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PHARMACOLOGICAL CHARACTERIZATION OF THE ORPHAN RECEPTOR GPR150

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ABSTRACT

GPR150 is an orphan G protein coupled receptor (GPCR), its physiological ligand is therefore unknown. It belongs to the class A rhodopsin-like family of GPCRs, in particular to the vasopressin-like subfamily. It is mainly expressed in the brain and in the reproductive system, but these may not be the only sites where this receptor is found. Its direct involvement in any pathology is yet unknown, although the silencing of its gene is associated with the progression of ovarian cancer.

The aim of this research is to pharmacologically describe GPR150 to find out which pathways characterize its signaling, in order to gain a more thorough understanding of which physiological and potentially pathological processes it may be involved in. Previous phylogenetic analysis linked this orphan receptor to the family of vasopressin and oxytocin receptors, based on homology of their most evolutionarily conserved sequences, which are crucial to their functioning.

Based on this evidence, bioluminescence resonance energy transfer assays were performed to investigate the activation of the receptor upon stimulation with vasopressin, oxytocin and peptides from different plant libraries. G proteins - Gs, Gi and Gq - and β-arrestin2 were analyzed as potential effectors of the signaling of GPR150, however neither G protein dissociation nor β-arrestin recruitment were detected upon ligand stimulation. All assays were carried out after molecular cloning of GPR150 sequence in the pEGFP-N1 plasmid, followed by transfection and transient expression of the cloned plasmid in HEK cells. Previous research showed that GPR150 might be constitutively active in the Gi pathway, this hypothesis was therefore explored in this project by performing luciferase reporter gene assays. Results suggested compatibility with previous findings, showing in fact higher luminescence signal in cells treated both with forskolin (which stimulates adenylate cyclase activity, regardless of Gi activation) and pertussis toxin (which inhibits Gi protein), compared to cells only treated with forskolin. These findings reinforce the hypothesis that GPR150 might be intrinsically active, however further research is needed to define the molecular mechanisms that regulate its activity and that determine its physio-pathological role in living organisms.

RIASSUNTO

GPR150 è un recettore orfano accoppiato a proteine G (GPCR), il suo ligando fisiologico è perciò ancora sconosciuto. Appartiene alle classe A di GPCR simili alla rodopsina, in particolare alla sottofamiglia di recettori simili al recettore della vasopressina. È principalmente espresso nelle cellule del cervello e del sistema riproduttivo, tuttavia questi potrebbero non essere gli unici siti di espressione di questo recettore. Il suo coinvolgimento diretto in meccanismi patologici è ancora sconosciuto, sebbene la silenziazione del suo gene sia associata alla progressione del cancro ovarico.

L'obiettivo di questa ricerca è la caratterizzazione farmacologica del recettore GPR150 per scoprire quali vie di segnalazione sono coinvolte nella sua attivazione, al fine di comprendere più a fondo i processi fisiologici e potenzialmente patologici in cui potrebbe essere coinvolto. Studi filogenetici hanno collegato questo recettore orfano alla famiglia dei recettori della vasopressina e dell'ossitocina, sulla base dell'omologia di sequenza nei siti più conservati, cruciali per il loro funzionamento. In virtù di queste evidenze sono stati eseguiti saggi basati sul trasferimento di energia per risonanza di bioluminescenza, in modo da testare l'attivazione del recettore in seguito a stimolazione con vasopressina, ossitocina e librerie di peptidi derivati da estratti vegetali. Le proteine G - Gs, Gi, Gq - e la β-arrestina2 sono state analizzate come potenziali effettrici della segnalazione del recettore GPR150, tuttavia non è stata rilevata alcuna attività significativa. Tutti i test sono stati effettuati grazie al clonaggio della sequenza GPR150 nel plasmide pEGFP-N1, seguita dalla sua trasfezione ed espressione transiente in cellule HEK. Ricerche precedenti hanno evidenziato come GPR150 potrebbe essere costitutivamente attivo nella via di segnalazione accopiata alla proteina Gi, tale ipotesi è stata quindi approfondita tramite saggi con il gene reporter della luciferasi. I risultati sono in accordo con le precedenti evidenze, infatti il segnale di luminescenza prodotto dalle cellule trattate con forskolina (che attiva l'adenilato ciclasi indipendentemente dall'attività delle proteine G) e con la tossina della pertosse (che inibisce la proteina Gi) era più intenso rispetto al segnale prodotto dalle cellule trattate solo con forskolina. Queste osservazioni rinforzano l'ipotesi che GPR150 sia intrinsecamente attivo, tuttavia è necessaria ulteriore ricerca per definire i meccanismi molecolari che ne regolano l'attività e per stabilire il suo ruolo fisiopatologico negli organismi viventi.

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LIST OF ABBREVIATIONS

GPR150	Orphan G protein coupled receptor GPR150
GPCR	G protein coupled receptor
pEGFP-N1	Plasmid EGFP-N1
cAMP	Cyclic adenosine monophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
AMP	Adenosine monophospate
ATP	Adenosine triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
CRE	cAMP response elements
CREB	cAMP response elements binding protein
PLCβ	Phospholipase C β
PIP_2	Phosphatidyl inositol biphosphate
IP_3	Inositol triphospate
DAG	Diacylglycerol
GRK	G protein coupled receptor kinase
PDE	Phosphodiestarase
DGK	Diacylclycerol kinase
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinases
JNK	c-Jun-NH_2-terminal kinase
CNS	Central nervous system
PNS	Peripheral nervous system
PAM	Positive allosteric modulator
NAM	Negative allosteric modulator
GLP-1	Glucagon-like peptide 1
GIPR	Gastric inhibitory polypeptide receptor
V1Ar, V1bR, V2r	Vasopressin receptors
AVP	Vasopressin
OXTR	Oxytocin receptor
OXT	Oxytocin
NPSR	Neropeptide S receptor
GnRHR	Gonadotropin-releasing hormone receptor
EST	Expressed sequence tag
GPR154	Oprhan G protein coupled receptor GPR154
DG	Dentate gyrus
LTP	Long term potentiation

PCR	Polymerase chain reaction
PTSD	Post traumatic stress disorder
PD	Parkinson's disease
TH	Tyrosine hydroxylase
HTS	High throughput screening
RLU	Relative light units
BRET	Bioluminescence resonce energy transfer
GFP	Green fluorescent protein
FSK	Forskolin
PTX	Pertussis toxin
Nluc-β-arrestin2	Nanoluciferase-tagged β-arrestin2
FSH	Follicle stimulating hormone
LH	Lutenizing hormone
MCS	Multiple cloning site
KOR	Kappa opioid receptor
DYN	Dynorphin
SD	Standard deviation
CHO	Chinese hamster ovary cells
HEK	Human embryonic kidney cells
LGR4/LGR5	Leucine-rich repeat-containing G-protein coupled receptor 4 and 5
5-HT_{2A} receptor	Serotonin receptor

1. INTRODUCTION

1.1 G PROTEIN COUPLED RECEPTORS

G protein coupled receptors are integral membrane proteins constituted by seven transmembrane α-helices, one extracellular N-terminus, one intracellular C-terminus, three intracellular and three extracellular loops [1] [2]. They are one of the largest family of proteins and take up most part of the human genome, with more than 800 genes accounting for their expression in many different tissues [3]. Their spread presence across the whole human body makes their malfunction the main cause for many diffused and often severe pathologies that affect the world's population. This also makes GPCRs the pharmacological targets of about one third of current marketed drugs [4] [5] [6].

There are five different GPCR classes: Rhodopsin (A), Secretin (B1), Adhesion (B2), Glutamate (C), Frizzled (F) and Taste2 (T) [7]. About 90% of all GPCRs belong to the rhodopsin family [8]. This classification is based on the degree of similarity between these receptors, given by sequence alignment of their amino acid residues [9]. Their structure defines their selectivity towards many different ligands, therefore explaining the large spectrum of physiological functions exerted by GPCRs. The transmembrane α-helices are the most conserved fragments that allow to classify GPCRs in the five different classes based on the difference between certain amino acids at specific function-related residues [10]. The conservation of the same amino acids at these positions is fundamental to maintain their ability to recognize, bind to their specific ligands and activate the receptor. A mutation at these residues can determine the impairment of the receptor's function, leading to a potential pathology [11]. Mutations are indeed one of the causes of physiological malfunction and GPCR-related diseases that often involve the central nervous system, the cardiovascular system and many other physiological processes [12]. GPCRs are involved in sight, taste, mood regulation, behavior, immune system regulation and many other functions [13] [14]. It is therefore fundamental to acquire further information regarding their mechanism of action and the unknown function of some receptors, namely orphan GPCRs. By testing the activity of vasopressin, oxytocin and libraries of plant peptides on the orphan GPCR in question, we aimed at discovering the signaling mechanism that characterizes its function and the physio pathological roles it may be connected to.

