR than dynorphins and enkephalins, which binds with higher affinity to KOR or DOR respectively (Cuitavi et al. 2021). One special class of endogenous opioids is endomorphin. We describe two types of endomorphins, 1 and 2. Endomorphins are also neuropeptides; however, their precursor has not been identified yet (Gu et al. 2017).

2.2.3 MOR structure

There is only one copy of the μ -opioid receptor gene identified, and it is called *OPRM1*. Since there is only one copy of the OPRM1 gene for MOR, the hypothesis of different splice variants of OPRM1 is more evident. We may now describe three subtypes

of splice variants: full-length seven transmembrane (7TM) domain C-terminal variants, truncated six transmembrane (6TM) variants, and truncated one transmembrane (1TM) variants (Liu et al. 2021).

The OPRM1 gene consists of 4 exons and 3 introns when exons 1, 2, and 3 encode the 7TM variant of MOR, and the C terminal tail is encoded by alternative splice variants of another exon. Interestingly, different splice variants of the C-terminal tail may influence the binding pocket of MOR for some endogenous opioid peptides (Abrimian et al. 2021).

The general structure of MOR consists of seven transmembrane domains that are connected by 3 extracellular loops (ECL1-3) and by 3 intracellular loops (ICL1-3). The binding pocket is surprisingly largely exposed to the extracellular surface and ligand like β-funaltrexamine makes contact, especially with TM 3, 5, 6, and 7 (Manglik et al. 2012). The active and inactive structure of MOR is very similar, except for TM6, which moves outward upon activation (Huang et al. 2015). The main interaction between the active MOR and the Gi protein is provided through ICL2 and ICL3 of MOR. Concretely, ICL2 interacts primarily with two helices of Gi (αN and α5) and ICL3 stabilizes the complex by adding an interaction with β6 strand of Gi (Koehl et al. 2018).

2.2.4 MOR signaling

As mentioned above, MOR is coupled with the Gi protein. Upon activation of MOR by its agonist, the heterotrimeric Gi protein is activated and the $G_{\alpha i}$ subunit dissociates from $G_{\beta\gamma}$ subunits. The role of the $G_{\alpha i}$ subunit is to inhibit the membrane enzyme called adenylyl cyclase (AC), which is responsible for creating the second messenger cAMP. The $G_{\beta\gamma}$ subunits activate the G protein-coupled inward rectifying potassium channels (GIRK) and inhibit the voltage-gated calcium channels (VGCC). However, this is not a complete story of MOR signaling. After the activation of MOR and its Gi proteins, phosphorylation of the C tail of MOR occurs due to the GRKs (Cuitavi et al. 2021). Together, 11 possible phosphorylation sites are exposed on the C tail of MOR and some additional serine or threonine are in ICL of MOR. We can distinguish two phosphorylation cassettes in the C tail of MOR as ^{354}TSST^{357} and ^{370}TREPHSTANT^{379} region (Lau et al. 2011). The study by Doll et al. confirms that the recruitment of different GRK and the phosphorylation barcode of MOR is ligand-dependent using siRNAs for different GRKs. Whereas the partial agonist morphine is capable of recruiting only GRK5 and causing Ser375 phosphorylation, the full agonist DAMGO works together with GRK2/3 and causes rapid phosphorylation of the

^{370}TREPHSTANT^{379} region followed by slow rate phosphorylation of the ^{354}TSST^{357} region (Doll et al. 2012). The phosphorylation of MOR is the essential step for the recruitment of β-arrestin which disrupts further activation of Gi protein and causes desensitization of MOR.

It is known that MOR can recruit both isoforms of β-arrestins. However, similarly to the recruitment of GRKs, the recruitment of β-arrestin1 or β-arrestin2 is dependent on the agonist. Morphine promotes the recruitment only of the β-arrestin2 and DAMGO recruits both isoforms of β-arrestins. Moreover, β-arrestin1 has been shown to be related to MOR ubiquitination together with MOR dephosphorylation (Groer et al. 2011).

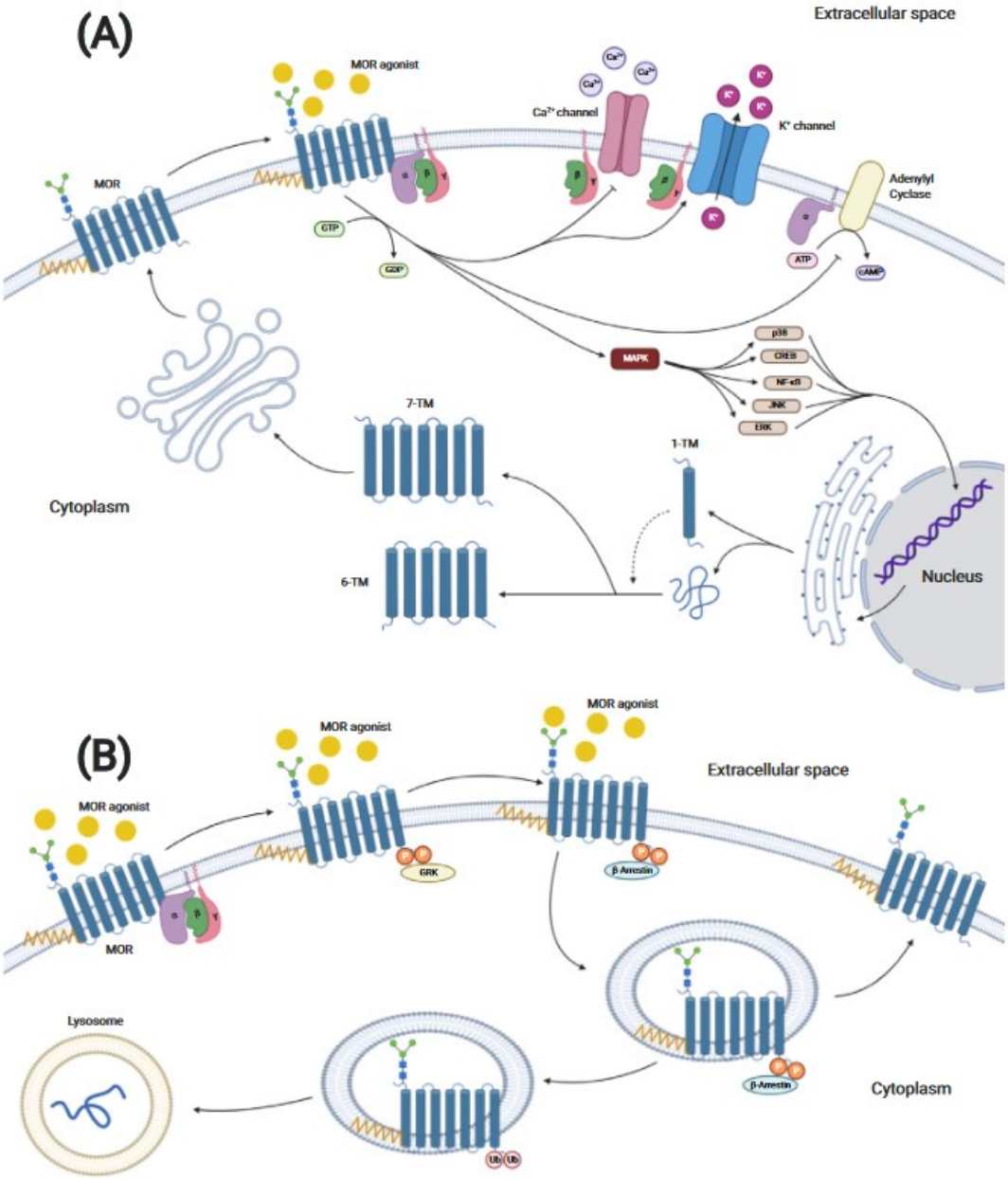

Figure 3 *MOR life cycle. (A)Different splice variants of MOR mRNA are translated to the 1TM, 6TM or 7TM variant of MOR. 7TM variant of MOR goes through the Golgi apparatus to the plasma membrane and its activation by the agonis leads to the activation of heterotrimeric Gi protein. The $G_{\alpha i}$ subunit inhibits the AC and decrease of cAMP production. $G_{\beta\gamma}$ presynaptically inhibits the VGCC channels and postsynaptically activates the GIRK channels. Activation of MOR can also lead to the MAPK signaling cascade. (B) Upon activation of MOR the GRKs are responsible for the MOR phosphorylation and recruitment of β-arrestin to the MOR. β-Arrestin is responsible for internalization of MOR. Internalized MOR might be restored back to the plasma membrane or depredated in lysosomes (Cuitavi et al. 2021).*

2.2.5 Biased agonism of MOR

Since MOR shows coupling not just only with Gi protein but also with β-arrestins researchers came up with the hypothesis of biased agonism for MOR. First, the study using genetically modified mice lacking β-arrestin1 or β-arrestin2 presented interesting results where mice lacking β-arrestin2 showed enhanced and prolonged antinociception induced by morphine using a hot plate test (Bohn et al. 1999). Additionally, following the study observed mice lacking β-arrestin2 do not develop tolerance to antinociception after chronic treatment with morphine. However, mice with β-arrestin2 deletion still built a physical dependence on the drug (Bohn et al. 2000). Surprisingly, even side effects induced by morphine such as respiratory depression or constipation were decreased in mice lacking β-arrestin2 (Raehal et al. 2005). Taken together, the β-arrestin2 pathway could be responsible for lower antinociception and the origin of tolerance induced by morphine (Fig. 4).

These findings led to a search for the agonist with a biased towards the G protein which could solve the problem of side effects caused by morphine. A small synthetic molecule called TRV130 or oliceridine. TRV130 showed high G protein coupling and was less potent in inducing the internalization of MOR. Furthermore, TRV130 causes a more robust analgesic effect than morphine (DeWire et al. 2013). After clinical trials, TRV130 was approved by the FDA approved TRV130 as a new opioid for moderate to severe acute pain treatment (Viscusi et al. 2019; Azzam and Lambert 2022).

Interestingly, two recent studies attempted to reproduce the original study in knockout mice described above. Both studies focused on the respiratory depression side effect caused by morphine and found that β-arrestin2 is not connected to this specific side effect (Kliewer et al. 2020; Bachmutsky et al. 2021). In addition, the problem of addiction to opioids might not be still resolved. A very recent study presented striking results, in which they pointed out that β-arrestin2 is not responsible for compulsive drug-seeking behavior in the case of morphine (Felth et al. 2023). It is necessary to investigate the biased signaling of MOR and shed more light on these very different findings.

As it is mentioned above, there are several opioids such as synthetic or non-synthetic opioids or endogenous opioids. It is known that different opioids may signal through distinct pathways and may result in different cellular environments. Comprehensive studies compared more than 20 opioids and their ability to activate the

G protein or recruit β-arrestin. Interestingly, endogenous opioids such as endomoprhins showed a higher bias towards β-arrestin2 together with etorphine or alfentanil (McPherson et al. 2010; Rivero et al. 2012).

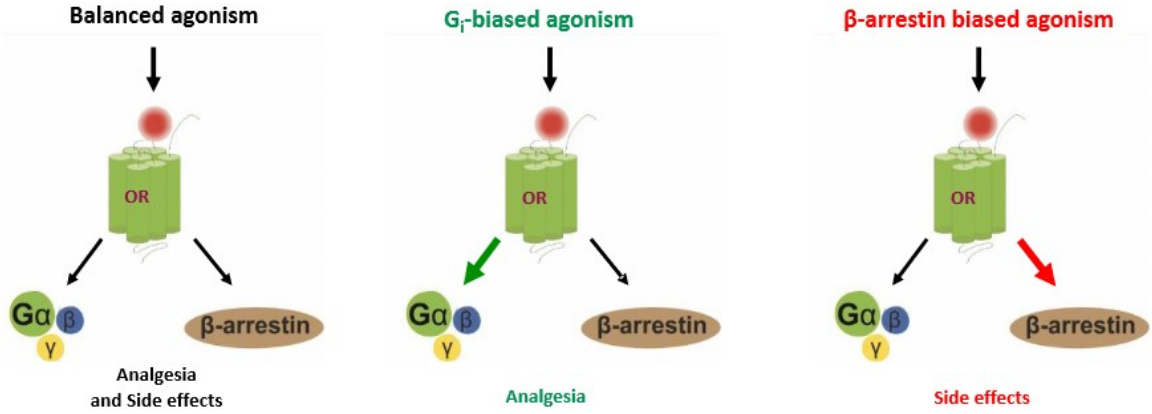

Figure 4 *Opioids and their bias. Balanced opioid agonist causes the analgesia together with side effects. Gi protein biased agonist leads to the hypothesis of beneficial effects as analgesia without the side effects. Contrary, β-arrestin biased agonist might primarily cause side effects (Faouzi et al. 2020).*

2.3 TRPV1

TRPV1 belongs to the TRPV (vanilloid) subfamily of transient receptor potential (TRP) channels together with 6 other subfamilies such as TRPC (canonical), TRPM (melastin), TRPML (mucolipin), TRPP (polycystin), TRPA (ankyrin) and TRPN (nompC). The TRPV family consists of 6 channels such as TRPV1-6. Members of this family can be activated by heat, mechanical stimulation, changes in pH, or ligands (Gees et al. 2012).

TRPV1 is the non-selective cation channel with high permeability for Ca^{2+}. In addition to the activators mentioned above, TRPV1 might be activated and modulated by inflammatory agents, protons, bioactive lipids, anandamide, and by its most known exogenous ligand capsaicin, part of chili peppers (Caterina et al. 1997). TRPV1 expression includes the central and peripheral nervous system, mainly sensory neurons in the dorsal root ganglion (DRG) and hypothalamus. Except for the nervous system, we may find TRPV1 in arteriolar smooth muscle, lung, kidney, or liver (Cavanaugh et al. 2011; Sasase et al. 2022). TRPV1 is involved in pain sensation, especially acute thermal nociception when mice lacking TRPV1 displayed no reaction to pain and vanilloid-evoked pain. In addition, inflammatory mediators are released upon tissue damage, and they can sensitize the TRPV1 channel which decreases the pain threshold (Caterina et al. 2000).

The TRPV1 structure contains six transmembrane domains S1-S6 when the N and C tail is intracellular. The S1-S4 domains are voltage-sensing domains. Domains S5-S6 form the tetrameric assembly that creates the central ion pore. The pore is wide open at the outer site of the channel. At the N terminus, we find six ankyrin repeat domains that are connected to the S1 domain and play a significant role in binding ATP and calmodulin (Liao et al. 2013).

2.3.1 TRPV1 signaling and regulation

TRPV1 is mostly known as the ion channel that responds to noxious heat and chemical stimuli. After applying noxious heat to the sensory neurons, TRPV1 gets activated and it opens its pore for especially calcium and in a less manner for sodium. The influx of these ions leads to the depolarisation of neurons and activation of voltage-dependent sodium channels which cause the creation of action potential. Additionally, activation of TRPV1 at the end of sensory neurons results in the release of substance P, neurokinin A, and calcitonin-gene-related peptide (CGRP). A cocktail of these neuropeptides causes the reaction of several distinct cells, including immune cells, endothelial, or epithelial cells, and gives the creation of neurogenic inflammation.

Calcium is an important second messenger, and it has another important function in cells. Calcium influx after capsaicin or resiniferatoxin is related to the activation of the activator protein-1 (AP-1) transcription factor through the ERK1/2 pathway (Backes et al. 2018).

Regulation of TRPV1 is driven via its phosphorylation at the intracellular part of TRPV1. The phosphorylation of TRPV1 robustly enhances the sensitivity of TRPV1 and the activation temperature threshold decreases to the body level. One of the kinases that phosphorylates the TRPV1 is protein kinase C (PKC). Several studies confirmed PKC as a crucial kinase for the potentiation of TRPV1 activation by heat, protons, and agonists (Premkumar and Ahern 2000; Vellani et al. 2001; Premkumar et al. 2004). Another important kinase for the regulation of TRPV1 is protein kinase A (PKA). PKA is activated by the second messenger cAMP and directly phosphorylates the TRPV1 at the Ser116 residue with the help of scaffold protein A-kinase anchoring protein (AKAP) and reduces the desensitization of TRPV1 (Bhave et al. 2002; Jeske et al. 2008). Interestingly, repeated and long exposure of capsaicin to sensory neurons leads to the desensitization of TRPV1. It was shown that the desensitization of TRPV1 is dependent on the activity of protein

phosphatase 2B called calcineurin and that calcineurin is responsible for the dephosphorylation of TRPV1 and its desensitization (Docherty et al. 1996). On the other hand, phosphorylation of TRPV1 by Ca^{2+}-calmodulin-dependent kinase II (CaMKII) improves the binding of vanilloids like capsaicin (Jung et al. 2004). It is obvious that the balance between phosphorylation and dephosphorylation of TRPV1 is crucial to controlling the TRPV1 activity.

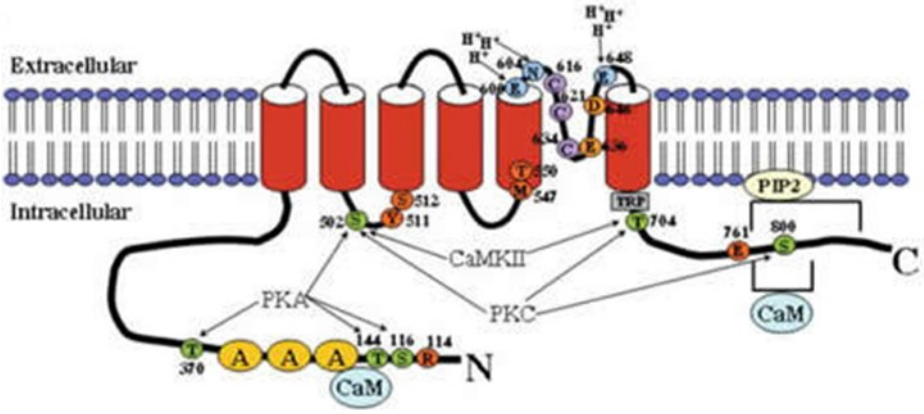

Figure 5 *A scheme of TRPV1 in the plasma membrane. Transmembrane domains are showed in red and should form a tetramer. Orange residues are involved in vanilloid binding. Green residues are suitable for phosphorylation. Blue residues represent the protonatable amino acids. Ankyrin repeats are shown in yellow with "A" (Rosenbaum and Simon 2007).*

2.3.2 TRPV1 and β-arrestins

β-Arrestins are mostly known for their regulation of GPCRs and their signaling cascades. However, we may find evidence that β-arrestins can influence TRPV1. A study from 2012 showed for the first time the association between the β-arrestin2 and TRPV1 in the plasma membrane of sensory neurons and transfected cells. The role of β-arrestin2 is to enhance TRPV1 desensitization. It has been shown that the expression of β-arrestin2 is responsible for the localization of the phosphodiesterase PDE4D5 to TRPV1 in the plasma membrane. PDE4D5 causes degradation of cAMP followed by a decreased activity of PKA. Furthermore, the coexpression of β-arrestin2 reduces the affinity of the agonist for TRPV1 (Por et al. 2012). The subsequent study observed a higher association between TRPV1 and β-arresttin2 together with the activation of PKA and PKC. Furthermore, the phosphorylation of β-arrestin2 at Thr(382) is crucial for the association with TRPV1 (Por et al. 2013).