1.1.1 MECHANISM OF G PROTEIN COUPLED RECEPTORS

As the name suggests, the mechanism of GPCRs involves G proteins (guanine nucleotide binding proteins) as transducers of the signal brought by their physiological ligand. Once the ligand binds to the receptor, a conformational change occurs in its structure, creating a pocket for the recruitment of G protein and triggering its dissociation [15]. G proteins consist of three subunits: α, β and γ. Altogether they constitute a trimer that dissociates into α and the dimer βγ, once activated. The α subunit of G protein in the inactive state is bound to a GDP molecule. When the conformational shift in the receptor structure occurs upon ligand binding, G protein is recruited and a GDP molecule leaves its place to a GTP molecule, initiating activation and subsequent dissociation of the α subunit from the βγ dimer [16]. G proteins can be of different nature according to the specific pathway that characterizes the signaling of a given receptor. The difference is found in the α subunit, which can be of three different kinds: Gαs, Gαi, and Gαq. Each subunit interacts in a specific way with different second messengers, according to the pathway related to the specific receptor. Gαs has a stimulatory role: when activated, it stimulates the activity of the enzyme adenylyl cyclase, which role is to synthesize cyclic AMP from an ATP molecule. This causes the level of cAMP to increase and activate protein kinase A (PKA) [17]. This serine-threonine kinase is constituted by two regulatory subunits and two catalytic subunits. The regulatory subunits of PKA sense the increase of cAMP in the cytosol and dissociate from the two catalytic subunits, causing their activation. Their role is to phosphorylate the cAMP response element binding protein (CREB), that will consequently be able to migrate into the nucleus and bind the cAMP response elements located in the DNA (CRE), thereby initiating DNA replication and receptor internalization processes to terminate GPCR signaling [18]. Gαi, on the other hand, has an inhibitory role. The second messengers involved in this pathway are the same as for Gαs, but the transduction of signal exerts opposite effects. The inhibitory α subunit inhibits adenylyl cyclase, causing a decrease in basal cAMP levels and blocking the phosphorylation operated by PKA. The Gαq subunit is still activated by the exchange of GDP for a GTP molecule, but this triggers the activation of phospholipase C β (PLCβ). This enzyme cleaves a phosphatidylinositol biphosphate molecule (PIP2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Both molecules act as second messengers. The increase in cytosolic levels of DAG activate protein kinase C (PKC); this kinase

phosphorylates Ca^{2+} binding proteins, resulting in the overall increase of intracellular Ca^{2+} levels [19]. The same effect is achieved by IP_3, which role is to directly increase Ca^{2+} in the cytosol by interacting with its receptors in the endoplasmic reticulum and triggering its release. Although the α subunit is the main actor of signal transduction of GPCRs, the dimer βγ also interacts with the same subcellular effectors as the α subunit (adenylyl cyclase and PLCβ) [20]. The cellular response culminates with the exchange of GTP for GDP and the reassociation of the α subunit with the βγ dimer, therefore confirming the termination of signal transduction.

G protein, however, is not the only transducer of messages brought by external stimuli. The so-called G protein independent signaling pathway is an equally important mechanism of signal transduction that depends on different effectors and accounts for the desensitization and internalization of the receptor. This process is initiated by phosphorylation at the receptor's C-terminus and occurs through the work of G protein receptor kinases (GRKs). The main effectors of this pathway are β-arrestins, which bind to the receptor only in its phosphorylated form and trigger different intracellular signaling processes, from internalization of the receptor to recruitment and association of distinct protein complexes that mediate the activation of further second messengers. β-arrestins mediate different cellular responses depending on the different GRK isoform that phosphorylates the receptor at the C-terminus. Distinct phosphorylation patterns trigger specific conformational changes in the β-arrestin structure, therefore activating different pathways of downstream signaling or receptor trafficking [21]. β-arrestins are adaptor proteins, which means that their role is to favor the association of effector proteins into larger signaling complexes that account for further signal transduction pathways. One of their main roles is to bind the phosphorylated receptor and desensitize it by sterically hindering the interaction of G proteins at the intracellular C-terminus of the receptor. β-arrestin also promotes recruitment of the phosphodiesterase (PDE) and diacylglycerol kinase (DGK) family of enzymes to the C terminus of the receptor. These enzymes are responsible for the degradation of second messengers produced upon activation of G proteins. PDE4D3 and PDE4D5 convert cAMP in AMP, therefore decreasing PKA activity. DKG, on the other hand, phosphorylates diacylglycerol (DAG), decreasing PKC activity. When phosphorylated, DAG is then converted into phosphatidic acid, a molecule that acts both as substrate for the

synthesis of phospholipids but also as second messenger in further signaling pathways. The recruitment of either one enzyme or the other depends on the specific Gα subunit and on the downstream messenger produced by it. The internalization and trafficking of the receptor depend on the binding of effector protein complexes, such as AP-2, to the activated β-arrestin, which in return favors the formation of a vesicle and its coating with clathrin. The early endosome originated by this process will then be redirected to either recycling and re-expression of the receptor on the membrane or to the formation of a lysosome for the degradation of the receptor. Generally, this sorting is operated after the ubiquitination of the GPCR, a process also mediated by β-arrestin. Finally, β-arrestins are also involved in further signaling processes mediated by mitogen-activated protein kinases (MAPK) such as ERK, (extracellular signal-regulated kinases) leading to cell proliferation or differentiation, and JNK (c-Jun-NH_2-terminal kinase), involved in cell apoptosis [22]. The multitude of pathways and components described above belongs to β-arrestin dependent mechanisms and remarks the importance of investigating the extent to which these mechanisms affect the signaling of a given receptor.

1.2 GPCR SIGNALING, BIASED AGONISM AND ALLOSTERIC MECHANISM

The spread presence of GPCRs across different cells and tissues of living organisms justifies the wide variety of different ligands and functions that these receptors exert. From neurotransmission to metabolism, from cell growth to secretion to immune responses, GPCRs act as converters of extracellular stimuli into intracellular signals, leading to the final cellular response and adaptation [2]. Each receptor, according to its specific amino acid sequence and tridimensional structure, interacts and gets activated by a specific agonist, known as its putative or physiological ligand. The function exerted upon binding of the agonist is the physiological response to the external messages brought to the cell. There is a wide variety of ligands that bind to specific GPCRs, and their nature can be very different: from small molecules such as hormones, peptides, nucleotides and chemokines to larger proteins, and even ions and photons. Each ligand interacts with its cognate receptor and triggers a conformational change, activating preferential signaling pathways between G proteins and β-arrestin [23]. When the same agonist activates more than one pathway but with different magnitudes of effect and by exerting different intracellular responses, it is called a biased agonist [24] [25]. Biased agonists are also molecules that can activate one pathway while inhibiting the other at

the same time, even though they're both linked to the same receptor. For example, carvedilol is a β adrenergic receptor blocker that acts as antagonist in the G protein pathway while behaving as an agonist in the β arrestin pathway [26]. This means that different ligands can alternatively activate pathways of the same receptor by establishing distinct structural interactions with precise residues, therefore triggering specific conformational changes that translate into preferential recruitment of either G proteins or β-arrestin. Each signaling pathway is linked to different downstream effects: when the ligand is a pharmacologically active molecule, its binding can lead to the recruitment of both G proteins and β-arrestin, therefore exerting multiple downstream effects, both therapeutic and side effects. Biased agonism can be exploited in drug design to pharmacologically engineer active molecules that only exert the desired therapeutic effect by exclusively activating one pathway without activating the other, which is responsible for side effects. A representative example of the advantages of biased agonism in drug design is given by ongoing research and discovery of safer therapeutics acting on opioid receptors for the treatment of acute and chronic pain in severe injury, cancer or terminally ill patients. Opioid receptors belong to class A of GPCRs and they are found in the CNS (central nervous system), PNS (peripheral nervous system) and other tissues of the body. The main functions they exert through their putative ligands are analgesia, mood regulation and stress response, which is why they're the first-choice targets in drug design for the treatment of multiple conditions [27]. However, there are many other side effects related to the activation of these receptors through pharmaceuticals. Euphoria, dysphoria, sedation and respiratory depression are among the side effects mediated both by prescribed opioids (morphine, oxycodone, hydrocodone, etc.) and non-pharmaceuticals (heroin, fentanyl, etc.), especially at high concentrations administered in the context of substance abuse caused by high addiction induced by opioids [28]. The study of biased agonism on opioid receptors for the treatment of acute and chronic pain could lead to discovery of novel biased ligands that only exert analgesic effects and avoid the dangerous side effects through the preferential activation of one pathway over another [29]. Another important strategy for regulation of the effects exerted by specific signaling pathways involved in GPCRs is the mechanism involving allosteric modulators. These are small peptides, hormones, neurotransmitters and larger proteins that bind to the receptor in a different position

from the orthosteric site, where the physiological ligands usually bind and interact with the receptor. The allosteric site is a pocket that hosts a molecule, the allosteric modulator, which structure can be very different from the sequence and 3D structure of the agonist. Despite this, when the modulator interacts with residues in this cavity it triggers conformational changes that alter the structure of the receptor and influence the interaction between agonist and receptor, therefore affecting the activation of downstream signaling pathways [30]. The allosteric mechanism can be exploited in drug design to control and regulate the intensity of effects triggered upon binding of a ligand to the receptor. The advantages of this strategy reside in the higher specificity and selectivity of allosteric pockets compared to the orthosteric site of a receptor [31]. The ladder is usually a cavity that presents highly conserved amino acid residues among receptors of the same subfamily (e.g.: vasopressin and oxytocin receptors), which lead to low specificity of ligand binding, both in the case of physiological ligands and therapeutic compounds. The high specificity of allosteric sites for each single type of receptor, instead, efficiently modulates the on-site effects and improves subtype selectivity while minimizing off-target interactions and significantly reducing side-effects [32]. Each allosteric modulator can either positively or negatively regulate the receptor's signaling, depending on the favorable conformational modifications of the interaction between receptor and agonist [33]. This concept is currently becoming a wide-spread approach in GPCRs-related drug design. There are currently 5 approved therapeutics acting as allosteric modulators on GPCRs, while 25 are in clinical trials [34] (**Table 1**).

Allosteric drugs	Condition	GPCR target	Action	Signaling
Cinacalcet	Hyperparathyroidism	CasR	PAM	Ca^{2+} mobilization
Ticagrelor	Stroke, acute coronary syndrome	$P2Y_{12}$	NAM	Gi
Ivermectin	Parasitic roundworm infections	GABAb	PAM	Unclear
ATx-201	Viral and bacterial infections, atopic dermatitis, cancer, rheumatoid arthritis	NPY4	PAM	Gq
Avacopan	ANCA-associated vasculitis	C5aR1	NAM	Gi/β-arrestin2

***Table 1**: list of approved allosteric drugs targeting GPCR. PAM: positive allosteric modulator; NAM: negative allosteric modulator. Taken from: doi: 10.3389/fendo.2023.1137604.*

1.3 THERAPEUTIC RELEVANCE OF GPCRs

There are currently 516 drugs targeting GPCRs on the market in North America, Europe, Asia and Australia and they constitute around 36% of the totality of approved drugs. Furthermore, 337 agents among new and repurposed drugs are now being investigated in different phases of clinical trials for the treatment of multiple diseases, with growing attention towards neoplasms, metabolic and nervous system diseases. The spectrum of disorders related to GPCRs is astonishingly wide, explaining why this family of receptors is being largely investigated for the treatment of severe and impactful diseases that range from major depressive disorder to obesity, to type 2 diabetes. The top selling drugs right now are in fact agonists targeting GLP-1R and GIPR, both receptors involved in mechanisms that favor the release of insulin and the decrease of glucose blood levels for the treatment of type 2 diabetes mellitus. GLP-1-like compounds such as dulaglutide and semaglutide also decrease glucagon secretion and slow the emptying of the stomach, making them ideal adjuncts to diet and exercise in the treatment of severe obesity. Most part of currently marketed drugs acting on GPCRs are small molecules of synthetic origin, although the trend in new drug research shows an increase of interest towards biomolecules, ranging from small peptides to larger proteins like antibodies, thanks to the advancements in drug delivery technologies that could help overcome the poor drug-like properties of these biomolecular compounds. The strongest trend, however, shows allosteric modulators and biased agonists as main actors of new pharmacological research, with an increase of 50% in allosteric ligands in the last 7 years and 14 new allosteric modulators currently in phases I-III of clinical trials. Challenges in translation of in vitro results in clinical profiles significantly hinder research on biased agonists, but their strong potential as selective drugs with improved therapeutics and side-effect profiles encourages the progression of studies that could overcome these hurdles. GLP-1-like drugs like tirzepatide showed indeed biased signaling towards the G protein pathway; another example of biased drug is LSD, which, upon interaction with 5-$HT_{2A,}$ preferentially activates the β-arrestin pathway and subsequently displays potent antidepressant effects while avoiding psychedelic activity [35]. Despite the great emphasis drawn to research on new GPCR targets, the whole GPCRome still presents a large percentage of uninvestigated receptors that could be potential therapeutic targets for the novel treatment of wide-spread pathologies. A substantial portion of unexplored

receptors is of course constituted by orphan GPCRs (**Figure 1**), since any mechanism and molecular actor involved in their signaling remains yet undiscovered. Their potential therapeutic role is therefore still untapped, and this remarks on the importance of the pharmacological characterization of orphan receptors.

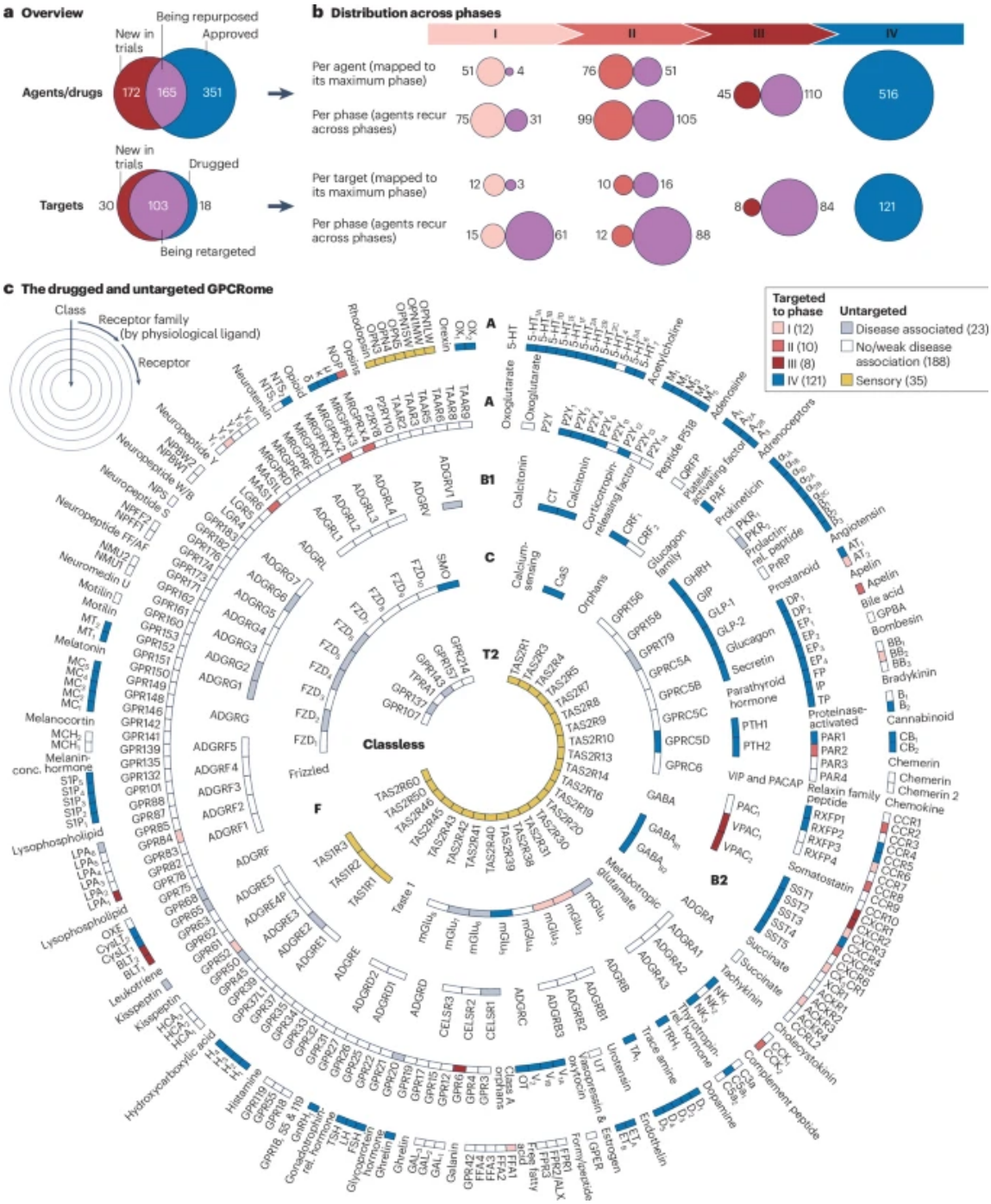

***Figure 1**: **a.** Overview of agents and drugs of new and drugged G protein-coupled receptors (GPCRs). **b.** Distribution across the highest and all clinical phases of new and repurposed agents and new and retargeted GPCRs. **c.** Status of GPCRs according to their most advanced compound. Modified and adapted from: https://doi.org/10.1038/s41573-025-01139-y*

1.4 ORPHAN G PROTEIN COUPLED RECEPTOR GPR150

The aim of this project is to pharmacologically characterize the pathways involved in the signaling of the G protein coupled receptor GPR150. This receptor is orphan, which means that its putative ligand and physiological function are yet unknown [36] [37]. There are less than ten articles mentioning this receptor on PubMed, and most of them date back to about twenty years ago. The known literature is therefore dated, and the starting information of this project is scarce.

The human orphan receptor GPR150 is encoded by the gpr150 gene, located in the chromosome 5 (location: 5q15) of the human genome. This receptor belongs to the class A rhodopsin-like family of GPCRs, specifically to the vasopressin and oxytocin subfamily of GPCRs. The silencing of the gpr150 gene is associated with the progression of ovarian cancer and it is triggered by the enhanced methylation of this gene. The human receptor is a protein of 434 amino acids and 46.353 Da [38]. According to the Human Protein Atlas databases [39], RNA transcripts relative to gpr150 were mainly found in the brain and in the pancreas, however protein expression was not detected (**Figure 2**). Regarding RNA transcripts, both single cell type and cancer line specificity are low, although pancreas expression is specific for pancreatic exocrine cells.