Moving further in the signaling cascade, β-arrestin2 translocases to activation of the nucleus upon the TRPV1 and increases the RNA polymerase I activity together with the inhibition of the level of tumor suppressor p53 and decrease of the outgrowth of neurites (Hassan et al. 2021).

2.4 TRPV1, MOR and TLR4

In this chapter, I would summarise the cooperation between known information about the MOR and TRPV1 in cells. First, natural co-expression of MOR and TRPV1 was shown in the primary afferent neurons in the dorsal root ganglion (DRG) and neurons in ventrolateral periaqueductal grey (VL-PAG) (Endres-Becker et al. 2007; Maione et al. 2009). Additionally, opioids such as morphine significantly inhibited TRPV1 in DRG neurons by decreasing the capsaicin-induced TRPV1 current (Endres-Becker et al. 2007).

Interestingly, some clinical studies pointed out that patients using opioids for the treatment of chronic pain for a long time or in high doses suffer from hyperalgesia and allodynia (de Conno et al. 1991; Sjøgren et al. 1993; Jacobsen et al. 1995). Since the discovery of this unpleasant side effect of morphine, researchers have tried to find out the mechanism behind the creation of hyperalgesia. First, the use of powerful capsaicin called resiniferatoxin caused the loss of TRPV1 expression in afferent neurons. Loss of TRPV1 turns out to enhance the morphine-induced analgesic effect (Chen and Pan 2006). Furthermore, rats lacking TRPV1 in afferent neurons showed no development of morphine-induced tolerance together with sustained G protein coupling with morphine-activated MOR (Chen et al. 2007). The study in TRPV1 knockout mice observed no development of hyperalgesia in prolonged morphine together with an observation that sustained morphine-induced hyperalgesia could be diminished using the antagonist of TRPV1 (Vardanyan et al. 2009). Taken together, these results suggest the close connection between TRPV1 and MOR especially in morphine-induced hyperalgesia.

These observations lead to the question of what the molecular mechanism of MOR-TRPV crosstalk is. Chen et al. (2008) observed that chronic morphine exposure increased TRPV1 immunoreactivity of TRPV1 in various parts of the nervous system like the DRG, spinal cord, dorsal horn, and sciatic nerve. In addition, an increase in the phosphorylation of various MAPK as p38, ERK1/2, and JNK was also observed after chronic morphine treatment. Sustained morphine treatment results in tolerance and hyperalgesia could be

associated with higher expression of TRPV1 through the MAPK signaling pathway (Chen et al. 2008).

Previous studies dealt with prolonged exposure to morphine. The other view came with opioid withdrawal. Hyperalgesia does not arise only from prolonged opioid treatment; however, hyperalgesia might arise also from opioid withdrawal (nicely reviewed in (Angst and Clark 2006)). During opioid withdrawal, the activity of AC in the plasma membrane is robustly increased, which causes higher activity of PKA. A study on transfected HEK293 cells and neurons from DRG showed that during opioid withdrawal the level of cAMP increased and TRPV1 activity was more robust after capsaicin treatment. TRPV1 appears to play a significant role in opioid withdrawal hyperalgesia (Spahn et al. 2013).

Since β-arrestins play a crucial role in MOR and TRPV1 signaling separately, it raises the question of the role of β-arrestin in MOR-TRPV1 crosstalk. As mentioned above, β-arrestin2 has a negative effect on TRPV1 activity of TRPV1 and causes its desensitization. Trigeminal ganglion neurons naturally co-express MOR and TRPV1. In this system, one study pointed out that activation of MOR by morphine or DAMGO sensitizes the TRPV1 channel in β-arrestin2 dependent manner. Upon MOR activation β-arrestin2 is recruited to MOR and TRPV1 is free of β-arrestin2-induced desensitization (Rowan et al. 2014). On the other hand, a more recent study represented data in which TRPV1 activation with capsaicin drives the β-arrestin2 into the nucleus. This shift of β-arrestin2 into the nucleus prevents the desensitization and internalization of MOR caused by β-arrestin2 (Fig. 6) (Basso et al. 2019).

Taken together, there is evidence that crosstalk between MOR and TRPV1 is important in several physiological processes such as opioid-induced hyperalgesia or morphine tolerance. The balance between the activation and inhibition of MOR and TRPV1, respectively, is crucial. A very recent study created a dual compound that is able to agonize MOR and antagonize TRPV1 at the same time. This compound did not cause side effects such as hyperthermia and tolerance to analgesics (Gao et al. 2023).

Toll-like receptor 4 (TLR4) and its function in the immune system are well established. TLR4 is an important mediator of the immune reaction by creating pro-inflammatory cytokines (Roy et al. 2016). Briefly, TLR4 is a transmembrane protein with the cytosolic domain (TIR domain) responsible for signal transduction and the recognition of pathogens in the extracellular domain. The natural expression of TLR4

occurs in antigen-presenting cells, myocytes, or adipocytes. Furthermore, TLR4 is found in the central nervous system, especially in microglia and astrocytes (Vaure and Liu 2014). Activation of TLR4 in spinal microglia is critical for pain induction and the release of pro-inflammatory molecules (Tanga et al. 2005). Interestingly, TLR4 is expressed in primary sensory neurons and trigeminal neurons together with TRPV1 (Wadachi and Hargreaves 2006). The activation of TLR4 with lipopolysaccharide (LPS) has been shown to cause sensitization of TRPV1 and increased expression of TRPV1 in the plasma membrane (Diogenes et al. 2011; Filippova et al. 2018).

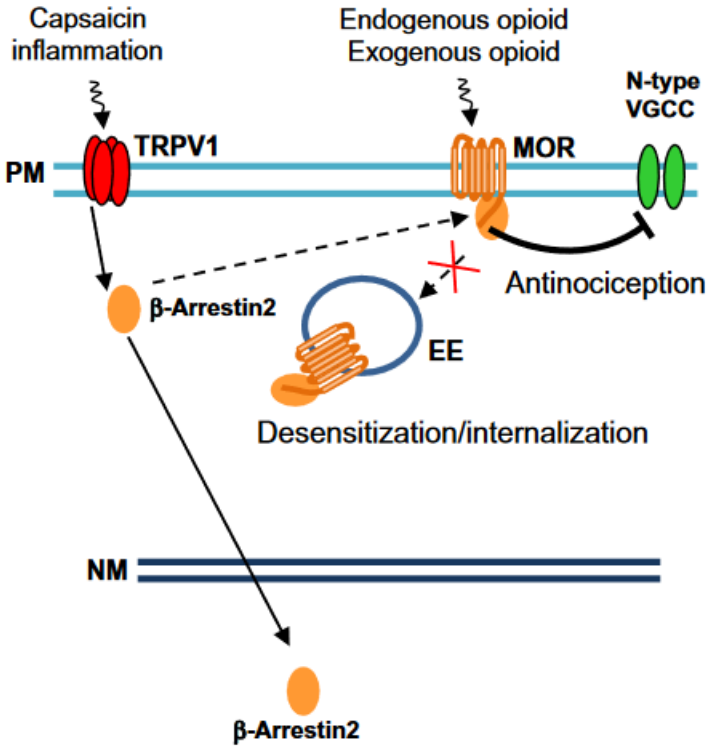

Figure 6 *Possible mechanism of TRPV1 role in MOR desensitization. Upon the TRPV1 activation, β-arrestin2 shifts to the nucleus. Afterwards, the activation of MOR leads to the higher antinociception by inhibition of N-type VGCC channels (Basso et al. 2019).*

3 Aims

- To downregulate the expression of β-arrestin1, β-arrestin2 or $G_{\alpha s}$
- To explore the differences between two isoforms of β-arrestin
    - To investigate the distinct roles of both isoforms in MOR lateral mobility, internalization, and function
    - To examine the differences between the effects of both β-arrestin isoforms on the AC function
    - To delineate the possible interaction between the AC and β-arrestin
- To measure the lateral mobility of MOR and TRPV1 and the possible involvement of β-arrestin2 in MOR-TRPV1 crosstalk in the plasma membrane
- To investigate the role of β-arrestin2 in the signaling cascade of MOR and TRPV1
- To explore the crosstalk between the TRPV1 and TLR4

4 List of publications

4.1 List of publications connected with this thesis

1. Melkes, B., Markova, V., Hejnova, L., & Novotny, J. (2020). β-Arrestin 2 and ERK1/2 Are Important Mediators Engaged in Close Cooperation between TRPV1 and μ-Opioid Receptors in the Plasma Membrane. *International Journal of Molecular Sciences*, *21*(13), 4626.
2. Markova, V., Hejnova, L., Benda, A., Novotny, J., & Melkes, B. (2021). β-Arrestin 1 and 2 similarly influence μ-opioid receptor mobility and distinctly modulate adenylyl cyclase activity. *Cellular Signalling*, *87*, 110124.

4.2 List of other publications

1. Lanznaster, D., Massari, C. M., Marková, V., Šimková, T., Duroux, R., Jacobson, K. A., Fernández-Dueñas, V., Tasca, C. I., & Ciruela, F. (2019). Adenosine A_1-A_{2A} Receptor-Receptor Interaction: Contribution to Guanosine-Mediated Effects. *Cells*, *8*(12), 1630.
2. Melkes, B., Markova, V., Hejnova, L., Marek, A., & Novotny, J. (2020). Naloxone Is a Potential Binding Ligand and Activator of the Capsaicin Receptor TRPV1. *Biological & Pharmaceutical Bulletin*, *43*(5), 908–912.
3. Bondar, A., Rybakova, O., Melcr, J., Dohnálek, J., Khoroshyy, P., Ticháček, O., Timr, Š., Miclea, P., Sakhi, A., Marková, V., & Lazar, J. (2021). Quantitative linear dichroism imaging of molecular processes in living cells made simple by open software tools. *Communications Biology*, *4*(1), 189.

4.3 Author´s contribution on the publications

1. Melkes, B., Markova, V., Hejnova, L., & Novotny, J. (2020). β-Arrestin 2 and ERK1/2 Are Important Mediators Engaged in Close Cooperation between TRPV1 and μ-Opioid Receptors in the Plasma Membrane. *International Journal of Molecular Sciences*, *21*(13), 4626.

The author performed the cAMP assay and contributed on the rest of the experiments specifically transfection of the HMY-1 cell line, preparation of crude membranes and samples for further electrophoresis, and Western blots.

2. Markova, V., Hejnova, L., Benda, A., Novotny, J., & Melkes, B. (2021). β-Arrestin 1 and 2 similarly influence μ-opioid receptor mobility and distinctly modulate adenylyl cyclase activity. *Cellular Signalling*, *87*, 110124.

The author did most of the work including transfection of HMY-1 or HEK293 cells, the FRAP and line-scan FCS experiments, all the cAMP assays, internalization, and confocal microscopy, and contributed on co-immunoprecipitation experiments. The author prepared all the graphs and images and made the statistic. The author contributed on writing the first draft of the manuscript.

I hereby confirm that the above-stated information about the contribution of Vendula Nagy Marková to all articles is correct.

Doc. RNDr. Jiři Novotný, DsC.

5 Methods

5.1 Materials

Fetal Bovine Serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Lipofectamine 3000 and Lipofectamine RNAiMAX reagents were from Invitrogen (Carlsbad, CA, USA). [^{3}H] DAMGO was purchased from American Radiolabeled Chemicals (Saint Louis, MO, USA), the HTRF cAMP kit was from Cisbio Bioassays (Codolet, France), and the Dynabeads Co-Immunoprecipitation kit was from Life Technologies (Carlsbad, CA, USA). The nitrocellulose membrane was purchased from GE Healthcare (Chicago, IL, USA) and the SuperSignal West Dura chemiluminescent detection reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). β-Arrestin 1, ERK1/2, p-ERK1/2, JNK, and pJNK antibodies were from Cell Signaling Technology (Beverly, MA), β-Arrestin 2 antibody was from Invitrogen, AC antibody, HA-probe, p38, and p-p38 antibodies were from Santa Cruz Biotechnology. All other chemicals and ligands were obtained from Sigma-Aldrich (St. Louis, MI, USA) and were of the highest purity available.

5.2 Cell culture

In this work, the human embryonic kidney cell line (HEK293) was used. This cell line was purchased from Sigma -Aldrich (St. Louis, MI, USA). The HEK293 cell line was transfected with the MOR-YFP plasmid. Using selection antibiotics and a cell sorter, a cell line stably expressing the MOR-YFP (HMY-1) (Melkes et al. 2016). HEK293 and HMY-1 cell lines were cultivated in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotic antimycotic solution (AAS, Sigma-Aldrich) and in case HMY-1 cell line geneticin as selection antibiotic was used. Cells were cultivated at 37°C in 5% CO_2-humified atmosphere.

5.2.1 Ligands

As an agonist for MOR, endomorphin-2, DAMGO, and morphine were used at a concentration of usually 1 μM if not stated otherwise. As an antagonist, naloxone was used in the 10 μM concentration for 10 minutes prior to the agonist addition. Cells were

exposed to the agonist for 5-10 minutes as an acute administration, and an experiment was performed afterward.

In the case of TRPV1, capsaicin was used as an agonist in 0.5 μM or 1 μM concentration for 5 minutes before measurement. Capsazepine was used as an antagonist of TRPV1 in 10 μM concentration for 10 minutes prior to adding the agonist.

Isoprenaline was used as an agonist of the β-adrenergic receptor (β-AR) at a concentration of 1 μM for 10 minutes if not stated otherwise.

5.2.2 Pertussis toxin

HMY-1 cells were incubated with 25 ng/ml pertussis toxin (PTX) for 24 hours prior to the experiment. Pertussis toxin was used to inactivate the $G_{\alpha i}$ signaling pathway.

5.3 Transient transfection

Cells were plated in multiwell plates depending on the type of experiment. After 24 hours, when cells reached around 60% of confluence, cells were transfected using Lipofectamine 3000 reagent. Lipofectamine 3000 reagent was diluted in Opti-MEM medium and mixed with diluted DNA in Opti-MEM and P3000 reagent. The final mixture was incubated for 15 minutes at room temperature, followed by the addition of the mixture to the cells. Cells were used for further experiment 24 hours post-transfection. The TRPV1-CFP plasmid was a gift from Dr. Leon D Islas (National Autonomous University of Mexico). The pcDNA3-TLR4-YFP was a gift from Doug Golenbock (Addgene plasmid # 13018 ; http://n2t.net/addgene:13018 ; RRID:Addgene_13018)

5.4 siRNA transfection

Cells were plated in multiwell plates, as it is mentioned above. The transfection reagent Lipofectamine RNAi MAX was diluted in Opti-MEM medium and mixed with diluted siRNA oligos or scrambled siRNA as a negative control in Opti-MEM. The mixture was incubated for 5 minutes at room temperature and added directly to the cell culture. After 24 hours, cells were used for the next experiment. siRNAs to silence the expression of selected proteins such as β-arrestin1 (sc-29741), β-arrestin2 (sc-29208) and $G_{\alpha s}$ (sc-29328) were purchased from Santa Cruz Biotechnology. Select Negative Control No.2 (#4390847) was purchased from ThermoFisher Scientific.

5.5 Fluorescent recovery after photobleaching (FRAP)

HMY-1, HMY-1/TRPV1 or HEK293/TRPV1/TLR4 cells were seeded in a glass bottom multi-well dish. Cells were cultivated in phenol red-free DMEM supplemented with 10% FBS. During the FRAP experiment, cells were incubated at 37 ° C with 5% CO_2. The FRAP experiment was performed on an inverted fluorescent confocal microscope ZEIS LSM 880 (Carl Zeiss AG, Oberkochen, Germany) equipped with the internal spectral detection unit and 40x/1.2 WDICIII C Aprochromat objective lens. For the excitation of fluorophore CFP, the 405 nm laser was used and for the excitation of fluorophore YFP, the 514 nm laser was used. All FRAP experiments were performed on the plasma membrane adjacent to the glass bottom of the well. The diffusion data were collected as a rule of 15 pre-bleach images, and immediately after the photo-bleach point, the 400 post-bleach images were scanned. To bleach the YFP we used a 488 and 514 nm Argon laser at 100% power and for CFP bleaching the 458 and 488 nm 40 mW Argon laser operated at 100% power. To monitor the redistribution of the YFP or CFP the circle region with a diameter of 2 μm was selected. The post-bleach images were scanned at 2% of the maximum laser power with a sampling rate of 2 ms. The recovery curves obtained at least from 50 cells (3 independent experiments) were analyzed using the easyFRAP, MATLAB-based software (Rapsomaniki et al. 2012) and calculated according to the following equation:

$$recovery\ (\%) = \frac{(I_{bleach} - I_{bckg})}{(I_{ref} - I_{bckg})} \times 100$$

Where I_{bleach} is the fluorescent intensity of the bleached spot, I_{bckg} is the fluorescent intensity of the background area, and I_{ref} is the fluorescent intensity of the control area of another cell. The apparent diffusion coefficient (D) for the receptors was calculated according to the following equation:

$$D = \frac{0.224 \times \omega^2}{t_{1/2}}$$

Where ω is the radius of the bleached spot and $t_{1/2}$ is the half-life of fluorescent recovery (Soumpasis, 1983).

The effects of the agonist on the apparent diffusion coefficient of MOR-YFP or TRPV1-CFP were investigated using morphine, endomorphin-2, DAMGO as MOR agonist, and capsaicin as an agonist of TRPV1 in cells lacking β-arrestin2.

5.6 Line-scan fluorescent correlation spectroscopy (FCS)

Data for line-scan fluorescence correlation spectroscopy (FCS) were collected using a Zeiss LSM 880 inverted point scanning confocal microscope. A 63× NA1.4 oil immersion objective was used along with 5 μW of the 488 nm laser at the sample plane. A 32-channel GaAsP spectral detector was operated in single-photon counting mode, and a pinhole size of 1 AU was used. The cell was first located using standard CLSM imaging, and an appropriate area of the plasma membrane was selected, focused, and zoomed in at a size of 6 × 6 μm2. A 6 μm long line was chosen for a fast xt scan, with data collected from at least 45 cells in 3 independent experiments. The acquired data were exported as *.lsm5 files and analyzed using home-written "LS-FCS data analysis" software with a user interface developed in LabVIEW2016 (NI) and a custom-written dll library for fast calculation of spatiotemporal correlations developed in C/C++ (MVS2015, Microsoft). The xt scan image data were converted into a single photon stream and further processed according to the method outlined in Benda et al. 2015 (Benda et al. 2015). No weighted NLSF was used to fit the resulting spatiotemporal correlations, assuming one component of free lateral (2D) diffusion, a Gaussian point spread function, and fast photophysical dynamics as described in Equation (1).

$$g(t,\delta) = g_{\infty} + \frac{1}{N \times \left[1 - F_{pp}\right]} \times \left[1 - F_{pp} \times \left(1 - e^{\frac{t}{t_{pp}}}\right)\right] \times \frac{1}{4Dt + \omega^2} e^{\left(\frac{-\delta^2}{4Dt+\omega^2}\right)}$$

where t is the correlation time, δ is the distance, g∞ is the constant offset, usually equal to 1, N is the average number of diffusing entities within the Gaussian detection area, D is the diffusion coefficient, ω is the radius of the Gaussian profile, Fpp is the fraction of molecules in the dark state and tpp is the time constant for switching between bright and dark states. The read-out parameters include the absolute lateral diffusion coefficient, the size of the detection area, the concentration of labeled diffusing particles, and when combined with intensity trace, the brightness of the diffusing particles.

5.7 Assessment of internalization

Internalization of MOR was assessed using radioactively labeled DAMGO. HMY-1 cells were seeded in a 24-well plate. The next day, cells were transfected with relevant siRNA, and a day after transfection, cells were incubated for different time points (0, 5, 10, 20, and 30 minutes) with relevant ligands such as morphine, DAMGO, or endomorphin-2

at 1 μM concentration at 37 ° C. Subsequently, cells were placed on ice to slow down the cell processes and washed with cold PBS. As the next step, 1-minute incubation on ice was performed with ice-cold acid/salt solution (0.5 M NaCl, 0.2 M acetic acid; pH 2.5) to remove the ligands from the cell surfaces. Subsequently, cells were washed twice with cold PBS followed by incubation with 10nM [^{3}H] DAMGO diluted in serum-free DMEM supplemented with 1% BSA for 2 hours on ice. As a non-specific binding, the 100 μM naloxone was used in parallel wells. After incubation, the cells were harvested by a Brandel cell harvester (Gaithersburg, MD, USA). Using liquid scintillation counting, the radioactivity was determined.

5.8 cAMP determination

For the determination of the cAMP level in cells, homogeneous time-resolved fluorescent (HTRF) technology was used (Cisbio). To observe the inhibition or stimulation of AC, the cAMP-Gi kit or cAMP-Gs kit was used, respectively. AC stimulation or inhibition of AC was carried out using isoprenaline, forskolin or DAMGO, endomorphin-2, and morphine, respectively. Cells were transfected with relevant siRNA, and 24 hours after transfection cells were redistributed in the 384-well microplate and cultivated in DMEM supplemented with 1% FBS. The next day, the relevant ligand was diluted at different concentrations (if not stated otherwise) in stimulation buffer supplemented with 0.5 mM IBMX (to inhibit phosphodiesterase activity) and added to the cells. The cells were incubated with the ligand for 20 minutes at 37 ° C. After incubation, forskolin was used at a final concentration of 2 μM and cells were incubated for another 45 minutes at 37 ° C for the cAMP-Gi kit. During the incubation period, d2-labeled cAMP and the monoclonal anticAMP antibodies labeled with Europium-cryptate were diluted in lysis buffer according to the manufacturer's instructions. Subsequently, the d2-labeled cAMP and anti-cAMP antibodies were added to the cells and incubated for 1 hour at RT. Subsequently, the lysed cells were transferred to a 96-well white low-volume plate. Using the Clariostar plus microplate reader (CLARIOstar, BMG Labtech), the fluorescent signal was read at 620 nm and 665 nm. To observe the FRET signal the ratio of emission of acceptor (665 nm) and donor (620 nm) was calculated. Using the calibration curve, the FRET ratio could have been converted to the cAMP concentration. The baseline concentration of cAMP was considered 0 % and forskolin itself was considered as the maximum response in the case

of the cAMP-Gi kit. Isoprenaline or forskolin in their highest concentration was granted as 100 % in the case of the cAMP-Gs kit.

5.9 Co-immunoprecipitation

To detect the possible interaction between β-arrestins and AC we performed the co-immunoprecipitation assay using the Dynabeads Co-IP kit. First, HEK-293 cells were transfected with β-arrestin1-HA tag or β-arrestin2-HA tag which were generous gifts from Robert Lefkowitz (pcDNA3 barr1 HA: Addgene plasmid #14693; http://n2t.net/addgene :14693; RRID: Addgene_14693; pcDNA3 barr2 HA: Addgene plasmid #14692; htt p://n2t.net/addgene:14692; RRID: Addgene_14692) (Luttrell et al. 1999). The plasmids were transfected using Lipofectamine 3000 as described in the chapter Transient transfection.

The Co-IP kit was used according to the manufacturer's instructions. First, to proceed with Co-IP, the specific antibodies must be covalently coupled to the Dynabeads® M-270 epoxy beads. Using the AC antibody (sc-377243; Santa Cruz Biotechnology), we performed the covalent binding of the antibody with 7 µg/mg of Dynabeads®. The antibody was diluted in C1 buffer and mixed with Dynabeads® diluted in C2 buffer. The mixture was incubated overnight on a rotatory shaker at 37 ° C. The next day, the Dynabeads® were collected and washed several times using the DynaMag™-2 magnet as it was written in the manufacturer´s protocol. Subsequently, the transfected cells were harvested and pelleted. The cell pellet was mixed with the lysis buffer (1x IP, 200nM NaCl, 0.5% Triton X-100 and the Complete EDTA-free protease inhibitor cocktail) in a 1:9 ratio (weight of the cell pellet:volume of the lysis buffer). The lysis was completed on ice for 15 minutes. As a next step, the lysed pellets were centrifuged at 2600 x g for 5 minutes at 4 ° C to remove cell debris and nuclei. The supernatant was mixed with 1.5 mg of the beads and the mixture was rotated for 30 minutes at 4 ° C. Thereafter, the beads were washed in extraction buffer and last wash buffer (LWB) using the magnet. In the last step, the beads were incubated with elution buffer (EB) for 5 minutes at RT on the rotator. Eluted purified proteins in the supernatant were used directly for SDS-Page electrophoresis and Western blot. The immunosignal was normalized to the weight of the cell lysates.

5.10 Isolation of a plasma membrane fraction

HMY-1 cells were transfected with TRPV1-CFP plasmid using Lipofectamine 3000 in 80 cm^2 flasks. After 24 hours after transfection, relevant ligands were applied for 5 minutes. Subsequently, cells were placed on ice, harvested, and homogenized in cold TMES (20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose; pH 7.4). To isolate the plasma membrane fraction, the Percoll® self-forming gradient was used. First, homogenized cells were centrifugated for 1000 g, 3 minutes at 4 ° C to obtain the post-nuclear supernatant (PNS). PNS was loaded onto 18% Percoll diluted in TMES buffer. Thick polycarbonate tubes with 30% Percoll and PNS on the top were centrifugated on a Beckman Ti50 for 15 minutes at 60 000 g. Two layers were formed, the upper layer containing the plasma membrane was aspirated and diluted in TME buffer (20mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA; pH 7.4) and centrifugated in Beckman Ti50 for 150 000 g, 1 hour. The formed pellet was collected and resuspended in TME buffer, frozen with liquid nitrogen, and stored at -80°C.

5.11 SDS-PAGE electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the effect of different siRNAs, detect the possible protein-protein interaction after Co-IP, and observe the phosphorylation of different MAPK.

Cells were transfected with relevant siRNAs or with TRPV1-CFP using Lipofectamine RNAiMAX or Lipofectamine 3000 respectively. After 24 hours post-transfection cells were harvested in cold PBS supplemented with a protease inhibitor cocktail and phospho-stop and homogenized by sonication (in the case of Co-IP the eluted proteins were used). Samples were diluted in Laemmli sample buffer at the final protein concentration of 1 μg/μl. The samples were loaded onto 10% polyacrylamide gel (20 μg of protein per well). Electrophoretic protein separation was performed under 200 V for 45-60 minutes.

5.12 Western Blot

Separated proteins on the gel were transferred to a nitrocellulose membrane using the wet blot technique (100 V; 60 minutes). The membrane was blocked with 5% non-fat dry milk diluted in TBS-T buffer (10mM Tris, 150 mM NaCl; pH 8, 0.3% Tween-20) for

30 minutes. Subsequently, the primary antibodies were diluted in 2.5% non-fat dry milk in TBS-T. The membrane was incubated with the relevant primary antibody against ERK1/2 (137F5; Cell Signaling), p-ERK1/2 (197G2; Cell Signaling), p38 (sc-535; Santa Cruz), p-p38 (D3F9; Cell Signaling), JNK (C80C3; Cell Signaling), p-JNK (81E11; Cell Signaling), AC (sc-377,243; Santa Cruz Biotechnology), HA-probe (sc-7392; Santa Cruz Biotechnology), β-arrestin1 (D8O3J; Cell Signaling) or β-arrestin2 (PA1-732; Invitrogen), and rocked overnight at 4 C. The next day, the membrane was washed three times for 10 minutes in TBS-T and subsequently incubated with the relevant secondary antibody diluted in 2.5% fat-free dry milk in TBS-T for 1 hour at RT. Followed by three times 10-minute washes in TBS-T. Using the enhanced chemiluminescence (ECL) method, the protein bands were visualized and developed on the film. The blots were scanned with high-resolution CCD scanner and evaluated using ImageJ software.

5.13 Statistic

The statistics were completed using GraphPad Prism software (versions 6.0 and 8.0). Data are shown as mean values ± standard error of the mean (S.E.M) of at least three independent experiments. The statistical significance of differences between the means of the relevant groups was assessed by one-way ANOVA followed by the Bonferroni test. Statistical significance was defined as p values: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, ****$p \leq 0.0001$.

6 Results

6.1 Knockdown of β-arrestins and $G_{\alpha s}$ protein

To verify the decrease in the expression level of the relevant protein, we used SDS-Page and western blot methods. Cells were transfected with relevant siRNAs (β-arrestin1, β-arrestin2, and $G_{\alpha s}$) and harvested. We obtained a decrease in relevant protein expression in all cases (Figure 7). As a loading control, we used Vinculin.

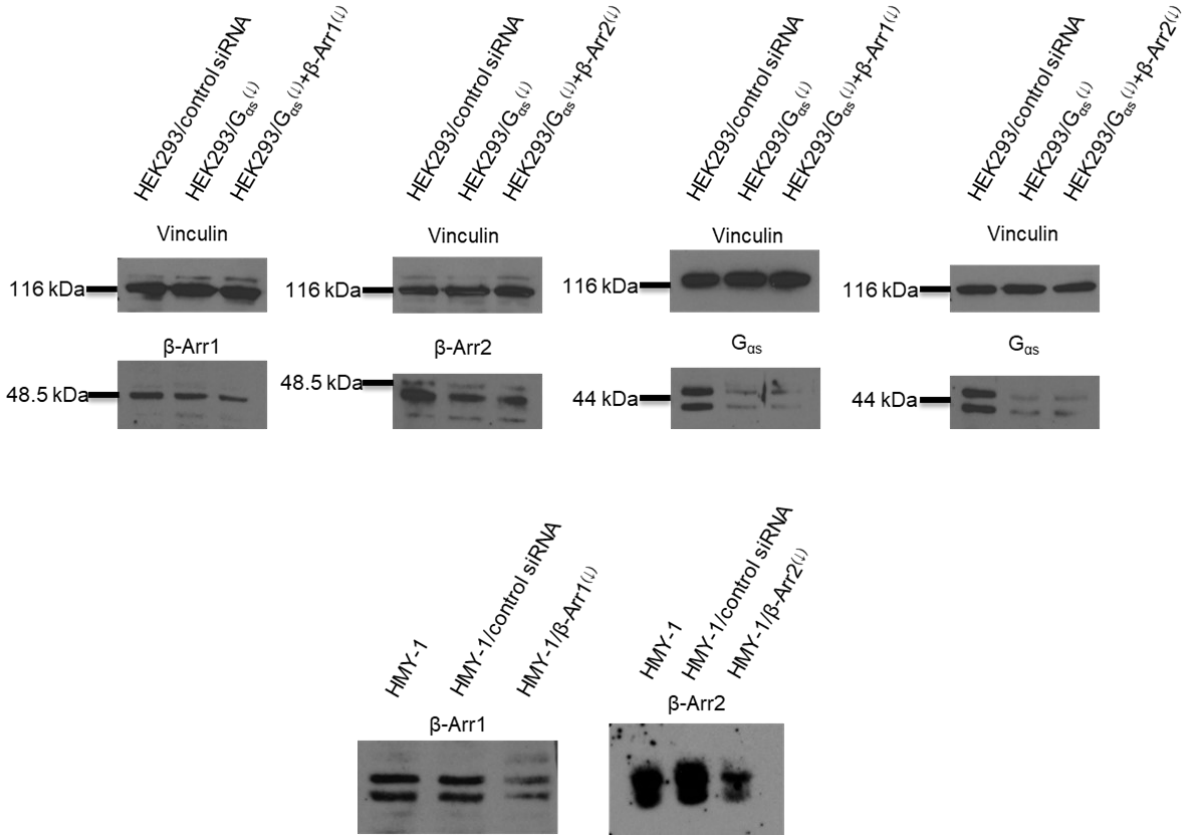

Figure 7 *Western blot images show the protein expression level in control cells and in cells transfected with relevant siRNA. HEK293 or HMY-1 cells were transfected with relevant siRNA and after 2 days cells were harvested, and SDS-Page and western blot was performed. Vinculin was used as loading control.*

6.2 β-Arrestins and their effect on the lateral mobility of MOR

To observe the lateral mobility of MOR-YFP in the plasma membrane in HMY-1 cells with lower expression of β-arrestin1 or β-arrestin2 we came up with two different

fluorescent microscopy approaches, such as Fluorescent Recovery After Photobleaching (FRAP) and Line-Scan Fluorescence Correlation Spectroscopy (FCS).

6.2.1 Fluorescent recovery after photobleaching

HMY-1 cells were seeded in a glass bottom well and the apparent diffusion coefficient of MOR-YFP was measured in a resting state and after activation of MOR-YFP by DAMGO, endomorphin-2 (End-2), or morphine at 1 µM concentration HMY-1 cells, HMY-1/β arr1$^{(\downarrow)}$ or HMY-1/β-arr2$^{(\downarrow)}$. Representative recovery curves of MOR-YFP in HMY-1 cells and HMY-1 cells lacking β-arrestin1 and β-arrestin2 are shown in Figure 8A, B, and C.

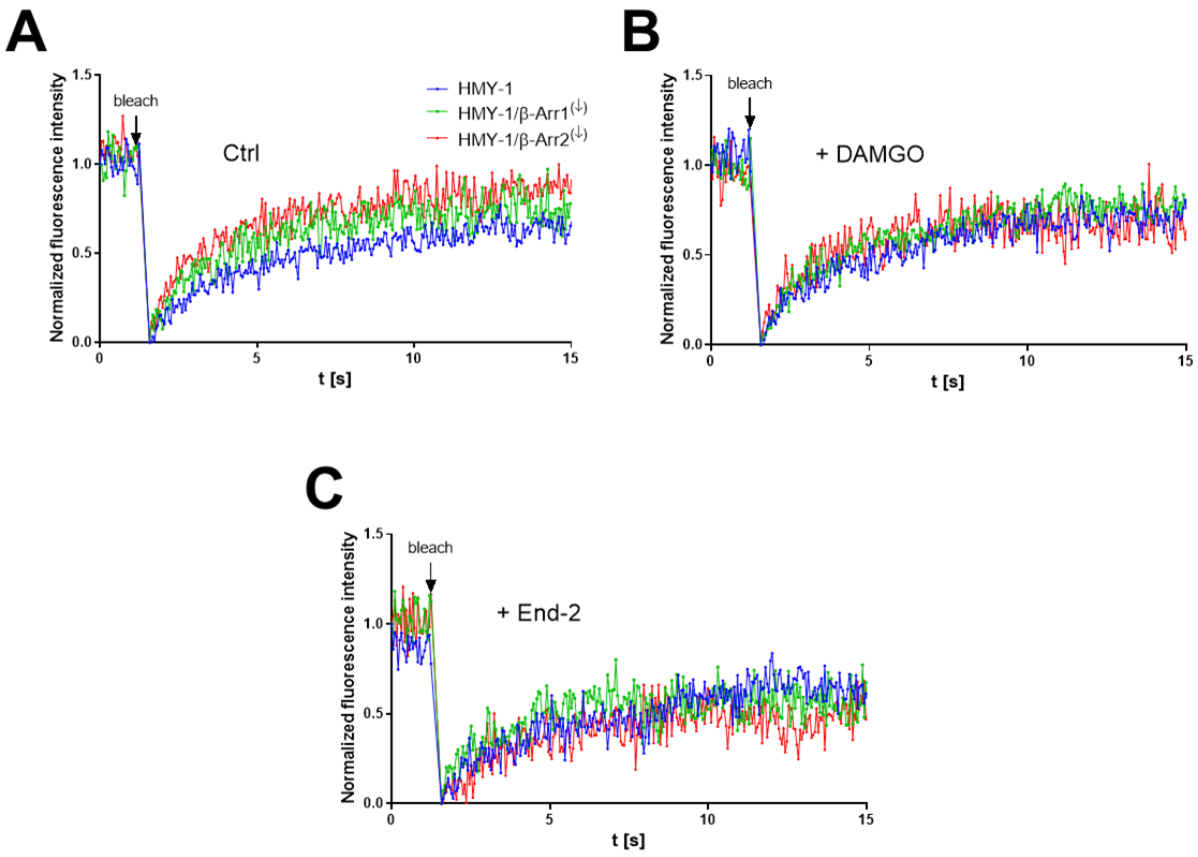

Figure 8 *Representative normalized recovery curves of FRAP measurements for the mobility of MOR-YFP in the plasma membrane and the effect of silencing β-arrestin1 and β-arrestin2. HMY-1 cells were seeded in glass bottom wells and transfected with relevant siRNA (β-arr1 or β-arr2). Activation of MOR was performed using 1 µM DAMGO or End-2 for 10 minutes. MOR mobility was measured on the bottom part of the plasma membrane close to the glass. 24 hours post transfection the MOR mobility was measured under resting conditions (A), treatment with DAMGO (B) or End-2 (C).*

From the recovery curves, we obtained the apparent diffusion coefficient of MOR-YFP (Figure 9A) and the mobile fraction of MOR-YFP (Figure 9B). The diffusion coefficient of MOR-YFP was significantly increased after activation of MOR-YFP with DAMGO (D = 0.53 ± 0.03 $\mu m^2/s$). In contrast, the lateral mobility of MOR-YFP in the plasma membrane was significantly decreased in the case of MOR-YFP activation with End-2 (D = 0.24 ± 0.03 $\mu m^2/s$). However, morphine did not cause any significant changes in the diffusion coefficient of MOR-YFP compared to the control.