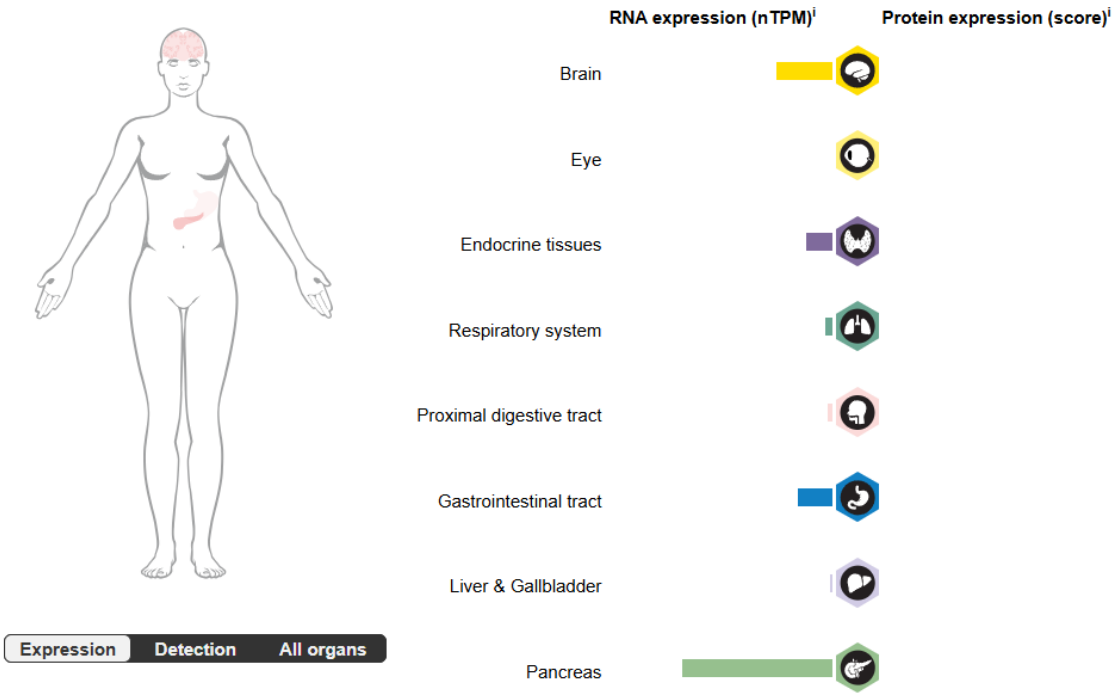

***Figure 2**: RNA and protein expression in the human body relative to the gpr150 gene, according to the Human Protein Atlas. Taken from: https://www.proteinatlas.org/ENSG00000178015-GPR150/tissue#expression_summary*

The relevant information acquired from existing literature mainly derives from studies on multiple orphan GPCRs and from phylogenetic research; this type of study connects orphan receptors to their closest family of GPCRs in terms of sequence homology and structure similarity, keeping into account the key structural characteristics and the degree of homology between highly conserved domains of GPCRs, i.e. the seven transmembrane α-helices. Phylogeny is fundamental to observe the evolution of a specific gene among different *taxa,* and it gives useful insight into the degree of homology between orphan receptors and their closest relatives, which descend from a common ancestor gene [40] [41].

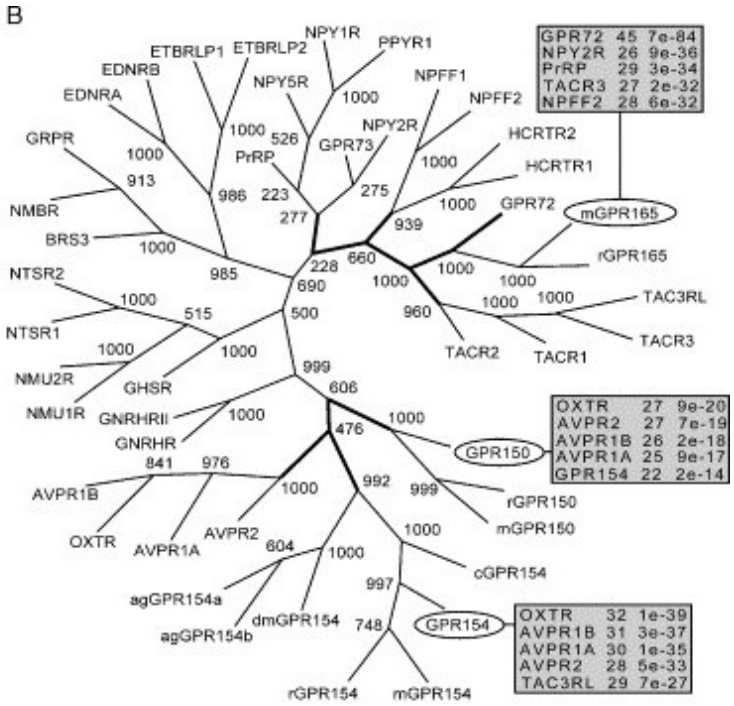

***Figure 3**: phylogenetic tree displaying the relationship between cognate rhodopsin GPCRs and three orphan receptors. Modified and adapted from: https://doi.org/10.1016/j.bbagen.2004.12.001*

In 2004, a set of orphan receptors were analyzed through the patterns of the rhodopsin family of GPCRs, identifying nine human orphan receptors as part of this family. The protein sequences were then compared to receptors of the NCBI database through the BLASTP (Basic Local Alignment Search Tool for Proteins) search tool to identify closely related receptors. A phylogenetic study was then carried out, placing the five highest-scoring BLAST hits for each orphan receptor into a phylogenetic tree. As shown in Figure 3, GPR150 shows the highest e-values for the BLAST search and shares the highest percentage of sequence homology with OXTR (oxytocin receptor), V2R, V1aR, V1bR (the three cognate vasopressin receptors) and GPR154. As mentioned above, the vasopressin subfamily of GPCRs is the closest relative of GPR150, although similarity scores are not very high (25-27% homology). GPR154 is another orphan receptor identified as part of the rhodopsin family of GPCRs and it displays 22% of homology with GPR150 (**Figure 3**). The study mentions

that the receptor GPR154 was found to be activated by arginine-vasopressin, however, after its publication, GPR154 was deorphanized and its physiological ligand was found to be neuropeptide S. GPR154 was therefore renamed NPSR1 (neuropeptide S receptor 1). The full genomic DNA sequences of the human and mouse receptors were subsequently screened against the human and mouse EST (expressed sequence tag) databases using BLASTN to compare nucleotide sequences and identify similarities between the receptor sequences and ESTs. ESTs are short genomic sequences corresponding to the receptor and are generated by isolating the receptor-related mRNA from a cell and reverse-transcribing it into cDNA (complimentary DNA) through the enzyme reverse transcriptase. In this way it is possible to obtain a DNA sequence containing exclusively the expressed sequences of the receptor, since it derives from an mRNA transcript. ESTs are relevant to identify the tissues where the corresponding receptors are expressed, since these transcripts are most likely to be found in the same tissue where the receptors are located. As shown in Figure 4, respectively 3 and 2 ESTs corresponding to GPR150 were found in both the human and mouse CNS (central nervous system), whereas the location of other 6 GPR150 ESTs in both species wasn't recognized. One further GPR150 EST was localized in the human reproductive organs (**Figure 4**) [42]. A more recent study from 2014 also investigated class A rhodopsin-like GCPRs through phylogeny, in this case by keeping into account not only sequence homology between receptors, but also the similarity of their structure. The sequence structure-based alignment

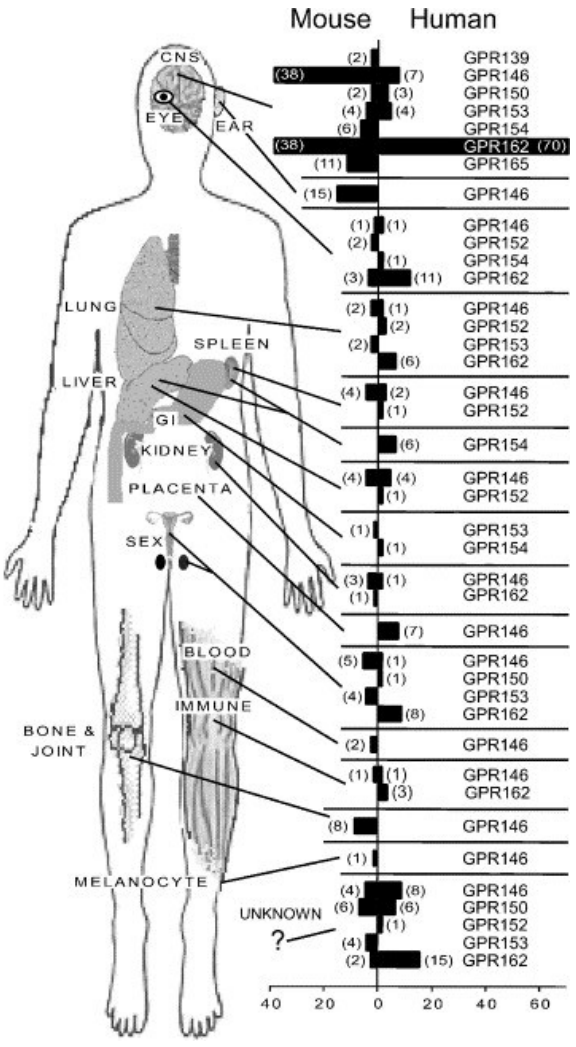

***Figure 4**: expression pattern of orphan receptors in different organs and tissues and relative number of ESTs. Modified and adapted from: https://doi.org/10.1016/j.bbagen.2004.12.001*

was performed by using the Promals3D program and these findings were used as input for the phylogenetic analysis obtained with the MEGA 5 software. The evolutionary history was inferred using the Neighbor-Joining method, with 1000 bootstrap replications [43]. Results showed the formation of 36 different clusters of receptors similar for both sequence and structure, and each cluster was then subjected to further analysis using the Maximum Likelihood method, in order to gain even more accuracy in results. This research linked a set of orphan class A GPCRs to different subfamilies of cognate rhodopsin-like GPCRs, and GPR150 was found to be closely related to cognate gonadotropin releasing hormone I and II receptors (GnRHI and GnRHII) (**Figure 5**). This discovery led to the hypothesis that GPR150 may be activated by gonadotropin releasing hormone-like agonists such as triptorelin and leuprolide and interact with antagonists like CMPD, antide, antarelix, NBI-4290 and WAY-207024 [43]. By comparison between the two mentioned phylogeny-based studies linking orphan GPCRs to cognate rhodopsin-like receptors, the ladder seems to offer a more accurate analysis based not only on

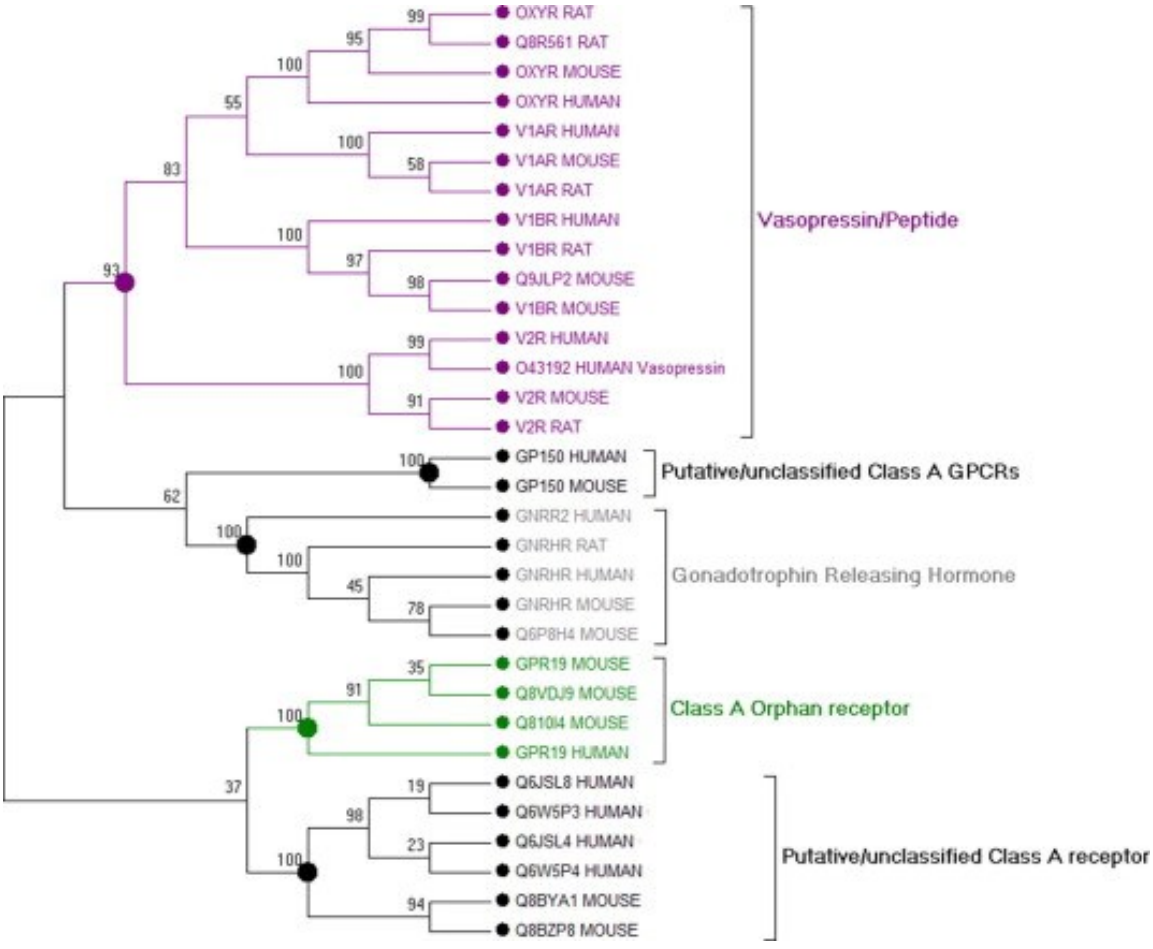

***Figure 5**: cluster of the phylogenetic tree linking human and mouse GPR150 to gonadotropin releasing hormone receptors and to the vasopressin subfamily of receptors, according to sequence homology and structure similarity. Modified and adapted from: https://doi.org/10.1016/j.ympev.2014.01.022.*

sequence homology but also on structure similarity, which is an equally important feature to keep into account in the research for the putative ligand of an orphan receptor, making results of this phylogenetic study more precise and reliable.

The most recent study mentioning the investigation of gpr150 (among other genes) inquired about the effects of amorphous curcumin, an antioxidant, antiangiogenic, anticancer and anti-inflammatory plant-derived polyphenol on the up- and down-regulation of the transcription of the rat genome. This screening identified a set of genes that were either hyper- or hypo-methylated, therefore affecting the degree of replication of such genes. Gpr150 was recognized as one of the genes that were hypermethylated, its expression was therefore downregulated (**Figure 6**). The administration of curcumin in the neurogenic niche of the hippocampus of rat offspring showed anxiolytic and antidepressant-like behaviors; these effects can be linked to the improvement in synaptic plasticity triggered by the altered methylation of different genes involved in the development of the rat hippocampus, specifically the dentate gyrus. By studying the expression patterns of each gene, this research found that GPR150 was mainly expressed in glial cells, especially astrocytes, of the dentate gyrus (DG) (**Figure 7**) [44]. The DG is the part of the hippocampus involved in neurogenesis, a process linked to the formation of new long-term

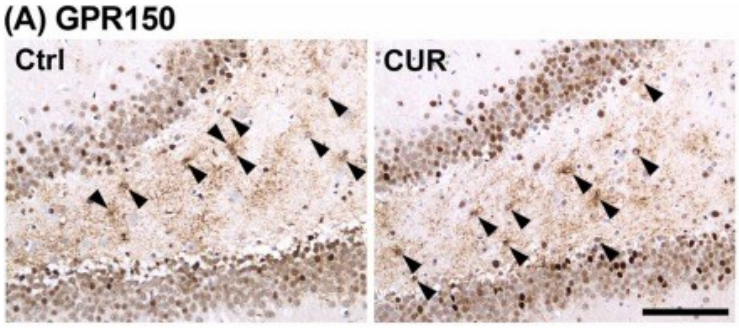

***Figure 6**: number of cells expressing GPR150 before and after curcumin administration. GPR150 expression after CUR exposure is downregulated, but the number of cells doesn't decrease. Modified and adapted from: https://doi.org/10.1016/j.jchemneu.2024.102414*

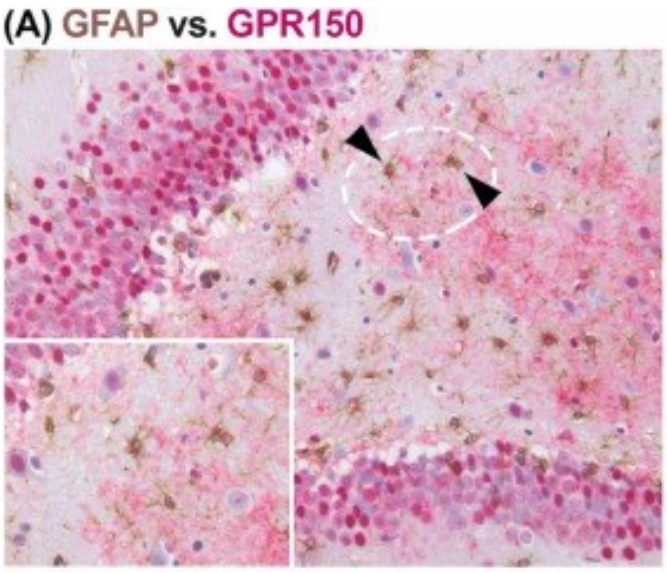

***Figure 7**: co-expression of glial fibrillary acidic protein (GFAP, brown) and G protein-coupled receptor 150 in astrocytes of the DG. Modified and adapted from: https://doi.org/10.1016/j.jchemneu.2024.102414*

memories from short-term ones, spatial memory and pattern recognition. The cells of this portion of hippocampus are divided into excitatory granule cells (which are directly involved in neurogenesis), inhibitory interneurons, mossy cells, stem cells and finally two types of glial cells: oligodendrocytes and astrocytes. Astrocytes are the most abundant cells in the brain and they're involved in different functions, ranging from metabolic, to neuroprotective, to structural and homeostatic functions. Especially in the DG, the main roles ascribed to astrocytes are neurogenesis and synaptic plasticity [45]. The increase in synaptic plasticity observed after curcumin exposure and down-regulation of GPR150 expression through enhanced methylation could lead to the conclusion that GPR150 could negatively influence the neural adaptability through an inhibitory pathway involved in the synaptic remodeling process for learning and memory, also known as long-term potentiation (LTP) [46]. There are multiple peptide ligands that bind to GPCRs and trigger an inhibitory effect which signaling mechanism has not been entirely elucidated, and GPR150 could be involved in such a mechanism through the interaction with the primary receptors of said ligands as a co-receptor that modulates this signaling process.