We examined the apparent diffusion coefficient of MOR-YFP in cells lacking β-arrestin1 or β-arrestin2. The lateral mobility of MOR-YFP in the plasma membrane significantly in HMY-1/β-arr1$^{(\downarrow)}$ (D = 0.49 ± 0.02 $\mu m^2/s$) and in HMY-1/β-arr2$^{(\downarrow)}$ (D = 0.5 ± 0.02 $\mu m^2/s$). Activation of MOR-YFP by opioids did not cause any significant changes in HMY-1/β-arr1$^{(\downarrow)}$ and HMY-1/β-arr2$^{(\downarrow)}$ compared to the control.

Activation of MOR-YFP by different ligands did not cause any significant changes in the mobile fraction of MOR-YFP in HMY-1. However, in HMY-1/β-arr1$^{(\downarrow)}$ and HMY-1/β-arr2$^{(\downarrow)}$ cells we observed a mobile fraction of about 10% decrease of MOR-YFP after using DAMGO and about 20% decrease of approximately 20% of the mobile fraction of MOR-YFP after using End-2 (Figure 9B).

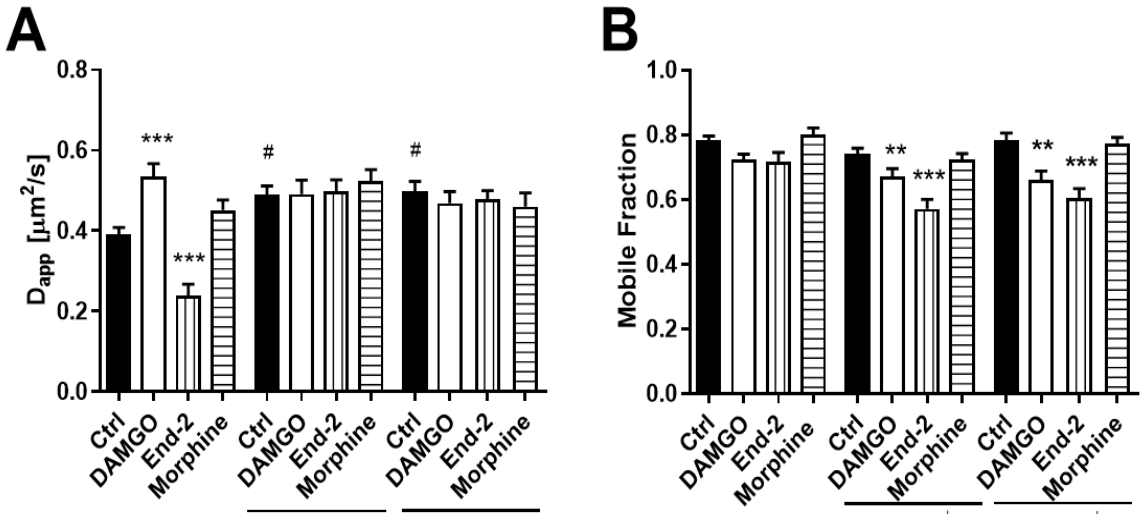

Figure 9 *Apparent diffusion coefficient (D_{app}) of MOR-YFP (A) and its mobile fraction (B) in HMY-1 cells lacking β-arrestin1 (β-Arr1$^{(\downarrow)}$) or β-arrestin2 (β-Arr2$^{(\downarrow)}$) and in presence of DAMGO, End-2 or Morphine. Data was collected at least from 60 cells in 3 independent experiments. Results are expressed as mean ± SEM (** p<0.01, *** p<0.001 versus respective control, # p<0.05 versus untreated control)*

6.2.2 Line-Scan Fluorescent Correlation Spectroscopy

The next method how to observe the movement of the selected receptor in the plasma membrane is Line-Scan Fluorescence Correlation Spectroscopy (FCS). The cells were treated in the same way as in the FRAP experiment. The spatiotemporal correlation function curves (STCF) are shown in Figure 10A for control HMY-1 cells and cells treated with DAMGO and End-2. Typically, the STCF curves represent the rate of diffusion of selected proteins, in our case MOR-YFP. The diffusion rate of MOR-YFP is shown at distances of 0 and 480 nm. Line scanning FCS allows us to determine the diffusion coefficient of MOR-YFP in the plasma membrane under our conditions (Figure 10B). We observed an increase in MOR-YFP movement in the case of DAMGO and a decrease in the case of End-2 in control cells. We found an approximately 22% increase in MOR-YFP diffusion in cells lacking β-arrestin1 and β-arrestin2. These results correspond directly to the FRAP results.

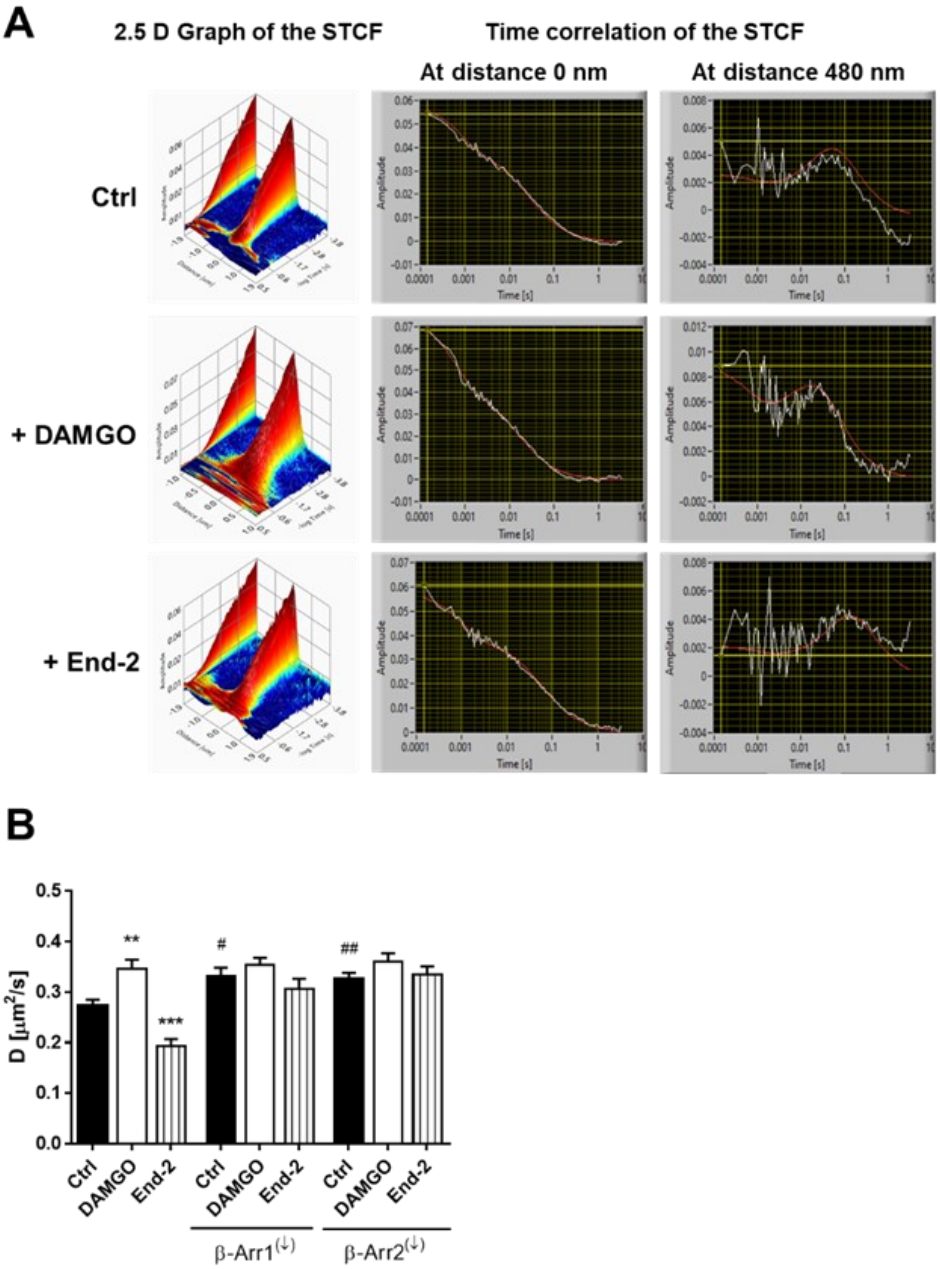

Figure 10 *Line-scan Fluorescent Correlation Spectroscopy (FCS) measurements and the effect of silencing of β-arrestin 1 and β-arrestin 2 in HMY-1 cells on the diffusion of MOR-YFP in the plasma membrane. HMY-1 cells were seeded on glass bottom wells and transfected with relevant siRNA for β-arrestin 1 (β-Arr1) or β-arrestin 2 (β-Arr2). 24 hours post transfection, FCS experiments were performed on control cells (Ctrl) and cells treated with DAMGO (1 μM) or endomorphin-2 (End-2, 1 μM) for 10 min. The lower plasma membrane adjacent to the glass was acquired and the total length of the scanned line was 6 μm. (A) Representative graphs showing the spatio-temporal time correlation function (STCF) in HMY-1 cells untreated and treated with DAMGO or End-2. (B) The summarized pooled data for the diffusion coefficient (D) of MOR under different experimental conditions. Data was collected from at least 60 cells and 3 independent experiments. Results are expressed as mean ± SEM (** $p < 0.01$, *** $p < 0.001$ versus respective Ctrl; # $p < 0.05$, ## $p < 0.01$ versus untreated Ctrl).*

6.3 Agonist-induced MOR internalization

6.3.1 Internalization of MOR observed by confocal microscopy

Activation of MOR with some opioids can cause internalization of MOR into vesicles. We performed confocal microscopy to detect the redistribution of MOR-YFP under the activation with 1 μM DAMGO (Figure 11A) and 1 μM End-2 (Figure 11B). After 5 minutes of treatment with DAMGO or End-2, we have observed slight redistribution of the fluorescent signal and after 20 minutes of treatment with both mentioned ligands, we have clearly seen the fluorescent signals in vesicles that should contain the MOR.

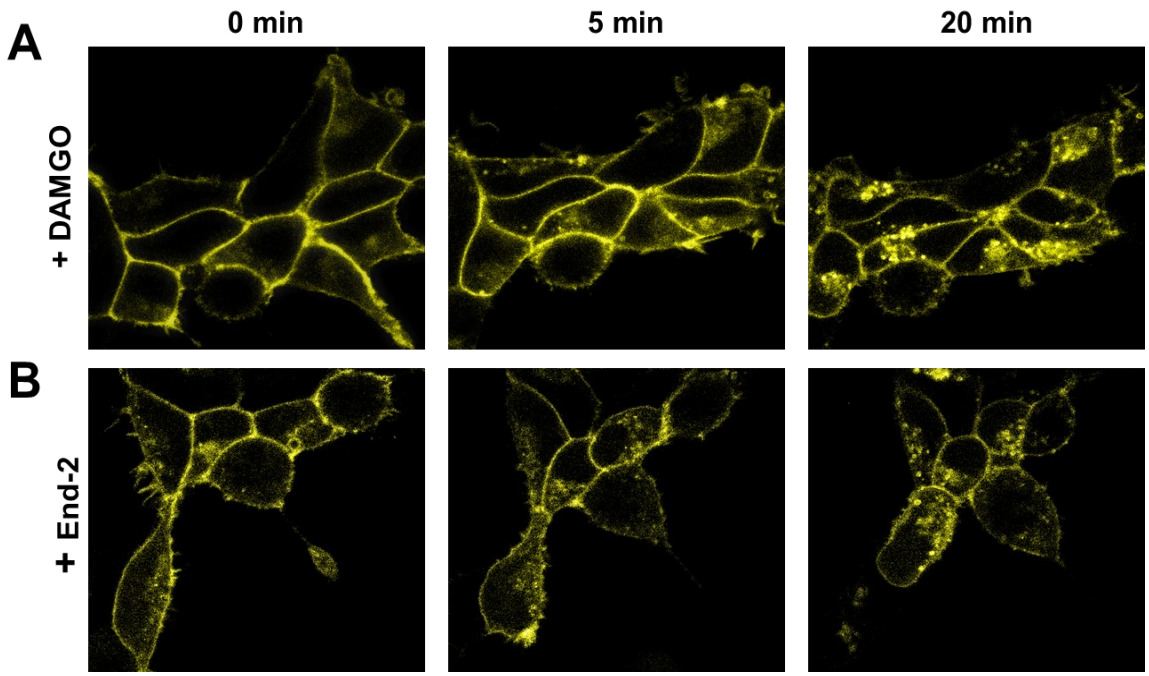

Figure 11 *Time series confocal images of the MOR-YFP internalization in HMY-1 cells. HMY-1 cells were treated with 1 μM DAMGO (A) or 1 μM End-2 (B). The images were acquired before the treatment (0 min) and after the activation of MOR-YFP with relevant ligands at 5 min and at 20 min.*

6.3.2 Effect of β-arrestin1 and 2 on MOR internalization

To quantify the amount of the internalized MOR-YFP receptors, we performed the radioligand binding assay using [^{3}H] DAMGO in HMY-1 cells and HMY-1 cells lacking β-arrestin1 or 2. Cells were stimulated with 1 μM DAMGO or with 1 μM End-2 in different time intervals (0, 5, 10, 20, and 30 min). First, both ligands differ strikingly in the induction

of the internalization of MOR. Treatment with DAMGO clearly caused internalization of MOR and after 30 minutes the number of MOR in the plasma membrane decreased by more than 50 % in HMY-1 cells. On the contrary, after 30 minutes of End-2 treatment, about 22 % of MOR disappeared from the plasma membrane (Figure 12).

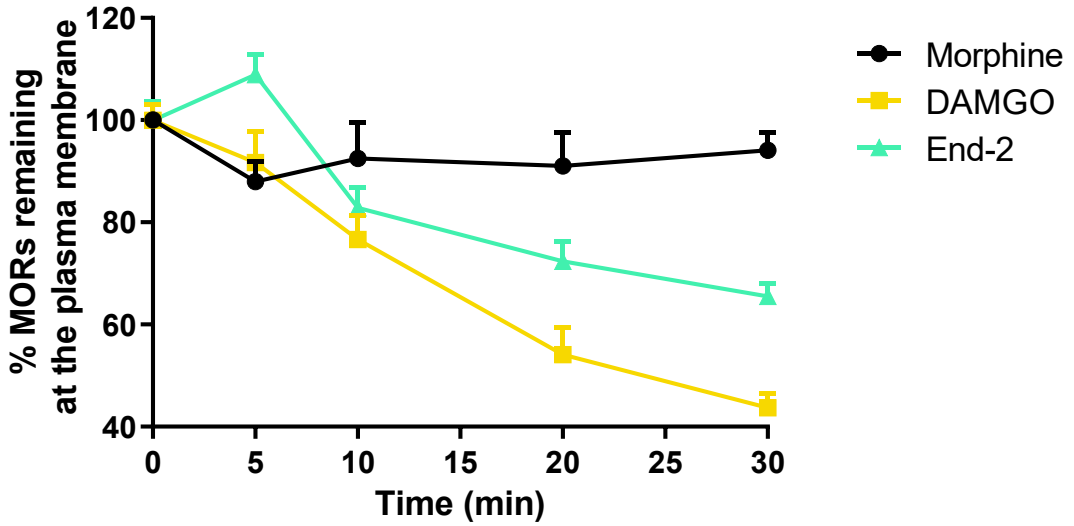

Figure 12 *Radioligand binding assay to observe the internalization of MOR-YFP after the treatment with morphine. DAMGO and endomorphin-2 (End-2). HMY-1 cells were seeded in 24 wells plate. After 24 hours HMY-1 cells were treated in different time intervals with 1 μM DAMGO or 1 μM End-2 and afterwards incubated with [^{3}H] DAMGO to observe the remaining MOR-YFP in the plasma membrane. Data are expressed as mean values ± SEM of three independent experiments.*

The lack of β-arrestin1 or 2 decreased the internalization rate of MOR and after 30 minutes of treatment with DAMGO about 77 % of MOR remained in the plasma membrane (Figure 13B). As a next ligand, we tested the ability of End-2 to trigger the MOR internalization. The lower expression of β-arrestin2 did not cause any significant change in the MOR internalization after 30 minutes of incubation with End-2 compared to control HMY-1 cells. However, the knock-down of β-arrestin1 diminished the MOR internalization in HMY-1/β-arr1$^{(\downarrow)}$ cells that were treated for 20 minutes with End-2 (Figure 13A).

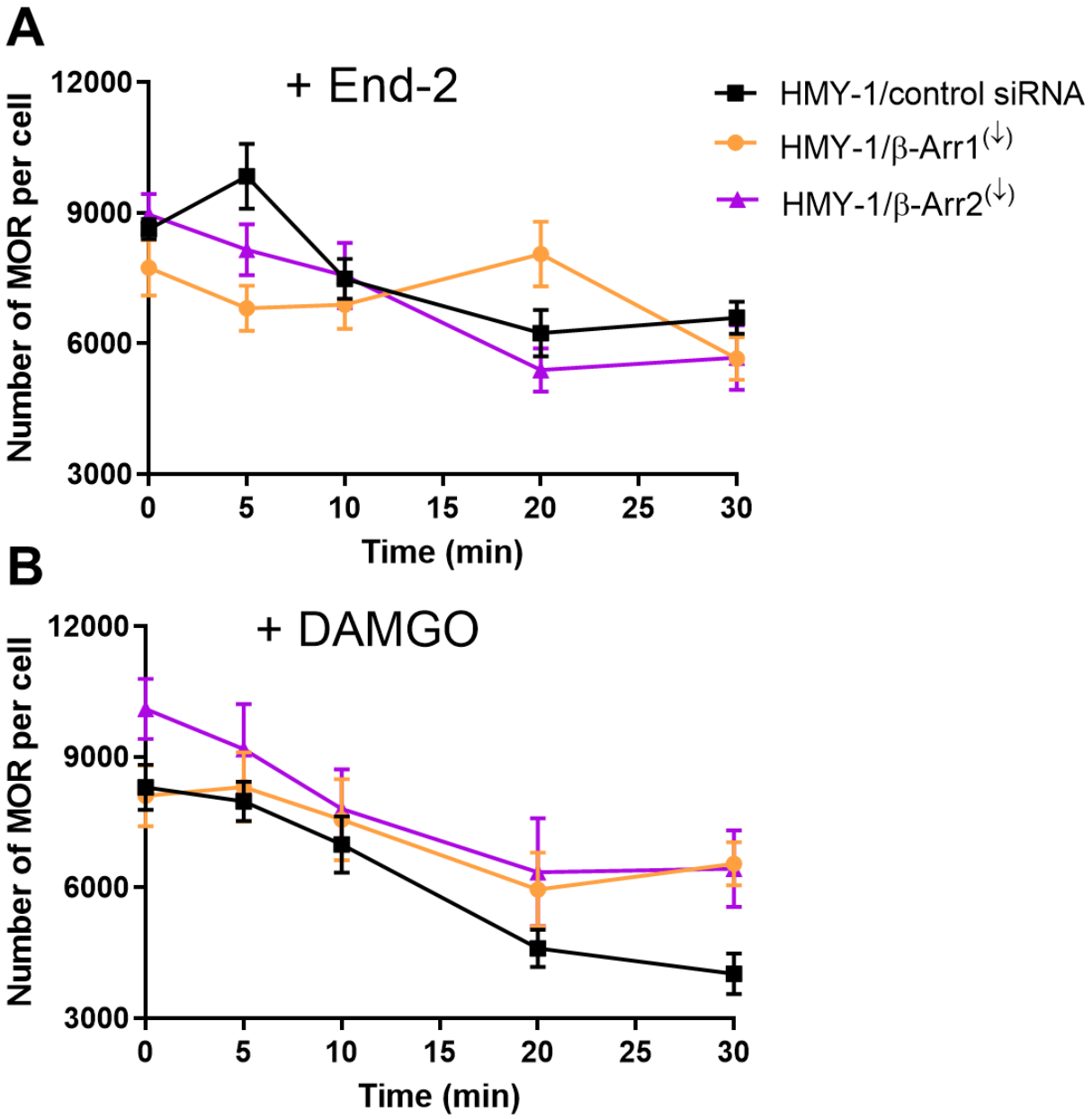

Figure 13 *Radioligand binding assay to observe the effect of β-arrestin1 or β-arrestin2 silencing on MOR-YFP internalization. HMY-1 cells were seeded in 24 wells plate and transfected with relevant siRNA (β-arr1 or β-arr2). 24 hours post transfection HMY-1 cells were treated in different time intervals with 1 μM End-2 (A) or 1 μM DAMGO (B) and afterwards incubated with [^{3}H] DAMGO to observe the remaining MOR-YFP in the plasma membrane. Data are expressed as mean values ± SEM of three independent experiments.*

6.4 Inhibition of AC by MOR agonists

MORs are coupled with the inhibitory class of G proteins. When the MOR is activated with the agonist, the $G_{\alpha i}$ subunit dissociates from the $G_{\beta\gamma}$ subunits and inhibits AC in the plasma membrane which results in lower production of the second messenger cAMP. Here, we looked at the ability of DAMGO, End-2, and morphine at concentration 1 μM to reduce the level of cAMP in HMY-1 cells, HMY-1/β-arr1$^{(\downarrow)}$, and HMY-1/β-arr2$^{(\downarrow)}$ cells. To measure inhibition of AC, first the direct activator of AC forskolin must be used. The response of AC to forskolin was calculated as a 100% response.

HMY-1 cells were treated with DAMGO and morphine, both opioids caused a significant 42% decrease in cAMP production compared to the control. We also obtained significant inhibition of AC after treatment the HMY-1 cells with End-2. However, End-2 treated HMY-1 cells showed only about a 28 % decrease in cAMP accumulation compared to the control (Figure 14A).

Knockdown of β-arrestin1 and β-arrestin2 significantly decreased the ability of forskolin to activate AC in HMY-1/β-arr1$^{(\downarrow)}$ and HMY-1/β-arr2$^{(\downarrow)}$ cells. Although the lower expression of β-arrestin1 completely abolishes the inhibition effect of individual opioids, the elimination of β-arrestin2 did not cause this effect and individual opioids significantly inhibited AC (Figure 14A).

To observe if MOR mediated $G_{\alpha i}$ activation is involved in AC activity in cells lacking both β-arrestins we pre-treated cells with pertussis toxin (PTX). PTX is responsible for the elimination of the $G_{\alpha i}$ activity. The results showed that the suppression of β-arrestin2 caused a significantly higher production of cAMP in PTX-treated cells compared to control cells and cells lacking β-arrestin1. These results could suggest that $G_{\alpha i}$ protein and its activation through MOR may play a role in changes in AC activity in HMY-1/β-arr2$^{(\downarrow)}$ (Figure 14B).

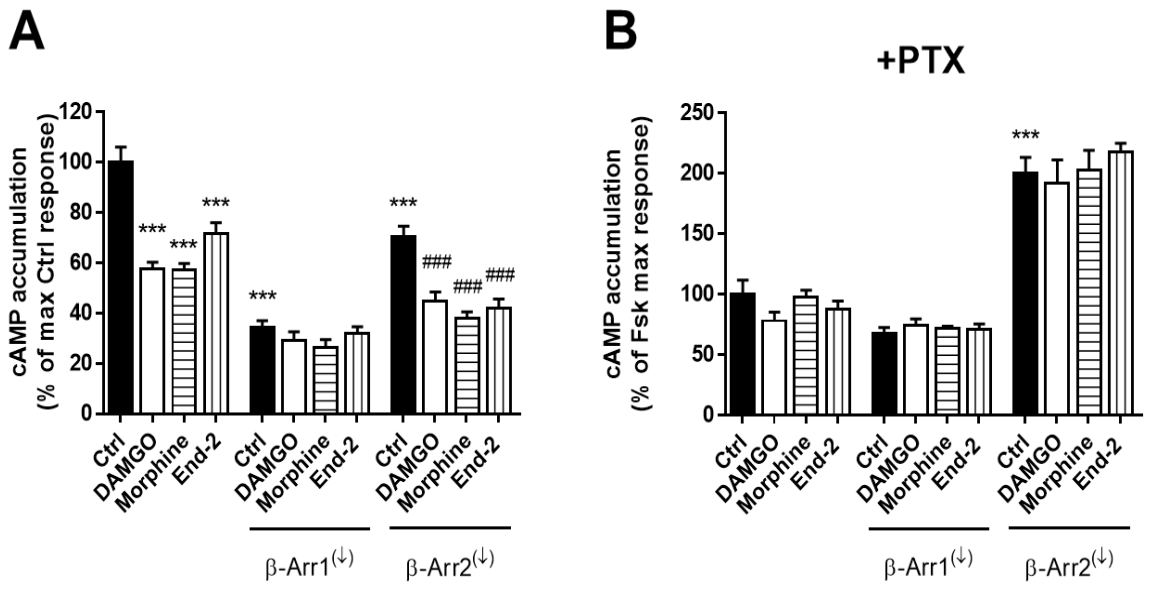

Figure 14 *The effect of silencing the β-arrestin1 or β-arrestin2 (A) and inactivation of $G_{\alpha i}$ by PTX (B) on the inhibition of AC by MOR agonists. In case of $G_{\alpha i}$ inactivation, 25 ng/ml of PTX was added to HMY-1 cells 24 hours prior the experiment. HMY-1 cells were transfected with relevant siRNA for β-arrestin1(β-Arr1$^{(\downarrow)}$) or β-arrestin2 (β-Arr2$^{(\downarrow)}$). HMY-1 cells were incubated in stimulation buffer supplemented with 0.5 mM IBMX in the presence of DAMGO or morphine or endomorphin-2 (End-2) for 20 minutes, at final concentration 1 μM and 37°C. Afterwards, forskolin was added at 2 μM concentration and incubated for 45 minutes at 37°C. Cells were lysed and accumulated cAMP was measured using the HTRF Gi cAMP kit form Cisbio. Results are represented as mean of ± SEM from 3 independent experiments. (*** $p < 0.001$ versus untreated Ctrl; ### $p < 0.001$ versus respective Ctrl)*

6.5 Stimulation of AC by forskolin or isoprenaline

As mentioned above AC can be activated by the direct activator forskolin. The second possibility of activating AC is through the GPCRs which are coupled with the stimulatory subunit of G_{α}. Here we decided for the β-adrenergic receptor (β-AR) endogenously expressed in HEK293 cells and its agonist isoprenaline to activate AC in the plasma membrane.

6.5.1 Effect of β-arrestin1 or β-arrestin2 knockdown

We investigated whether the lower expression level of β-arrestin1 or β-arrestin2 may affect the activity of AC in a dose-response dependent manner. In the case of the highest forskolin concentration, we observed an increase in cAMP accumulation of approximately 39 % in HEK293 lacking β-arrestin2 (Figure 7A). On the contrary, the

reduction of β-arrestin1 in HEK293 cells caused a decrease in forskolin-stimulated cAMP production of approximately 55 % (Figure 15A). Interestingly, cAMP accumulation was not affected by lower expression of β-arrestin2 after activation of β-AR with increasing concentration of isoprenaline (Figure 7B). However, HEK293 lacking β-arrestin1 and treated with isoprenaline showed a decrease in cAMP production by about 55 % compared to control cells (Figure 15B). The intrinsic efficacy (EC50) and potency (logEC50) of rising forskolin and isoprenaline concentration with statistics are shown in Table 1.

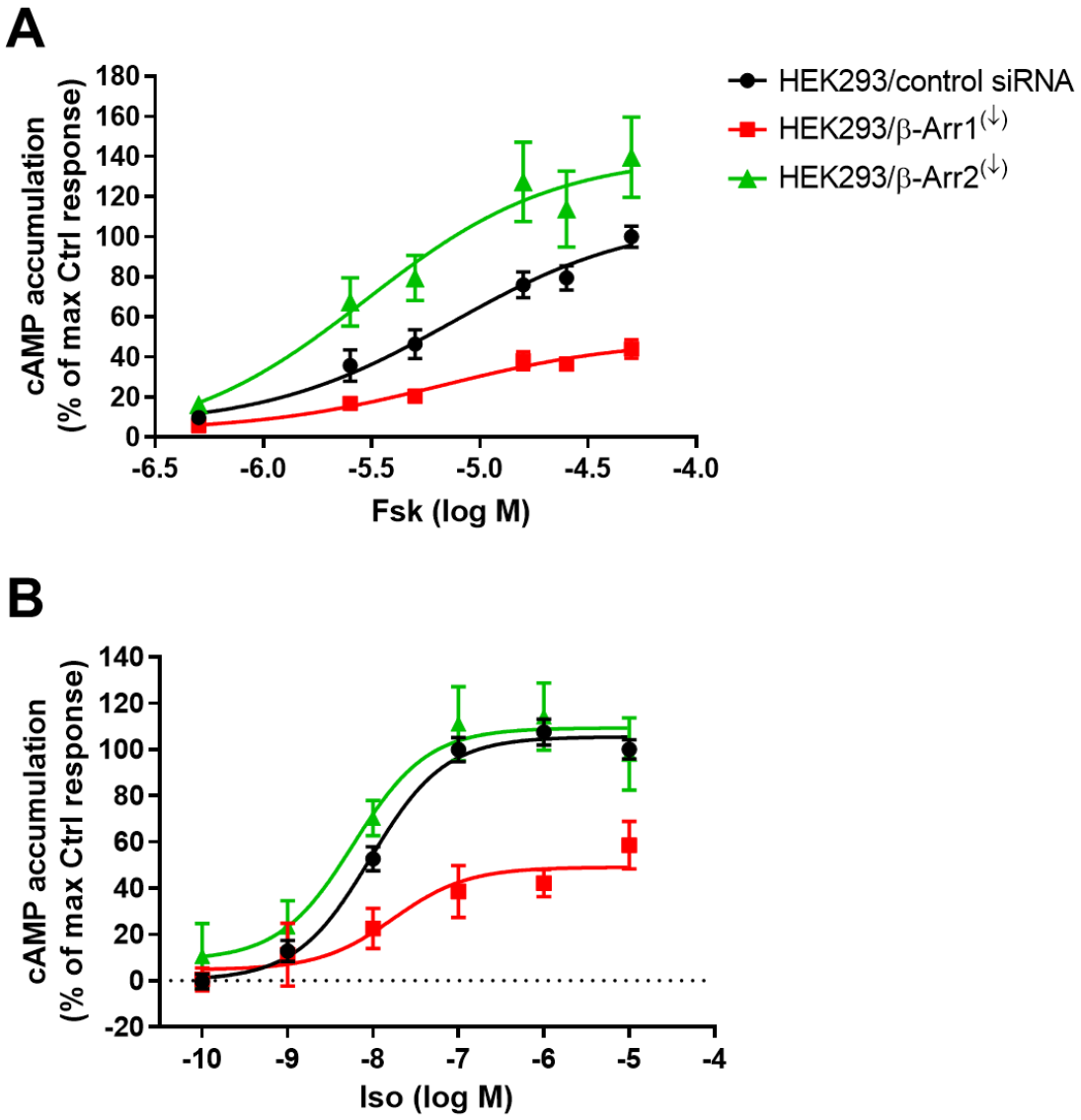

Figure 15 *Dose response curves of cAMP accumulation in HEK293 cells and the effect of silencing β-arrestin1 or β-arrestin2. HEK293 with silenced β-arrestin1 (HEK293/β-Arr1$^{(\downarrow)}$) or β-arrestin2 (HEK293/β-Arr2$^{(\downarrow)}$) were incubated with increasing concentration of forskolin (Fsk) (A) or isoprenaline (Iso) (B) for 30 minutes at 37°C. The cAMP accumulation was measured using the HTRF method (Gs kit, Cisbio). Data represents the ± SEM of three independent experiments.*

6.5.2 Effect of β-arrestin1, β-arrestin2 and $G_{\alpha s}$ knockdown

To test whether $G_{\alpha s}$ is involved in suppressed cAMP production in cells lacking β-arrestins we tested cAMP accumulation in cells transfected with $G_{\alpha s}$ siRNA to decrease the expression level of $G_{\alpha s}$ alone or in combination with one or another β-arrestin siRNAs. First, we tested the increasing concentration of forskolin and isoprenaline. First, as we expected, the low expression of $G_{\alpha s}$ in the HEK293 cell caused a decrease in cAMP accumulation after treatment with the highest concentration of isoprenaline (Figure 16).

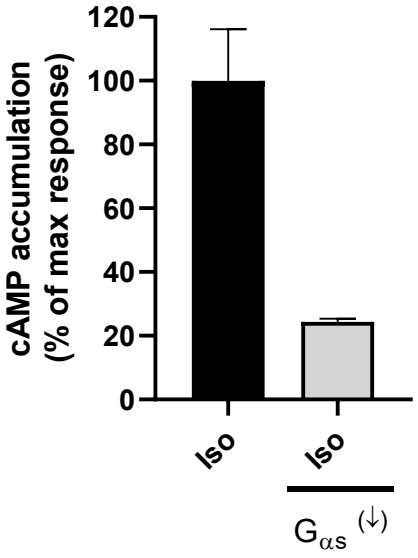

Figure 16 *Effect of silencing $G_{\alpha s}$ ($G_{\alpha s}{}^{(\downarrow)}$) on isoprenaline-induced cAMP accumulation. HEK293 cells were transfected with the $G_{\alpha s}$ siRNA and 24 hours post transfection cells were incubated with 10 μM concentrated isoprenaline for 30 minutes at 37°C. The cAMP accumulation was measured using the HTRF method (Gs kit, Cisbio). Data represents the ± SEM of three independent experiments.*

However, stimulation of HEK/$G_{\alpha s}{}^{(\downarrow)}$ with a raising concentration of Forskolin caused a decrease in cAMP accumulation by about 58 %. Interestingly, cells lacking $G_{\alpha s}$ and β-arrestin1 or β-arrestin2 showed an even greater decrease in cAMP production of approximately 79 % after treatment with 50 μM Forskolin (Figure 17). The statistics of the potency and intrinsic efficacy of forskolin under different conditions are shown in Table 1.