A study from 2015 investigated the constitutive activity of class A orphan GPCRs within the cAMP pathway by using reporter gene assays. Each orphan receptor was transfected and expressed in CHO cell lines together with a plasmid containing luciferase as reporter gene, which expression is under the control of cAMP response elements (CRE), which is a genomic sequence. The expression of luciferase is therefore a direct indicator of activation of the Gs or the Gi pathway, since they respectively increase or decrease cAMP levels in the cytosol and directly influence the activity of PKA and the phosphorylation of CREB, which in return migrates in the nucleus and binds to CRE to either promote or downregulate the expression of genes encoded in the CRE sequence, encoded in the CRE plasmid together with the reporter gene of luciferase. An increase in RLU (relative light units) is the indicator of constitutive activity within the Gs pathway, whereas a decrease in RLU translates into activation of the Gi pathway. GPR150, among other orphan GPCRs, showed a decrease in RLU signal above 80% compared to both controls, one presenting baseline luciferase expression and the other presenting forskolin-enhanced luciferase expression (**Figure 8**). Forskolin's role is to stimulate adenylyl cyclase activity and increase basal cAMP levels; its utility in reporter gene

assays and second messenger assays that investigate the Gi pathway is to observe the extent to which this pathway is activated and its efficacy in decreasing cAMP levels. This finding suggests that GPR150 may be constitutively active within the Gi pathway, and the binding of its unknown putative ligand may enhance the inhibitory effect on adenylyl cyclase, leading to a decrease in CRE-regulated gene expression [47]. This finding fits well with the abovementioned discoveries that suggest an inhibitory activity of GPR150 on synaptic plasticity. By joining the results of this and of the abovementioned research, it is reasonable to hypothesize that GPR150 may suppress the replication of genes involved in the formation and plasticity of new synapses through the Gi pathway of GPR150 receptors of astrocytes, at least in the early developmental stages of the neurogenic niche that originate the dentate gyrus.

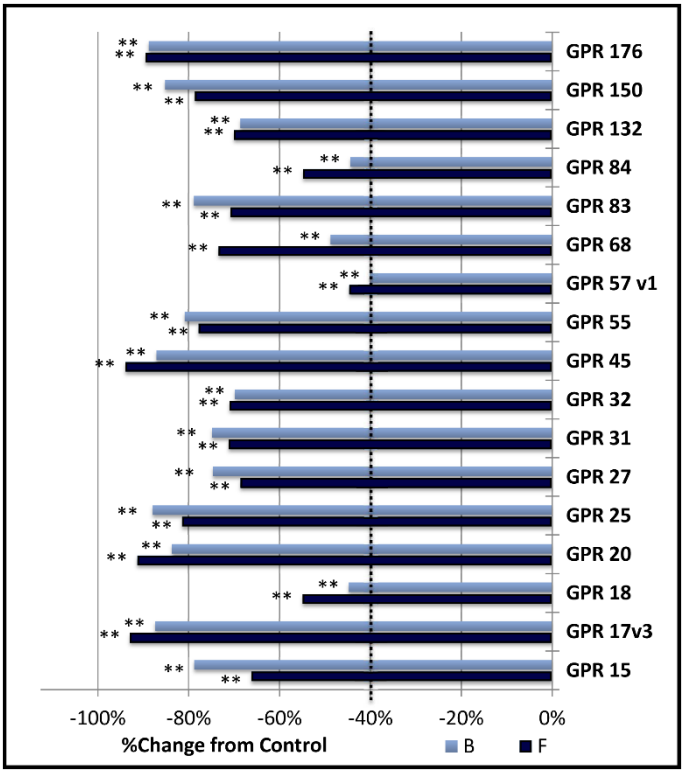

***Figure 8**: constitutive inhibition of both baseline (B) and forskolin stimulated expression (F) of luciferase for a set of class A orphan GPCRs; GPR150 shows a decrease of RLU reporter luciferase gene signal above 80% compared to both controls. Modified and adapted from: doi: 10.1371/journal.pone.0138463.*

In a study of 2007, gpr150 was identified among the other 33 genes as a tumor-suppressor gene in ovarian cancer. Ten ovarian cancer cell lines were analyzed by methylation-specific PCR and gpr150 was found to be hypermethylated in 8 of them (**Figure 9**). Furthermore, its expression was downregulated in 4 out of 15 primary ovarian cancer cell lines [48]. Therefore, the aberrant methylation of gpr150 could be considered as a possible candidate tumor marker to improve the early detection of ovarian cancer, which is especially important given the high mortality of ovarian cancer due to the lack of markers that allow its diagnosis at early stages. The involvement of gpr150 in the suppression of tumor cells suggests that it may negatively influence the cell proliferation mechanisms that underlie in cancer progression, and this may happen through GPCR

mechanisms that inhibit replication. The plausibility of this hypothesis would reconfirm the evidence of constitutive activity of GPR150 within the Gi pathway.

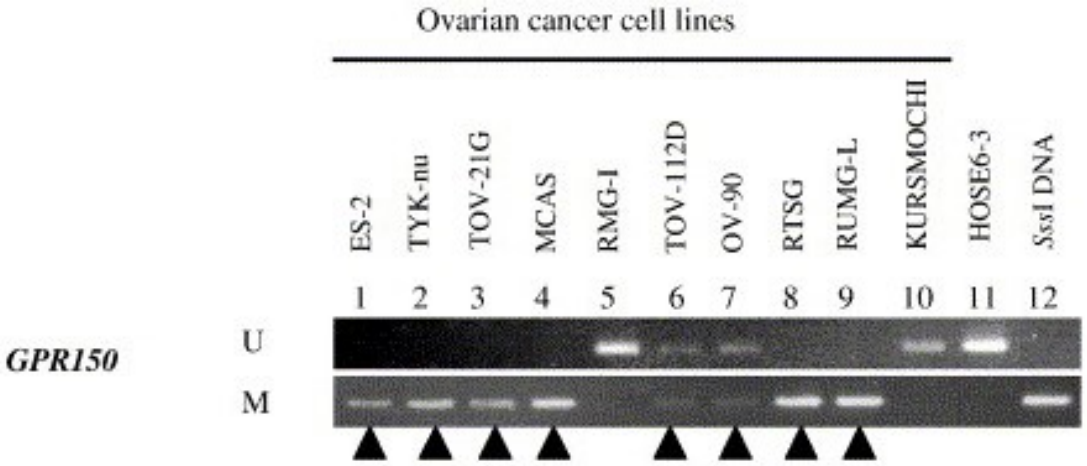

***Figure 9**: methylation of gpr150 in 10 ovarian cancer cell lines, HOSE6-3 (immortalized normal human ovarian surface epithelial cells) and DNA fully methylated by SssI methylase). U and M, primer sets specific to unmethylated and methylated DNA molecules, respectively. Modified and adapted from: https://doi.org/10.1016/j.lfs.2007.01.015*

1.5 VASOPRESSIN, OXYTOCIN, NEUROPEPTIDE S AND GONADOTROPIN RELEASING HORMONE AS CANDIDATE LIGANDS AND PHARMACOLOGY OF THEIR COGNATE RECEPTORS

The entire human genome sequence has been completely unraveled in 2004, thanks to low-stringency hybridization approaches, PCR-derived methods and bioinformatic analyses [49]. Since then, the number of human GPCRs increased to about 800, outnumbering the already known putative ligands and consequently creating a new class of GPCRs, namely orphan GPCRs [50]. The therapeutic potential hidden behind orphan receptors which malfunction could be involved in different pathological processes led to the development of novel deorphanization strategies to associate each orphan GPCR to its putative ligand. Some of these strategies include high throughput screening (HTS) of natural peptide and compound libraries of candidate ligands, in-silico HTS methods but most importantly cell and tissue-based assays. In cell-based assays, the orphan receptor is transfected and expressed into an adequate cell line to perform activation assays by either detecting G protein dissociation, β-arrestin recruitment (e.g., BRET assay) or second messenger production (e.g., IP_1 or cAMP assay) [51]. In tissue-based assays, different portions of animal tissue or organ which are known to express the orphan receptor in question are tested against a set of candidate ligands, and the

triggering of novel physiological responses compared to the control suggests the activation of the receptor upon the binding of a specific ligand. These approaches belong to the reverse pharmacology strategy, which has been developed in response to the lack of putative ligands for orphan receptors. Historically, ligands were discovered first, but the completion of the human genome reversed this trend, hence explaining the need for novel strategies such as reverse pharmacology to associate orphan receptors with their putative ligands [52]. Based on sequence homology and structure similarity analyses, a study from 2018 regarding deorphanization of GPCRs categorized GPR150 as a probable cognate receptor for peptide-like ligands, suggesting a set of physiological peptides as putative GPR150 ligands: this list of peptides mentions oxytocin, vasopressin, neuropeptide S and gonadotropin-releasing hormone, reconfirming results of previously mentioned phylogenetic studies [53]. Hereinafter is an insight into these ligands and the main features of their respective cognate receptors that may correlate to the main characteristics of GPR150, given their shared phylogenetic heritage. GPR150 is mostly presented as a member of the vasopressin-like subfamily of GPCRs, according to multiple phylogenetic studies. It shows around 25% of sequence homology to vasopressin and oxytocin receptors, and the pathways involved in this receptor's signaling may offer a useful comparison tool for uncovering the pharmacological characteristics of the orphan receptor GPR150.