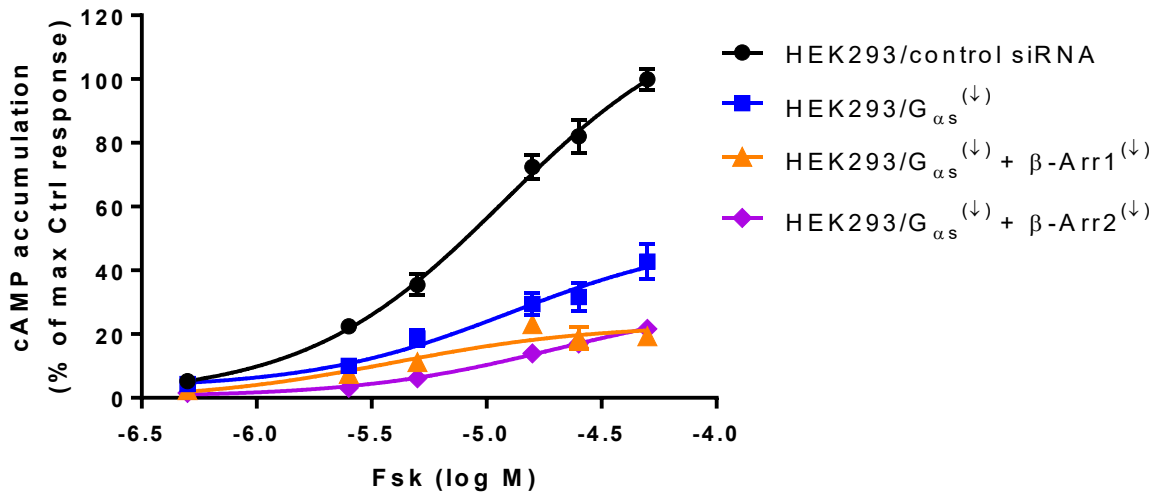

Figure 17 *Effect of silencing of $G_{\alpha s}$ or simultaneously $G_{\alpha s}$ (HEK293/$G_{\alpha s}^{(\downarrow)}$) and β-arrestin1 (HEK293/ $G_{\alpha s}$ $^{(\downarrow)}$+β-Arr1$^{(\downarrow)}$) or β-arrestin2 (HEK293/ $G_{\alpha s}^{(\downarrow)}$+β-Arr2$^{(\downarrow)}$) on cAMP accumulation induced by rising concentration of forskolin (Fsk) in HEK293 cells. HEK293 were incubated with different concentration of Fsk for 30 minutes at 37°C. cAMP accumulation was measured using the HTRF method (Gs kit, Cisbio). Data represents the ± SEM from three independent experiments.*

Table 1
Potency ($logEC_{50}$) and intrinsic efficacy (E_{max}) of forskolin and isoprenaline.

	Forskolin						Isoprenaline		
	Control	β-Arr1$^{(\downarrow)}$	β-Arr2$^{(\downarrow)}$	$G_{\alpha s}^{(\downarrow)}$	$G_{\alpha s}$ + β-Arr1$^{(\downarrow)}$	$G_{\alpha s}$ + β-Arr2$^{(\downarrow)}$	Control	β-Arr1$^{(\downarrow)}$	β-Arr2$^{(\downarrow)}$
$LogEC_{50}$	-4.9 ± 0.1	-5.2 ± 0.2	-5.4 ± 0.2	-5.3 ± 0.2	-5.4 ± 0.2	-4.7 ± 0.1	-7.9 ± 0.1	-7.7 ± 0.4	-8.2 ± 0.1
E_{max} (%)	120.8 ± 5.1	49.2 ± 7.6***	147.8 ± 15.3	42.1 ± 10.4***	19 ± 2***	31.2 ± 5.8***	103.5 ± 1.9	49.4 ± 8.4***	109.8 ± 6.1

*** $p < 0.001$ *versus* respective control.

6.6 Interaction of activated AC with β-arrestins

β-Arrestins clearly affect AC activity based on the previous results. We came up with a hypothesis of the possible interaction between the AC and β-arrestin. To test the hypothesis, we performed the co-immunoprecipitation assay. HEK293 were transfected with a plasmid encoding the β-arrestin1-HA or β-arrestin2-HA. The transfected cells were used for Co-IP either in the resting state or after treatment with isoprenaline. Both β-arrestins associate with AC in the resting state to a certain level. However, after activation of β-AR with isoprenaline, we did observe an increase in the association of β-arrestins-HA

with AC, especially a significant increase of the association in the case of β-arrestin1-HA (Figure 18).

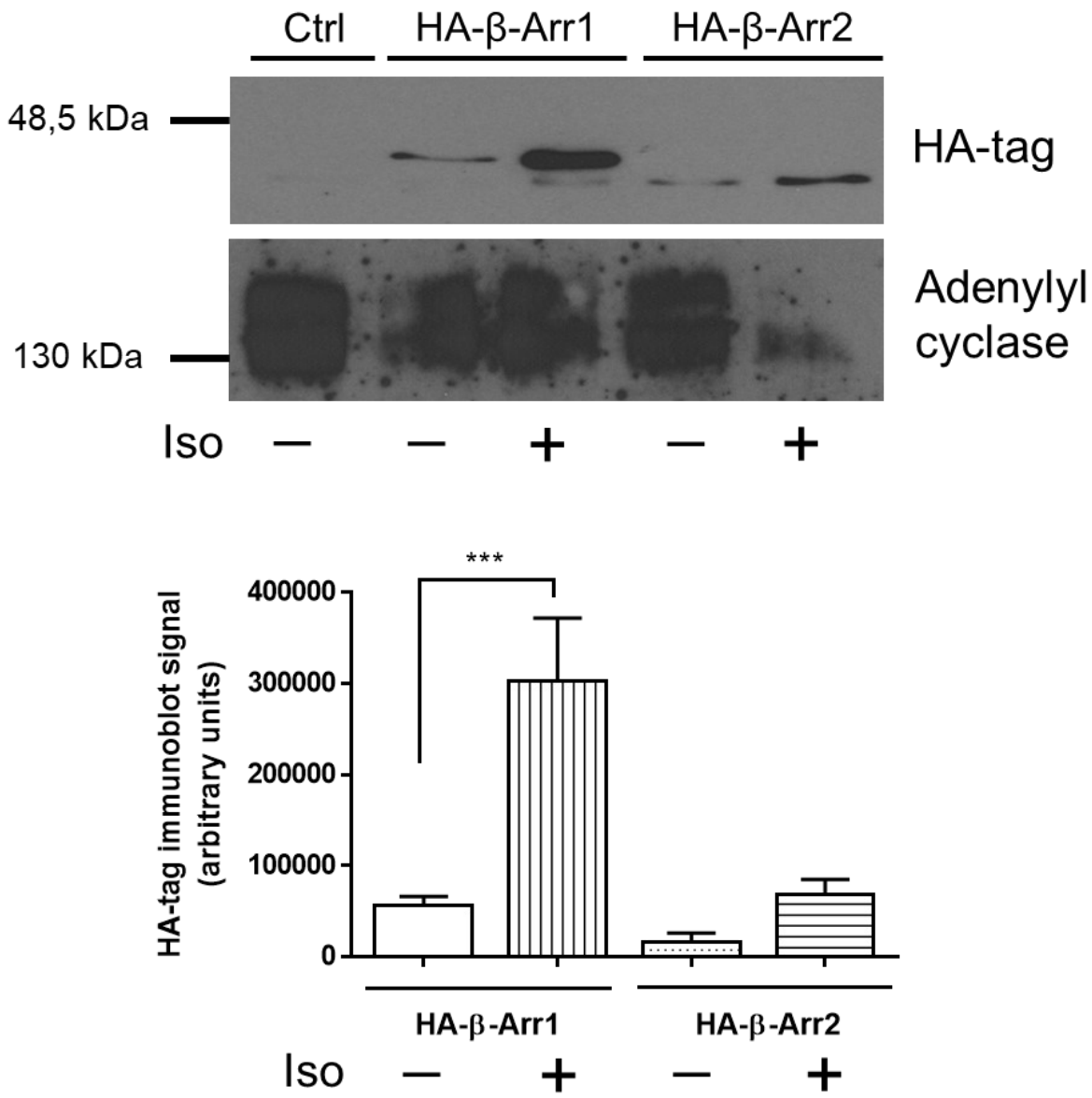

Figure 18 *Co-immunoprecipitation of β-arrestins with AC. HEK293 were transiently transfected with HA tagged β-arrestin1 (HA-β-Arr1) or HA tagged β-arrestin2 (HA-β-Arr2) one day prior the experiment. Next day, cells were treated with isoprenaline (Iso) for 10 minutes at 37°C. Whole cell lysates were used for the co-immunoprecipitation assay and lysates were immunoprecipitated using the anti-AC antibody. Afterwards, immunoblot was obtained using the anti-HA and anti-AC antibody. (A) representative immunoblot of HA tagged β-arrestins and AC. (B) Relative optical density of HA tagged β-arrestins immunoblot. Data represents ± SEM of three independent experiments.*

6.7 Expression of TRPV1 in the HMY-1 cell line

6.8 Functional studies of MOR in cells expressing TRPV1

Here, we tested the functionality of MOR in HMY-1 cells expressing the TRPV1 ion channel. First, we tested cAMP production in HMY-1 cells after treating the cells with End-2 and Capsaicin. We observed a significant decrease in cAMP production in HMY-1 cells treated with End-2 compared to control cells. As expected, capsaicin did not cause any significant decrease in cAMP accumulation in HMY-1 cells compared to the control. Equivalent conditions were tested in HMY-1/TRPV1 cells expressing the TRPV1 channel. Treatment of HMY-1/TRPV1 cells with End-2 caused significant inhibition of AC in the same manner as in HMY-1 cells. Capsaicin did not inhibit AC activity in HMY-1/TRPV1 cells (Figure 19).

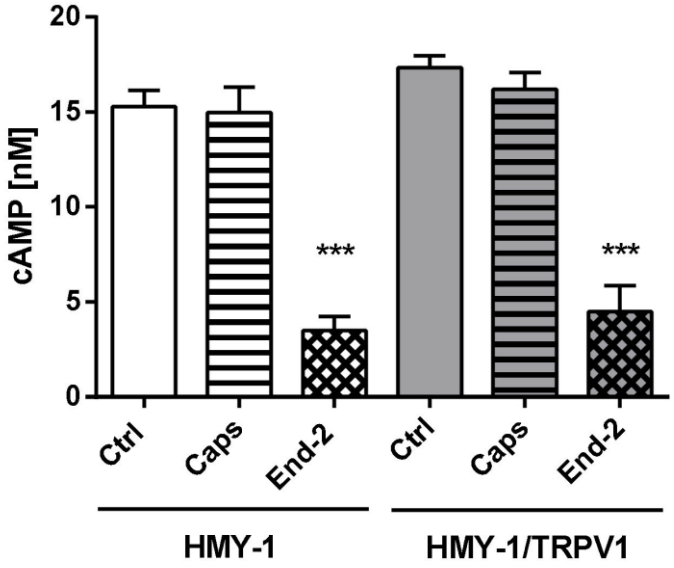

Figure 19 *Functional study of MOR in HMY-1 and HMY-1/TRPV1 cells. HMY-1 cells were transiently transfected with TRPV1-CFP plasmid. 24 hours post transfection cells were incubated with 1 µM of capsaicin (Caps) or 1 µM of endomorphin-2 (End-2) and later with forskolin to observe the inhibition of AC. Accumulation of cAMP was measured using the HTRF method. Data are expressed as means ± SEM of three independent experiments. (*** $p \leq 0.001$ compared to control.)*

6.9 Lateral mobility of selected receptors in HMY-1/TRPV1 cells lacking β-arrestin2

Here, we measured the diffusion coefficient of MOR-YFP or TRPV1-CFP in HMY-1/TRPV1 cell line under different experimental conditions. HMY-1 cells were seeded in a glass bottom chamber and transiently transfected with TRPV1-CFP. First, we observed a decrease in MOR-YFP lateral mobility in HMY-1/TRPV1 cells after End-2 treatment. Interestingly, TRPV1 agonist capsaicin caused an almost twofold increase in MOR-YFP diffusion rate. On the other hand, the diffusion coefficient of inactivated TRPV1-CFP in HMY-1/TRPV1 cells was higher than the diffusion coefficient of inactivated MOR-YFP. Activation of TRPV1-CFP with capsaicin resulted in a significant increase in the lateral mobility of TRPV1 in the plasma membrane. Nevertheless, End-2 had a similar effect on the diffusion of TRPV1-CFP in HMY-1/TRPV1 cells, however, to a less extent (Melkes et al. 2020).

6.9.1 Lateral mobility of MOR in the plasma membrane

We wondered if β-arrestin2 may play a role in the cooperation between MOR and TRPV1 at the plasma membrane level. We used the siRNA technique to decrease the expression level of β-arrestin2 in HMY-1/TRPV1 cells. First, we looked at the diffusion rate of MOR-YFP in HMY-1/TRPV1 cells. Knockdown of β-arrestin2 slightly decreases the diffusion coefficient of inactivated MOR-YFP by about 16 %. Capsaicin treatment caused a significant increase of MOR-YFP lateral mobility in cells lacking β-arrestin2 and simultaneously expressing TRPV1 Figure 20A).

The mobile fraction of inactivated MOR-YFP increased significantly in HMY-1/TRPV1 cells lacking β-arrestin2 compared to control cells. Activation by End-2

or capsaicin caused a significant decrease in the MOR-YFP mobile fraction compared to control cells lacking β-arrestin2 (Figure 20B).

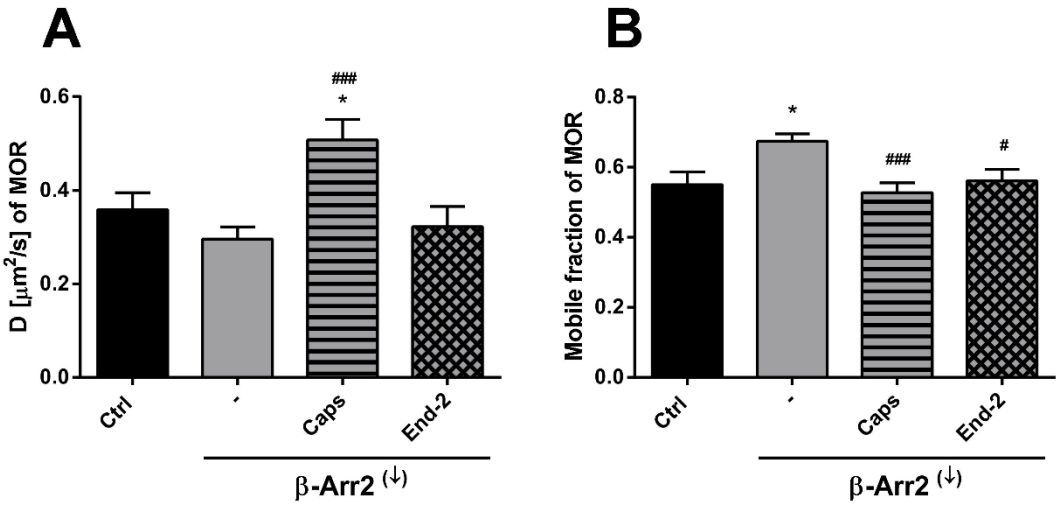

Figure 20 *Lateral mobility of MOR-YFP in HMY-1/TRPV1 cells lacking the β-arrestin2 (β-Arr2(↓)). HMY-1 cells were transfected with TRPV1-CFP and β-arrestin2 siRNA. 24 hours post transfection the apparent diffusion coefficient of MOR-YFP (A) and its mobile fraction (B) was measured using FRAP. The lateral mobility was measured in control cells and in cells treated with 0.5 μM capsaicin (Caps) or 1 μM endomorphin-2 (End-2) for 5 minutes before the measurements. FRAP data were collected from a bottom part of the cell membrane from three independent experiments and at least 50 cells. Results are expressed as means ± SEM. (* p≤0.05 compared to control; # p ≤ 0.05, ### p ≤ 0.001 compared to control β-Arr2(↓))*

6.9.2 Lateral mobility of TRPV1 in the plasma membrane

Under the same experimental conditions as for MOR-YFP lateral mobility, we tested the diffusion of TRPV1-CFP in cells HMY-1/TRPV1 lacking β-arrestin2. Interestingly, the removal of β-arrestin2 caused a significant decrease (by approximately 47%) in the diffusion of inactivated TRPV1-CFP on the plasma membrane, and the TRPV1-CFP diffusion rate of TRPV1-CFP was slightly increased (by approximately 25%) by adding capsaicin or End-2 (Figure 21A).

The mobile fraction of inactivated TRPV1-CFP increased significantly by about 25 % in cells lacking β-arrestin2 compared to control cells. By adding capsaicin or End-2 the fraction of mobile TRPV1-CFP decreased back to the control level (Figure 21B).

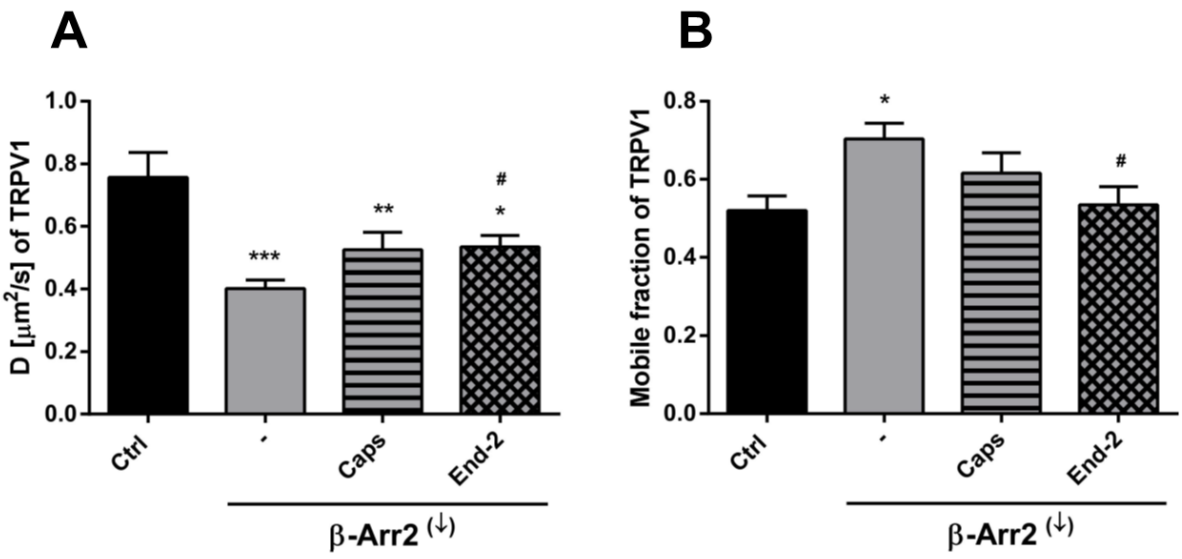

Figure 21 *Lateral mobility of TRPV1-CFP in HMY-1/TRPV1 cells lacking the β-arrestin2 (β-Arr2$^{(\downarrow)}$). HMY-1 cells were transfected with TRPV1-CFP and β-arrestin2 siRNA. 24 hours post transfection the apparent diffusion coefficient of TRPV1-CFP (A) and its mobile fraction (B) was measured using FRAP. The lateral mobility was measured in control cells and in cells treated with 0.5 μM capsaicin (Caps) or 1 μM endomorphin-2 (End-2) for 5 minutes before the measurements. FRAP data were collected from a bottom part of the cell membrane from three independent experiments and at least 50 cells. Results are expressed as means ± SEM. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to control; $^{\#}$ $p \leq 0.05$ compared to control β-Arr2$^{(\downarrow)}$)*

6.10 Level of β-arrestin2 on the plasma membrane after activation of MOR or TRPV1

From our FRAP results, we may know that β-arrestin2 might play an important role in the cooperation between MOR and the TRPV1 channel on the plasma membrane level. We decided to test the loss of β-arrestin2 from the plasma membrane in HMY-1 and HMY-1/TRPV1 cells after activation with capsaicin or End-2. We collected the plasma membrane fraction of control cells and cells activated with capsaicin or End-2 for 5 minutes. The relative level of β-arrestin2 in the plasma membrane was determined using Western blot analysis. We observed a significant loss of β-arrestin2 from the plasma membrane by about 50 % after the addition of capsaicin or End-2 to HMY-1/TRPV1 cells. However, this phenomenon was not observed in HMY-1 cells (Figure 22).