1.5.1 VASOPRESSIN AND OXYTOCIN

Vasopressin and oxytocin are neuropeptide hormones synthesized in neurons of the hypothalamus and released in the bloodstream from the pituitary gland [54]. They are

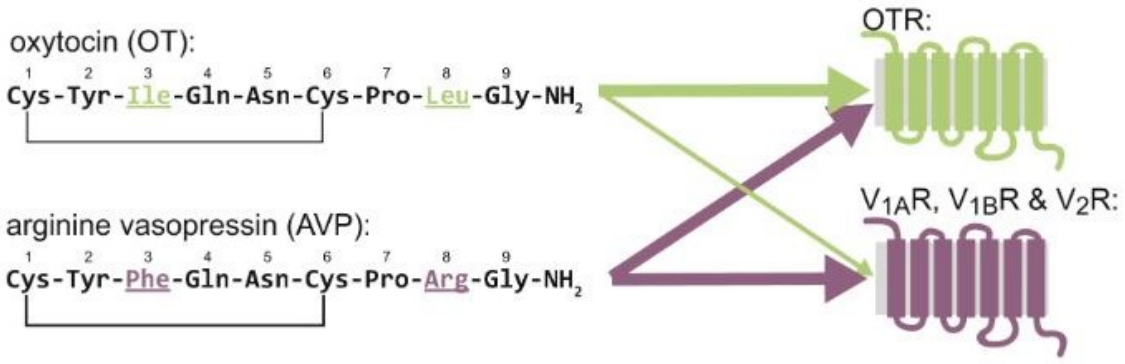

***Figure 10**: amino acid sequence of oxytocin and vasopressin peptides representing the difference in residues 3 and 8 and the disulfide bond between Cys1 and Cys6. Each ligand can activate both AVP and OXT receptors. Modified and adapted from: https://doi.org/10.1038/s41467-022-31325-0.*

both cyclic nonapeptides (with two cysteine residues forming a disulfide bond) that only differ for two amino acids in positions 3 and 8 [55]. The cognate receptors of vasopressin are V1aR, V1bR, V2R and the cognate receptor of oxytocin is OXTR [56]. The selectivity of the two ligands towards their own receptor is not exclusive, in fact oxytocin can also activate vasopressin receptors and vice versa (**Figure 10**).

The following table (**Table 2**) characterizes each receptor by its location in the organism, its physiological functions and diseases caused by its malfunction. Among others, we can find severe and wide-spread pathologies, ranging from hypertension to congestive heart failure, from anxiety to depression. These conditions require new and effective treatments, and this explains the importance of uncovering the function of orphan receptors such as GPR150, which could potentially be involved in oxytocin and vasopressin-activated signaling pathways [57].

	V1aR	V1bR	V2R	OXTR
Tissue expression	Vasculature	Pituitary CNS	Kidney	Uterus Mammary gland CNS
Physiological function	Smooth muscle contraction	ACTH release Behavior	Antidiuresis	Smooth muscle contraction Maternal and sexual behavior
Disease relevance	Hypertension Congestive heart failure	Anxiety Depression	Hyponatremia Congestive heart failure	Preterm labor Sexual dysfunction

***Table 2**: Physiological function, tissue expression and disease involvement of V1aR, V1bR, V2R and OXTR receptors. Taken from: Vasopressin and Oxytocin receptors, sigmaaldrich.com.*

There are only a few approved therapeutics acting on vasopressin and oxytocin receptors; oxytocin itself is used in clinic for the induction of labor and delivery, whereas desmopressin (a vasopressin analogue) is an approved V2R agonist for the treatment of diabetes insipidus [58]. A OXTR and V1aR antagonist, atosiban, is approved for the treatment of preterm labor and could reveal itself useful against hypertension by acting as an antagonist on V2R [59]. Atosiban also revealed itself to be a biased ligand by acting as an antagonist in the Gq coupled pathway but also as an agonist in the Gi signaling mechanism, activating anti-inflammatory pathways and inhibiting cell growth [60]. Other compounds targeting OXTR and V1bR are currently in clinical trials for the prevention of

preterm labor, major depressive disorder and generalized anxiety disorder [61] (**Figure 11**). The paucity of therapeutics acting on vasopressin and oxytocin receptors is not adequate to face the rising incidence of pathologies that involve OXT and AVP signaling pathways. It is therefore important to unveil the molecular mechanisms that allow the interaction between receptor and ligand that trigger conformational changes and lead to the activation of each specific pathway. The relevance of this research in this project is given by the phylogenetic relationship between GPR150 and OXTR/AVPR. It is important to investigate the sequence and structure of these receptors and compare the correspondent amino acid residues responsible for the binding and activation of the receptor, in order to see differences and similarities that might offer an insight into the molecular mechanism of activation of GPR150. As shown in the results section, a sequence alignment between GPR150 and OXT/VPR reports the homology in amino acid residues involved in the agonist-receptor interaction, revealing the differences in

Name	Type	Target	Status	Brand name/ Company[c]	Admin.	Indication
Oxytocin	P	OTR	Approved	Syntocinon, Pitocin	iv, im, in	Uterine contractions, postpartum bleeding or hemorrhage; lactation
Demoxytocin	P	OTR	Approved	Sandopart, Odeax, Sandopral	buccal[d]	Labor induction; lactation; postpartum mastitis
Carbetocin	P	OTR	Approved	Duratocin, Pabal Lonactene	iv, im	Uterine atony; postpartum bleeding or hemorrhage
Merotocin	P	OTR	Phase 2	Ferring	in	Lactation
Atosiban	P	OTR/V_{1a}R	Approved	Antocin, Tractocile	iv	Prevention of preterm labor
Barusiban	P	OTR	Phase 2	Ferring	sc, iv	*In-vitro* fertilization
Cligosiban	SM	OTR	Phase 2	Ixchelsis Limited	oral	Prevention of premature ejaculation
Epelsiban	SM	OTR	Phase 1/2	GlaxoSmithKline	oral	Prevention of premature ejaculation; embryo transfer; adenomyosis
Nolasiban	SM	OTR	Phase 3	ObsEva SA	oral	*In-vitro* fertilization
Retosiban	SM	OTR	Phase 3	GlaxoSmithKline	iv	Prevention of preterm labor
L-368,899	SM	OTR	Phase 2	Merck	oral	Prevention of preterm labor
L-371,257	SM	OTR	Phase 2	Merck	oral	Prevention of preterm labor
SSR126768A	SM	OTR	Phase 1	Sanofi-Aventis	oral	Prevention of preterm labor
Vasopressin	P	V_{1a}R, V_{1b}R, V_2R	Approved	Pitressin Vasostrict	sc, im, iv	Central diabetes insipidus (V_2R); postoperative abdominal distention, vasodilatory shock (V_{1a}R)
Desmopressin (dDAVP)	P	V_2R	Approved	DDAVP	iv, im, sc, in, oral, sl	Central diabetes insipidus; nocturnal enuresis; hemophilia A
Terlipressin	P	V_{1a}R, V_{1b}R, V_2R	Approved	Glypressin, Teripress	iv	Hypotension (V_{1a}R); hepatorenal syndrome (V_{1a}R)
Phenypressin	P	V_{1a}R, V_{1b}R, V_2R	Approved	Felypressin	iv	Vasoconstrictor additive for local anesthesia during dental procedures (V_{1a}R)
Velmupressin	P	V_2R	Phase 2	Ferring	oral	Nocturia
Lypressin	P	V_{1a}R, V_{1b}R, V_2R	Approved	Diapid	in	Central diabetes insipidus (V_2R), Cushing's syndrome
Selepressin	P	V_{1a}R	Phase 2	Ferring	iv	Septic shock
Conivaptan	SM	V_{1a}R/V_2R	Approved	Vaprisol	iv	Hyponatremia
Mozavaptan	SM	V_2R	Approved	Physuline	oral	Hyponatremia
Tolvaptan	SM	V_2R	Approved	Samsca Jynarque	oral	Hyponatremia; autosomal dominant polycystic kidney disease
Balovaptan	SM	V_{1a}R	Phase 2	Roche	oral	Post-traumatic stress disorder (PTSD)
SRX246	SM	V_{1a}R	Phase 2	Azevan Pharm	oral	Irritability in Huntington's patients, intermittent explosive disorder, PTSD
Pecavaptan	SM	V_{1a}R/V_2R	Phase 2	Bayer	oral	Heart failure
Balovaptan	SM	V_{1a}R	Phase 3	Roche	oral	ASD
Satavaptan	SM	V_2R	Phase 3	Sanofi-Aventis	oral	Hyponatremia; Ascites
Lixivaptan	SM	V_2R	Phase 3	Palladio Bio, CadioKine Inc	oral	Polycystic kidney disease; heart failure; hyponatremia;
TS-121	SM	V_{1b}R	Phase 2	Taisho Pharm	oral	Major depressive disorder
Balovaptan	SM	V_{1a}R	Phase 2	Roche	oral	Acute ischemic stroke
Nelivaptan	SM	V_{1b}R	Phase 2	Sanofi-Aventis	oral	Major depressive disorder; generalized anxiety disorder
Relcovaptan	SM	V_{1a}R	Phase 2	Sanofi-Aventis	oral	Raynaud's disease; dysmenorrhea; preterm labor; small cell lung cancer
ABT-436	SM	V_{1b}R	Phase 2	AbbVie	oral	Major depressive disorder; alcoholism
RO5028442	SM	V_{1b}R	Phase 1	Hoffmann-La Roche	iv	ASD

***Figure 11**: OTR and AVPR agonists and antagonists approved or currently investigate in clinical trials for the treatment or prevention of multiple conditions and disorders. Modified and adapted from: https://doi.org/10.1016/j.tibs.2024.01.010*

binding and activation mechanisms that characterize and distinguish oxytocin and vasopressin receptors from the orphan receptor GPR150. Even though homology is not high enough to suggest oxytocin or vasopressin as physiological ligands for GPR150, it is still relevant to verify whether either of these ligands trigger any activation of it.