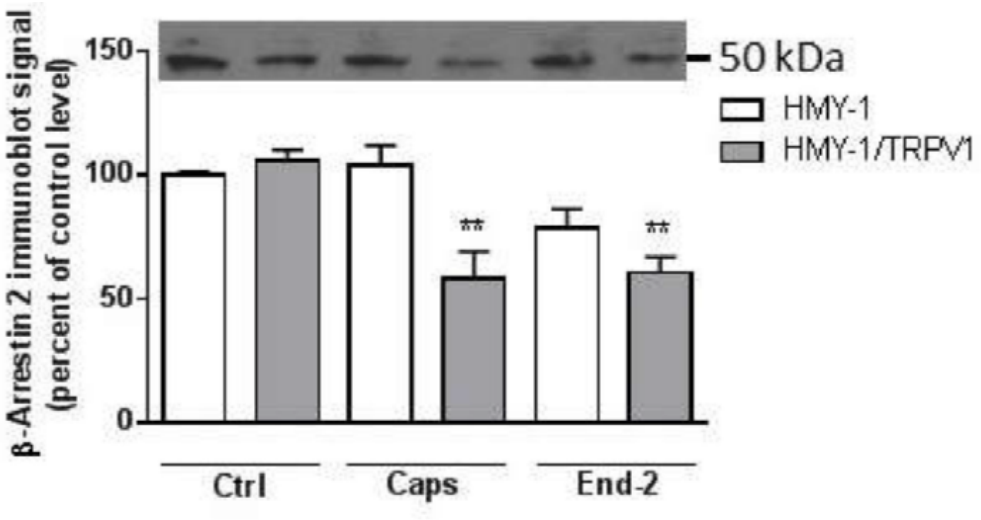

Figure 22 *Distribution of β-arrestin 2 in the plasma membrane fraction and the effect of capsaicin (Caps) and endomorphin-2 (End-2). Fraction of the plasma membranes was isolated from HMY-1 or HMY-1/TRPV1 cells without treatment (Ctrl) or after treatment with either 1μM capsaicin (Caps) or endormoprhin-2 (End-2) for 5 minutes. Subsequent immunoblot analysis of β-arrestin2 distribution was performed. Representative western blot is shown, and data are expressed from four independent experiments and represents the percentage of control. Results are expressed as ± SEM (** $p \leq 0.01$ compared to control).*

6.11 MAPKs involved in crosstalk between MOR and TRPV1

β-Arrestin is involved in the activation of MAPK signaling cascades. Here, we performed the western blot analysis of the phosphorylated forms of individual MAPK, specifically p-38, JNK, and ERK1/2 to reveal their potential involvement in HEK-293/TRPV1, HMY-1/TRPV1, and HMY-1 cells after addition of capsaicin or End-2.

We did not observe any significant changes in the phosphorylation forms of JNK or p38 in all the different combinations (Figure 23A, B).

We detected a significant change in the phosphorylated form of ERK1/2 (Figure 23C) in different combinations. First, capsaicin caused a significant increase in ERK1/2 phosphorylation in cells expressing TRPV1 (HEK293/TRPV1 and HMY-1/TRPV1). End-2 was able to cause the phosphorylation of ERK1/2 in cells HMY-1 however ERK1/2 phosphorylation was largely increased in cells HMY-1/TRPV1 after End-2 treatment. Interestingly, the difference in ERK1/2 phosphorylation in HMY-1/TRPV1 and HMY-1 cells after End-2 addition was decreased by the knockdown of β-arrestin2 (Figure 23D).

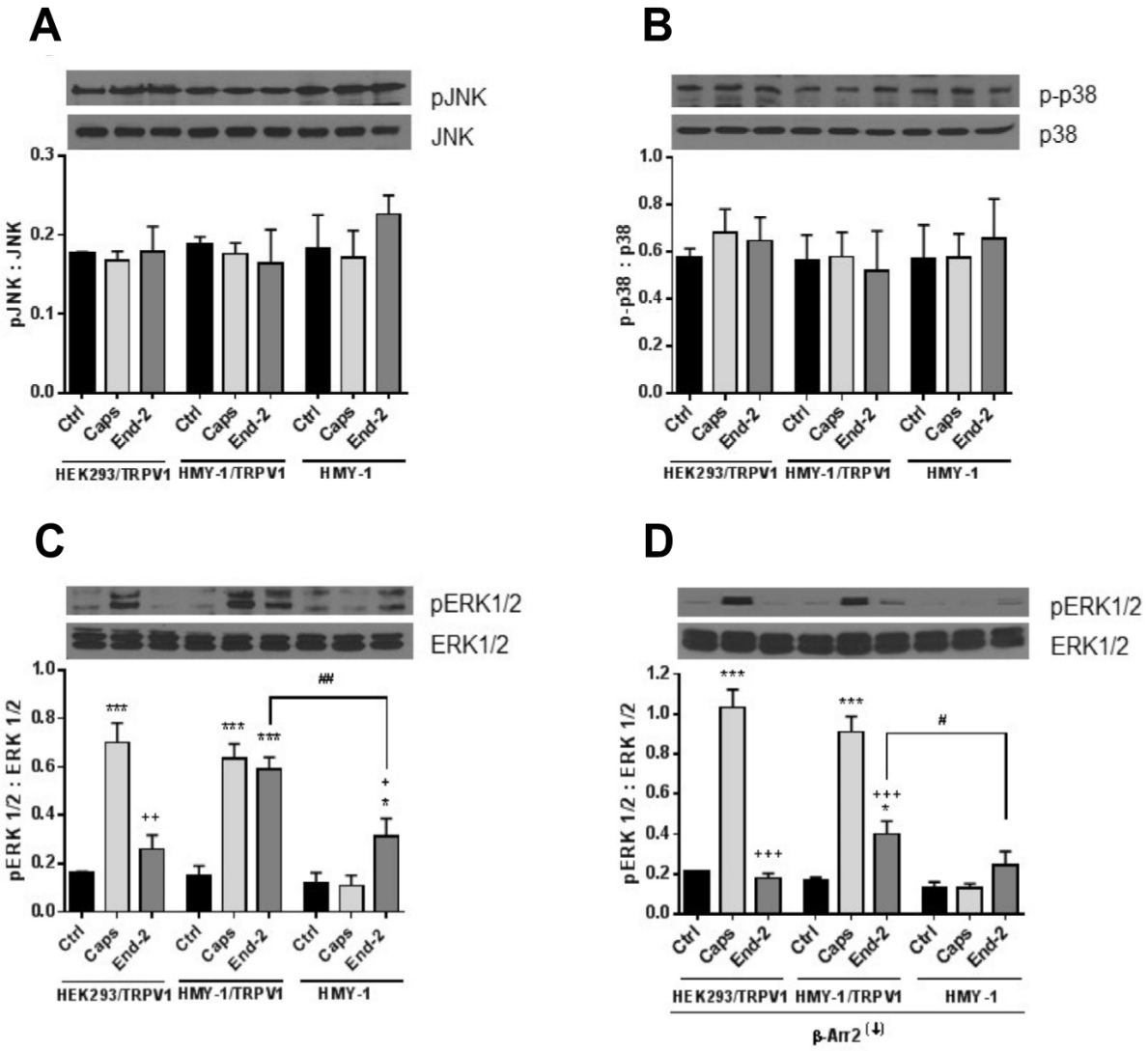

Figure 23 *Phosphorylation of MAP kinases in HEK293/TRPV1, HMY-1/TRPV1 and HMY-1 cells and the effect of capsaicin (Caps) and endomophin-2 (End-2). Cells were incubated for 5 minutes at 37°C in the absence (Ctrl) or presence of 1μM End-2 or Caps. β-Arrestin2 was silenced (β-Arr2$^{(\downarrow)}$) in some experiments 24 hours before the treatment with agonists. Samples were loaded on the poly-acrylamide gel and SDS-Page electrophoresis was performed. Specific antibodies against JNK and p-JNK (A), p38 and p-p38 (B) and ERK1/2 and p-ERK1/2 (C, D) were subsequently used after the protein transfer on the nitrocellulose membranes. Representative immunoblots are shown and data are expressed from four independent experiments. Results are expressed as means ± S.E.M. of the ratios of phosphorylated to unphosphorylated MAPK forms (* $p < 0.05$, *** $p < 0.001$ compared to corresponding control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared to HMY-1/TRPV1; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ compared to cells treated with Caps).*

6.12 Lateral mobility of TLR4 in cells expressing TRPV1

Here, we tested the possible crosstalk between TRPV1-CFP and TLR4-YFP at the plasma membrane level. Using FRAP, we observed the representative recovery curves of TLR4-YFP (Figure 24A). From the normalized recovery curves, we obtained the apparent diffusion coefficient and the mobile fraction of TLR4-YFP in the absence of any agonist or after activation of TRPV1-CFP with 1μM capsaicin (Figure 24B, C). The diffusion coefficient of TLR4-YFP increased significantly after the activation of TRPV1 ($D = 0.93 \pm 0.2$ $\mu m^2/s$) with capsaicin. Contrary, the mobile fraction of TLR4-YFP robustly dropped down ($Mf = 0.32 \pm 0.09$) in the presence of TRPV1 agonist capsaicin compared to the control.

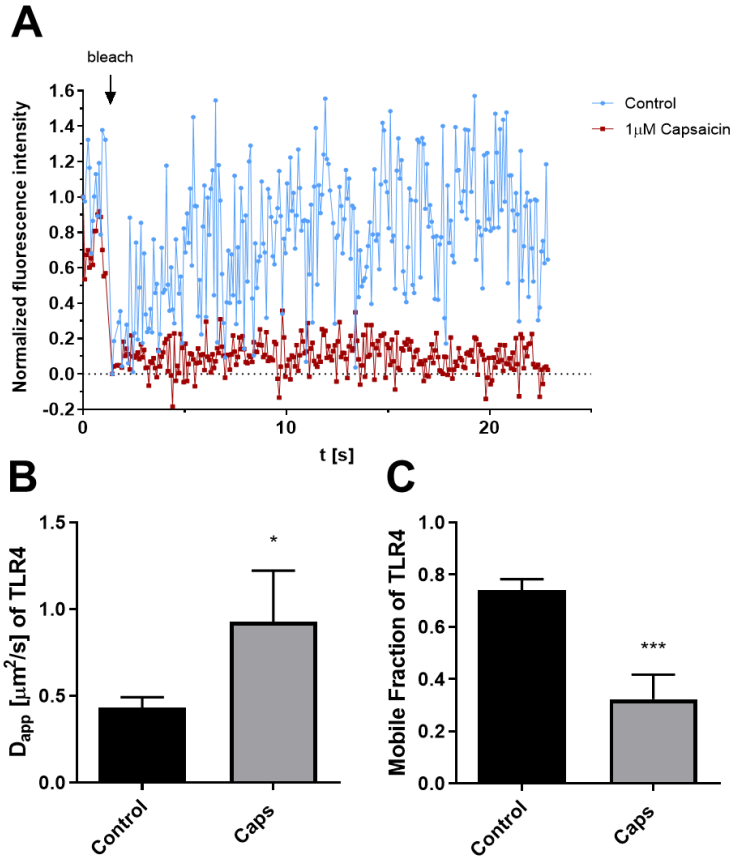

Figure 24 *Effect of TRP1-CFP activation on the lateral mobility of TLR4-YFP in the plasma membrane. HEK293 cells were co-transfected with TRPV1-CFP and TLR4-YFP and 24 hours post transfection the FRAP measurement was performed on the bottom part of the cell membrane. The recovery curves of TLR4-YFP were obtained (A) and the diffusion coefficient (B) and mobile fraction (C) of TLR4-YFP was measured in the absence (Ctrl) of any agonist and in the presence of 1μM capsaicin (Caps). Data are expressed as a mean ± SEM from three independent experiments. (* $p \leq 0.05$, *** $p \leq 0.001$ compared to control).*

7 Discussion

The purpose of this thesis was to investigate the role of β-arrestin in multiple signaling pathways, particularly those connected with the MOR and TRPV1 channel. In general, β-arrestin is a multifunctional protein involved in several cellular events such as the desensitization of receptors, scaffold function, and its signaling role. There are only two isoforms of β-arrestin (β-arr1 and β-arr2) compared to more than 800 types of GPCR (Lagerström and Schiöth 2008).

Here, we use the classical HEK293 cell line or the HMY-1 cell line derived from HEK293. The HMY-1 cell line stably expresses the MOR tagged with YFP. The mRNA level of different proteins related to GPCR signaling in the HEK293 cell line was observed in a study by Atwood 2011 (Atwood et al. 2011). Both isoforms of β-arrestins and $G_{\alpha s}$ are endogenously expressed in the HEK293 cell line. However, the expression of β-arrestin2 is two times higher than β-arrestin1 at the mRNA level. To observe the role of selected proteins in the signaling of MOR and TRPV1, we successfully used the siRNA method to down-regulate the expression of β-arrestin1, β-arrestin2, and $G_{\alpha s}$ in the HMY1 or HEK293 cell line.

Previously, the HMY-1 cell line was shown to be suitable for investigating MOR-YFP and its properties in the plasma membrane (Melkes et al. 2016). Using a FRAP method, we observed the lateral mobility of MOR-YFP in HMY-1 treated with different ligands such as DAMGO, morphine, and endormophin-2 (End-2), where DAMGO significantly increased, and End-2 significantly decreased the apparent diffusion coefficient of MOR-YFP in HMY-1 cells. These results correspond to the study of Melkes et al. 2016, where biased agonists of MOR regulate its lateral mobility in a diverse way (Melkes et al. 2016).

In the next step, we investigated the effect of β-arrestin isoforms on the lateral mobility of MOR-YFP in the plasma membrane. Silencing of both β-arrestin isoforms separately resulted in a significant increase of MOR-YFP lateral mobility in the plasma membrane. In addition, the effect of DAMGO, End-2, and morphine on MOR-YFP diffusion in control HMY-1 cells was abolished in cells lacking β-arrestin1 or β-arrestin2. To support our results with increased diffusion of MOR-YFP in the plasma membrane in cells lacking both β-arrestins, the study on the neuropeptide Y type 1 receptor (NPY1) with the mutation disrupting the β-arrestin recruitment revealed a significant increase in its

lateral mobility in the plasma membrane (Kilpatrick et al. 2012). In our study, we used two biased ligands as DAMGO and End-2, where DAMGO should be a G protein bias agonist of MOR, End-2, on the other hand, should lead the signal from MOR towards the β-arrestin2 and morphine as an unbiased ligand of MOR (Rivero et al. 2012). Despite this, we did not observe any significant differences between the two ligands and their effect on the lateral mobility in the plasma membrane in HMY-1 cells lacking both β-arrestins isoforms. Additionally, no significant differences were observed between the two isoforms of β-arrestins on the lateral mobility of MOR-YFP in the plasma membrane. Nevertheless, the mobile fraction of activated MOR-YFP by DAMGO or End-2 was significantly decreased in cells lacking β-arrestin1 or β-arrestin2. Both β-arrestins isoforms somehow affect the mobility of MOR-YFP in the HMY-1 cell line. Furthermore, several studies reported that the diffusion of GPCRs in the plasma membrane might be affected by their agonists or cognate signaling proteins, such as arrestins, G proteins, or trafficking proteins (Cézanne et al. 2004; Lalo et al. 2010; Saulière-Nzeh et al. 2010; Moravcova et al. 2018).

Using line-scan fluorescent correlation spectroscopy (line-scan FCS) as another approach to revealing the lateral mobility of MOR-YFP in the plasma membrane, we supported our data obtained from FRAP experiments. Although the values from the line-scan FCS might be slightly different from the values observed using FRAP, the trend of changes in the MOR-YFP diffusion coefficient was similar even under different conditions, such as different ligands or siRNAs. It is known that both methods are comparable. Moreover, the line-scan FCS method can provide even more precise values and information on membrane compartmentalization (Ries et al. 2009; Macháň et al. 2016). From our results of the MOR-YFP mobility is clear that both β-arrestins strongly affect MOR-YFP lateral mobility in the plasma membrane of the HMY-1 cell line and silencing of one or the other isoform of β-arrestin abolishes the effect of a distinct agonist on MOR-YFP diffusion coefficient of MOR-YFP.

It is known that β-arrestin plays a major role in the desensitization of GPCRs that results in receptor internalization. In the next step of our investigation of the differences between the two isoforms of β-arrestin, we looked at MOR internalization. β-Arrestins are necessary scaffold proteins to induce internalization. Internalization of MOR induced by increasing concentration of DAMGO, fentanyl, and loperamide was completely abolished in CRISPR/Cas9 edited HEK293 cells lacking both β-arrestin isoforms (Møller et al. 2020). β-Arrestins serve as a scaffold protein for the endocytic machinery that involves adaptor

protein 2 (AP-2) and clathrin (Laporte et al. 1999). Interestingly, it was observed that β-arrestin1 binds with a higher affinity to AP-2 compared to β-arrestin2. On the other hand, β-arrestin2 shows a higher affinity for clathrin (Laporte et al. 1999). Moreover, preferences between one or the other β-arrestin isoform are not only in the endocytic machinery. Apparently, receptors from class A GPCR (MOR, β2AR, dopamine D1A receptor D1A, etc.) prefer recruitment and binding with β-arrestin2. In contrast, no significant differences were observed in the recruitment of β-arrestin1 or β-arrestin2 for GPCR class B of GPCRs (V2R, angiotensin II type 1A receptor, neurotensin receptor 1, etc.). (Oakley et al. 2000). In our set of experiments focused on investigating the time interval of MOR internalization in control HMY-1 cells or HMY-1 cells lacking β-arrestin1 or β-arrestin2, we used two MOR agonists, DAMGO and End-2. First, the internalization of MOR induced by DAMGO was more efficient, and a higher number of MORs were internalized compared to the internalization of MOR induced by End-2. It is known, DAMGO is a synthetic ligand with a very high affinity for MOR. Additionally, DAMGO should stabilize the structure of the MOR which leads towards the G protein signaling pathway and End-2 has the opposite bias towards the β-arrestin2 (Rivero et al. 2012; Manabe et al. 2019). However, in most of the studies, DAMGO was used as a reference ligand to count bias for the other ligand of MOR and we cannot say with absolute certainty that DAMGO is biased towards the G protein (Conibear and Kelly 2019). Furthermore, a recent study revealed that End-2 is biased toward the cAMP signal (LaVigne et al. 2020). Together, the differences between both the agonists and their capability to induce the MOR internalization could be connected to their distinct bias. Individual silencing of β-arrestin isoforms resulted in less efficient internalization of MOR induced by DAMGO. Although one or another isoform of β-arrestin was silenced, there was still several internalized MOR after DAMGO binding explaining to some extent the equality of β-arrestin1 and β-arrestin2 which is in agreement with the study of Kouhout et al. 2001 (Kohout et al. 2001). In the case of End-2 induced internalization of MOR, there were no significant differences between the number of internalized MOR in HMY-1 cells lacking β-arrestin2. However, silencing of β-arrestin1 disabled the internalization of MOR induced by End-2 at 20 minutes. This result might suggest the possible engagement between the MOR internalization induced by End-2 and β-arrestin1 which is in line with the results of the study by Thompson et al. where they pointed out that End-2 recruits more efficiently β-arrestin1 than β-arrestin2 (Thompson et al. 2015). Taken together, biased ligands of MOR may cause a different structural state of

the MOR which may influence the following interaction of MOR with its cognate signaling proteins.

To further investigate the MOR signaling and the role of β-arrestin isoforms we focused on cAMP accumulation. cAMP is an important second messenger in cells that makes the signal even stronger and transmits it to subsequent partners. MOR is coupled with the inhibition subtype of G_{α} protein. To observe the inhibition of cAMP production by adenylyl cyclase (AC), it was necessary first to use a direct activator of AC called forskolin. All three used ligands of MOR (DAMGO, End-2, and morphine) significantly inhibited the forskolin-stimulated activity of AC. Surprisingly, the silencing of β-arrestin1 itself decreased forskolin-stimulated AC activity and also completely abolished the inhibitory effect of the individual MOR agonists on AC activity. Similar results were observed in HMY-1 cells lacking β-arrestin2 where forskolin-induced AC activity was slightly inhibited by silencing of β-arrestin2 itself. However, the inhibitory effect of all three agonists of MOR was preserved and did not change significantly compared to control HMY-1 cells. To observe the role of $G_{\alpha i}$ proteins in AC activity, we used pertussis toxin (PTX) which is known to inhibit the $G_{\alpha i}$ proteins (Burns, 1988). As we expected, the effect of all three MOR agonists on AC activity was abolished in HMY-1 cells treated with PTX and similarly in HMY-1 cells lacking β-arrestin1. However, the silencing of β-arrestin2 in HMY-1 cells treated with PTX resulted in a large increase in cAMP. These results might suggest the possible involvement of β-arrestin2 in AC activity and moreover, β-arrestin2 might be considered as a potential negative regulator of AC. Unfortunately, there are no supportive or non-confirming studies on the influence of both β-arrestin isoforms on the MOR signaling pathway relative to cAMP accumulation.

To discover in detail the roles of β-arrestins in the regulation of AC, we focused on AC stimulation of AC by two different pathways. First through the direct activator forskolin and second through classical G protein signaling using isoprenaline as an agonist of endogenously expressed β-adrenergic receptors (β-AR). It is necessary to mention that this set of experiments was performed on a classical HEK293 cell line. Using a rising concentration of forskolin or isoprenaline, we obtained a dose-response curve of cAMP accumulation in HEK293 cells lacking β-arrestin1 or β-arrestin2. In both forskolin- and isoprenaline-induced AC activity we observed a decrease in cAMP accumulation in HEK293 lacking β-arrestin1. These results correspond to the previous experiment. Furthermore, in case of an increase in forskolin, we observed an even higher accumulation

of cAMP accumulation in HEK293 cells lacking β-arrestin2. It is clear that β-arrestin1 and β-arrestin2 are important for AC activity in both cases as AC activation AC through the $G_{\alpha s}$ or directly through forskolin. However, both isoforms seem to have a distinct relationship with the AC and its activity. These results are supported by the study by Ahn et al. where they used HEK293 cell line and siRNA to downregulate the expression of one or the other isoform of β-arrestin. Using the cAMP accumulation assay, they observed a significant increase in cAMP accumulation after the treatment in cells lacking β-arrestin2. The elimination of β-arrestin1 caused a slight decrease in cAMP accumulation but the result was not significant. It is important to mention that the cAMP accumulation assay was performed using a 10 μM isoprenaline for only 2 minutes (Ahn et al. 2003). On the contrary, a study on mouse embryonic fibroblast (MEF) derived from knockout mice lacking β-arrestin1 or β-arrestin2 observed an increase in cAMP accumulation induced by isoprenaline in both cases of silencing β-arrestin isoforms. In this experiment, the accumulation of cAMP was measured using a 10 μM isoprenaline, and the cAMP accumulation was monitored for 60 minutes. Furthermore, the β-adrenergic receptor was overexpressed in MEF cells (Kohout et al. 2001). The differences between our results and results from other studies might be due to the use of different concentrations, incubation times, and methods.

Some previous studies showed the importance of $G_{\alpha s}$ subunit in forskolin-induced AC activity (Green and Clark 1982; Chen-Goodspeed et al. 2005). These results are in line with our observation of forskolin-induced cAMP accumulation in cells lacking the $G_{\alpha s}$ subunit. Furthermore, subsequent silencing of β-arrestin1 or β-arrestin2 even deepened the effect of decreased forskolin-induced AC activity. It is important to mention that study from 2013 observed a direct interaction of $G_{\alpha s}$ subunit and β-arrestin1. Furthermore, it was shown that β-arrestin1 regulates the $G_{\alpha s}$ activity and possibly promotes the binding and release of GTP on the $G_{\alpha s}$ subunit (Li et al. 2013). Clearly, β-arrestin1 affects the AC activity and our results from the co-immunoprecipitation suggest a potential direct interaction between the AC and β-arrestin1. Additionally, the interaction is enhanced by the activation of AC with isoprenaline, and to some extent, we observed possible interaction even between the activated AC and β-arrestin2. Since we know there is a direct interaction of β-arrestin1 with the $G_{\alpha s}$ subunit we may consider the creation of AC, $G_{\alpha s,}$ and β-arrestin1 complex. However, this idea would need further investigation.

The next part of the thesis was investigating the role of β-arrestin in the behavior and function of MOR and TRPV1. It is known that both these receptors are naturally co-expressed in primary sensory neurons and the brain and may closely cooperate (Endres-Becker et al. 2007; Maione et al. 2009). For the purpose of this study, we used the HMY-1 cell line stably expressing the MOR-YFP and transiently co-expressed the TRPV1 tagged with the cyan fluorescent protein (CFP). First, we tested whether the expression of TRPV1 in HMY-1 cells affects MOR function using the cAMP assay. As expected, End-2 treatment of HMY-1 cells or HMY-1/TRPV1 cells caused a significant decrease in cAMP production. Capsaicin did not affect MOR function in HMY-1 cells or HMY-1/TRPV1 cells. However, capsaicin had an effect on the lateral mobility of MOR in HMY-1/TRPV1 cells, and contrary End-2 affected the lateral mobility in HMY-1/TRPV1 cells (data not shown). Taken together, both End-2 and capsaicin could have changed the mobility of their cognate and even non-cognate receptors, but capsaicin did not affect the function of MOR in HMY-1/TRPV1 cells. Clearly, there is communication between both receptors, but the mechanism must be resolved.

β-Arrestin plays a crucial role in MOR and TRPV1 desensitization. In the case of MOR, β-arrestin is recruited to activated and phosphorylated MOR in the plasma membrane and disables further activation of G proteins. Desensitization of TRPV1 through β-arrestin has a different mechanism where β-arrestin2 decreases the activity of PKA, which results in lower phosphorylation of TRPV1 and its desensitization. It was shown, β-arrestin is an important part of MOR and TRPV1 crosstalk. A study from 2017 observed the potential involvement of GRK5 in the MOR and TRPV1 crosstalk. Specifically, TRPV1 activation drives the GRK5 into the nucleus which prevents the possible phosphorylation of MOR and its desensitization (Scherer et al. 2017). A more recent and supportive study revealed the translocation of β-arrestin2 into the nucleus after activation of TRPV1 which caused no internalization and desensitization of MOR (Basso et al. 2019). Clearly, β-arrestin has an emerging role in communication between the signaling pathways of both receptors. To observe the detailed function of β-arrestin2 in receptor crosstalk, we used the siRNA to silence the expression of β-arrestin2 in HMY1/TRPV1 cells and designed several experiments that could help us to better understand its role in the system.

First, we tested the lateral mobility of MOR-YFP and TRPV1-CFP and their mobile fraction in HMY-1/TRPV1 cells lacking β-arrestin2. Knockdown of β-arrestin2 itself significantly decreased the lateral mobility of TRPV1 and, in the case of MOR, there was

also a decreasing trend. Interestingly, the elimination of β-arrestin2 abolished any effect of End-2 and capsaicin on the diffusion of TRPV1 into the plasma membrane in HMY-1/TRPV1 cells. The lateral mobility of MOR in the plasma membrane of HMY-1/TRPV1 cells increased significantly after the treatment with capsaicin in cells lacking β-arrestin2. From the study of Melkes et al., we know that End-2 robustly decreased the lateral mobility of MOR in HMY-1 cells (Melkes et al. 2016). However, in the case of HMY-1/TRPV1 cells lacking β-arrestin2, End-2 did not cause any changes in MOR diffusion in the plasma membrane. We may conclude that TRPV1 plays a key role in the lateral mobility of MOR in cells lacking β-arrestin2. Furthermore, it was shown that the β-arrestin1 biased agonist of the angiotensin II receptor type 1 activates the TRPC3 channel through the GPCR-arrestin complex. The coupling between GPCRs and TRP channels might be arrestin-dependent (Liu et al. 2017).

The association of β-arrestin2 with the plasma membrane upon the activation of MOR or TRPV1 by their agonists for 5 minutes was studied using the plasma membrane fraction. It is known that MOR undergoes internalization upon its activation with End-2 or some other ligands, and the MOR-β-arrestin complex remains stable for several minutes (Horner and Zadina 2004). It corresponds to our observation that activation of MOR with End-2 did not cause the decrease of β-arrestin2 associated with the plasma membrane in HMY-1 cells. However, our results showed a decrease in the level of associated β-arrestin2 with the plasma membrane after activation with End-2 or capsaicin in HMY-1/TRPV1 cells. TRPV1 may possibly play a role in the β-arrestin2 association with the plasma membrane upon the activation of MOR or TRPV1. Interestingly, it was shown that interaction between the TRPV4 and β-arrestin appears after 2 minutes of angiotensin receptor (AT1aR) activation with angiotensin and disappears after 5 minutes of angiotensin treatment. This study showed a membrane complex consisting of AT1aR, TRPV4, and β-arrestins upon stimulation of AT1aR (Shukla et al. 2010).

The MAPK family of proteins, including ERK, p38, and JNK, is involved in a wide variety of cell physiological processes, including pain hypersensitivity (Obata a Noguchi 2004). It was previously described that activation of MOR by agonists such as morphine, DAMGO, or fentanyl induces the phosphorylation of ERK1/2 (Belcheva et al. 1998; Narita et al. 2002). Furthermore, morphine-induced phosphorylation of ERK1/2 was found to be dependent on β-arrestin2 (Macey et al. 2006). The phosphorylation of ERK1/2 is not only the job of MOR. TRPV1 was shown to signal through MAPK as ERK1/2 by the activation

of the protein kinase C (Mandal et al. 2018). All these data support our results, in which we observed enhanced phosphorylation of ERK1/2 after activation of TRPV1 with capsaicin or of MOR with endomorphin-2 in cells where the receptors were expressed alone. However, cells that expressed both receptors together showed almost twofold higher phosphorylation of ERK1/2 induced by endomorphin-2 treatment. This increase was reduced in cells lacking β-arrestin2 showing that MOR-induced phosphorylation of ERK1/2 depends on β-arrestin2 in cells expressing TRPV1. This observation provides us with a better and more detailed view of the cooperation between the MOR and TRPV1. Furthermore, the phosphorylation of ERK1/2 induced by TRPV1 activation was not affected by β-arrestin2 knockdown suggesting that the mechanism of ERK1/2 phosphorylation by TRPV1 differs from MOR-induced ERK1/2 phosphorylation.

The last part of our study focused on possible cooperation between TRPV1 and TLR4, which are important receptors that are involved in pain transduction (Caterina et al. 2000; Tanga et al. 2005). Since both receptors were found together in primary sensory neurons and trigeminal neurons it raised a question on possible crosstalk between TRPV1 and TLR4 (Wadachi and Hargreaves 2006). TLR4 activation with LPS is known to sensitize the TRPV1 channel and increases TRPV1 expression (Diogenes et al. 2011; Filippova et al. 2018). We focused on the possible cooperation between TLR4 and TRPV1 at the plasma membrane level. Using FRAP, we observed that activation of TRPV1 significantly increased the lateral mobility of TLR4. However, TLR4 became almost immobile after activation of TRPV1 with capsaicin. We may say that there is potential cooperation between both receptors at the plasma membrane level and this result is supported by a study where it was observed a direct interaction between TLR4 and TRPV1. This interaction is mediated through the TIR domain of TLR4 (Min et al. 2018).

In summary, we have presented a detailed view on the differences between the β-arrestin isoforms in MOR signaling and AC function. Furthermore, for the first time, we showed the potential direct interaction between the β-arrestin1 and AC upon AC activation. We revealed the importance of β-arrestin2 in the crosstalk between MOR and TRPV1.

8 Summary

In this work, our objective was to study the role of β-arrestin and the differences between its two isoforms in MOR signaling and AC function. Moreover, we focused on the role of β-arrestin2 in the crosstalk between MOR and TRPV1 which are important receptors involved in pain transduction.

We found that the lateral mobility of nonactivated MOR might be affected by its cognate signaling proteins like β-arrestins due to possible close cooperation between β-arrestins and the receptor. Above that, the lateral mobility of MOR is affected by both isoforms of β-arrestins even when the receptor is activated with its agonists. However, we did not identify any significant differences between β-arrestin1 and β-arrestin2 and their effect on the diffusion of MOR in the plasma membrane.

Receptor internalization is an important process in the regulation of MOR signaling. We showed that different agonists may induce a distinct level of internalization where DAMGO was the most efficient ligand to induce the MOR internalization followed by endomorphin-2. Morphine, as is known, did not cause any internalization of MOR. Moreover, we showed that there are differences in MOR internalization after silencing β-arrestin1 or β-arrestin2. In particular, silencing of β-arrestin1 disabled internalization induced by endomorphin-2 up to 20 minutes of treatment. On the other hand, internalization of MOR was observed in cells lacking β-arrestin2 but in a less severe manner.

We showed the importance of β-arrestin isoforms in the modulation of the activity of AC. Inhibition of AC through the MOR signaling pathway was highly affected by the silencing of β-arrestin1 as MOR agonists of MOR were not able to inhibit forskolin-activated AC. This phenomenon was eliminated using pertussis toxin (PTX) to block the activity of $G_{\alpha i}$ proteins. However, PTX had a completely opposite effect on AC inhibition in cells lacking β-arrestin2, and AC activity was significantly increased even after treatment with MOR agonists. These observations led us to explore the AC activity induced either by forskolin or through the $G_{\alpha s}$ protein. We found that the AC activity induced by forskolin or isoprenaline was decreased in cells lacking β-arrestin1 in a dose-response manner. On the contrary, silencing of β-arrestin2 resulted in the opposite effect, and the activity of AC was enhanced. Moreover, we demonstrated the possible importance of the $G_{\alpha s}$ subunit in this phenomenon.

Furthermore, we pointed out a very interesting fact between the AC and β-arrestins. We observed a potential interaction between the β-arrestin1 and AC in the resting state that increased significantly after the activation of AC through the $G_{\alpha s}$-mediated signaling pathway.

In the next part of this study, we focused on describing the possible involvement of β-arrestin2 in the cooperation between MOR and TRPV1. We showed that β-arrestin2 can affect the lateral mobility of non-activated TRPV1 in the presence of MOR and above that activation of TRPV1 with capsaicin affected the lateral mobility of MOR in the plasma membrane in cells lacking β-arrestin2.

The content of β-arrestin2 in the plasma membrane may serve as important information for the crosstalk between MOR and TRPV1. We found that the level of plasma membrane β-arrestin2 was decreased after activation of MOR or TRPV1 in the presence of both receptors. In addition, we showed that the β-arrestin2 is essential for the phosphorylation of ERK1/2 induced by endomorphin-2 in cells expressing MOR and TRPV1.

In the last part, we investigated the potential cooperation between TRPV1 and TLR4 in the plasma membrane and found possible crosstalk between these two receptors. However, this crosstalk needs further investigation.

Together, we have provided detailed information on the importance of the two β-arrestin isoforms for lateral mobility in the plasma membrane and their function together with AC activity. In addition, we found that β-arrestin2 plays a key role in the crosstalk between the MOR and TRPV1.

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