1.5.2 NEUROPEPTIDE S

The ligand neuropeptide S is a peptide of 20 amino acids which cognate receptor is the previously deorphanized GPR154 receptor, namely NPSR1. The physiological ligand of this receptor was found by following the "reverse pharmacology" strategy and by stably expressing it in cells and using it as a bait to purify its correspondent endogenous ligand from brain extracts, since the relative RNA transcripts were mainly identified in multiple cortical structures, in the hypothalamus and in the amygdala [62]. When bound to NPS, this receptor recruits Gαs and Gαq stimulatory transducers that trigger the subsequent increase of cAMP and the cytosolic release of Ca^{2+} [63]. The excitatory mechanism of NPS is therefore thought to be involved in synaptic transmission and plasticity in the hippocampus, and this hypothesis is also supported by observations of animal behavior after intra-amygdala administration of NPS, which exerted acute anxiolytic-like effects, accelerated fear extinction and enhanced learning and memory consolidation. Interestingly, anxiolytic effects were also observed after intranasal administration of NPS in mice, highlighting an effective and non-invasive prospect for the treatment of anxiety diseases such as PTSD [64]. Furthermore, knockout mouse models for NPSR1 and NPS showed attenuated arousal, deficits in learning and memory and slightly increased anxiety [65]. A recent study identified NPSR as a potential therapeutic target in the treatment of Parkinson's disease (PD). The receptor's mRNA transcripts were detected in the dopaminergic areas of the brain, such as substantia nigra pars compacta and mainly in tyrosine hydroxylase (TH) positive neurons, which are consistently involved in the pathophysiological processes that lead to the development of PD. TH is in fact the enzyme responsible for the biosynthesis of dopamine and other catecholamines, and the degeneration of TH positive dopaminergic neurons impairs the production and neurotransmission of dopamine[66], causing gradual advancement of motor dysfunctions and non-motor symptoms including constipation, urinary dysfunction, orthostatic hypotension and depression. Based on the colocalization of NPSR1 and TH in neurons,

NPS was centrally administered to observe the improvements of PD models in rodents. Results showed improvements in motor and non-motor dysfunctions, decrease in oxidative stress and slowed loss of TH-positive neurons. Furthermore, no changes or improvement in locomotor activity were observed after NPS administration in NPSR1 knock-out mice. These findings led to the hypothesis that the signaling of neuropeptide S might play a role in the modulation of the dopaminergic transmission, and it could represent a valuable target for the treatment of PD (**Figure 12**) [67].

The diversity of functions affected by the NPS signaling make it a relevant research opportunity and its similarities to the orphan receptor GPR150 need to be investigated to understand the mechanisms that characterize it. In the abovementioned phylogenetic studies, this orphan receptor showed 22% of sequence homology to GPR150 and 32% homology to OXTR. This means that it is highly unlikely, given the low sequence homology percentage, that either oxytocin or vasopressin will reveal themselves to be physiological ligands for the orphan receptor GPR150 since NPSR1 showed even higher homology to OXT and AVP receptors and its physiological ligand was found to be a 20 amino acid peptide with very different features in comparison to oxytocin and vasopressin. However, it is fundamental to analyze the similarities between GPR150 and OXTR/AVPR to assess which amino acid residues are crucial to the functioning of the receptor. It is also important to verify whether oxytocin or vasopressin show any binding and activation of the receptor to capture which pharmacological pathways are linked to the orphan receptor in question.

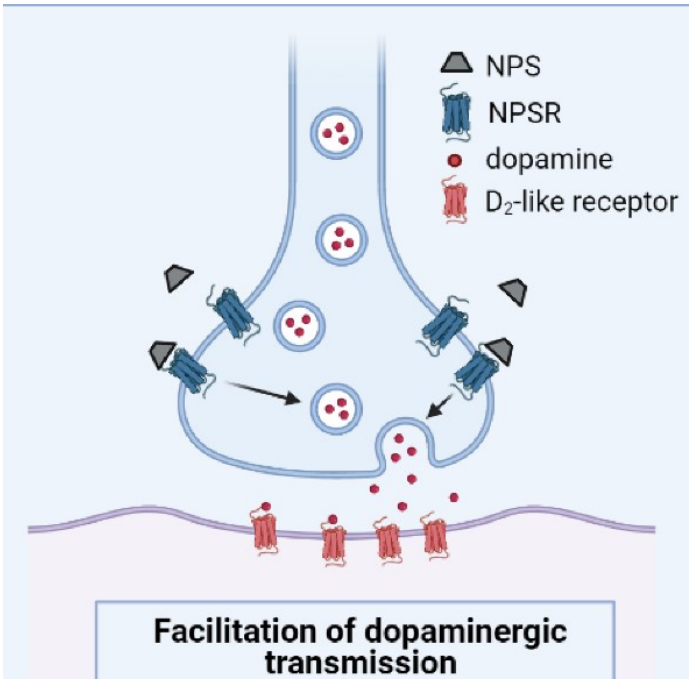

***Figure 12**: Putative mechanisms by which Neuropeptide S (NPS), activating Neuropeptide S receptor (NPSR), alleviates Parkinson's Disease (PD)-induced motor and non-motor dysfunctions. NPS increases dopaminergic transmission in mesolimbic and nigral dopaminergic pathway. Modified and adapted from: https://doi.org/10.3390/ph14080775*

1.5.3 GONADOTROPIN RELEASING HORMONE

Gonadotropin releasing hormone (GnRH) is a decapeptide secreted from the hypothalamus into circulation to reach the anterior pituitary and interact with its cognate receptor GnRHR on the membrane of pituitary gonadotropic cells, where it stimulates the production of the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). These two gonadotropins are subsequently released in the bloodstream and reach the reproductive system in males and females, where they interact with their physiological targets in testes and ovaries to regulate the production of hormones such as testosterone and estradiol to control the production of sperm in men and the maturation of the egg during the menstrual cycle in women. The gonadotropin releasing hormone receptor couples to the Gq protein pathway, therefore increasing the intracellular Ca^{2+} levels [68] [69]. The expression of this receptor was mainly detected in the gonadotropic cells of the pituitary gland but also in ovaries, prostate, heart and liver, to a minor extent. Genetic mutations of the receptor and dysregulation in the GnRH signaling can lead to delayed puberty, infertility and impaired sexual development due to the lack of sexual hormones, triggered by a condition recognized as hypogonadotropic hypogonadism [70]. The pharmacological agents currently approved for the treatment of such malfunctions are GnRHR agonists, which are synthetic analogues that help to regulate the production of FSH and LH by desensitizing and downregulating the receptor over time. Some agonists used in therapy are goserelin, leuprorelin and triptorelin. These are widely used for the treatment of spread pathologies like endometriosis, uterine fibroids and prostate cancer, to contrast sex steroid-dependent hyperplasia [71]. GnRHR antagonists are used in clinic for fertility treatment but also against precocious puberty. Equally important is the potential role of pharmacological chaperones, which bind to misfolded proteins and correct their folding to improve their trafficking and stability. For example, genetic mutations of the GnRHR can lead to its misfolding and retention in the endoplasmic reticulum; pharmacoperones are properly engineered to bind to the misfolded receptor, drag it to the cell membrane and restore its functionality. Studies were conducted on four different chaperones and all of them proved successful in the trafficking and expression of the functional receptor at the membrane [72].

The receptor GnRHR was found to be the closest relative receptor to GPR150, according to structure similarity. Their expression patterns in the brain differ, but GnRH might exert some activity on the orphan receptor in question. For this reason, this decapeptide was also included in this study to uncover the pharmacology behind GPR150.

1.5.4 DRUG DISCOVERY FROM PLANT PEPTIDE LIBRARIES

In this project, libraries containing peptides from plant extracts were also exploited to test the activation of the pharmacological pathways related to GPR150. Peptide libraries are in fact a precious resource for the discovery of novel peptides that can assist the design and creation of new compounds that could act as drug leads in the research and development of new therapeutics acting on GPCRs. The potential activation of GPR150 upon stimulation with any of the compounds from these libraries constitutes an important investigation opportunity to isolate specific peptides, analyze their structure and formulate a possible interaction mechanism between the isolated compound and the receptor. This process could lead to the identification of specific residues of the receptor that are involved in its conformational change and in the consequent communication with G proteins or β-arrestin as signal effectors. This type of approach, with the help of AI-based databases predicting the three-dimensional structure of large proteins like GPCRs, might help in the definition of the orthosteric pocket of GPR150, leading to its association with probable endogenous ligands based on simulated interactions with peptide-like compounds, derived from the originally tested libraries. These plant-derived libraries contain many natural peptides of different physicochemical properties that can be used as probes to test the activation of the four different pharmacological pathways related to GPR150, from G proteins to β-arrestin2. The possible activation of the receptor's signaling could give significant insight into the type of peptide structure that best fits and interacts with the active or allosteric sites of the receptor, and it would give precious information on the types of residues involved in its activation, given that there is indeed a putative ligand that occupies a specific pocket in the tridimensional structure of the orphan receptor in question.

1.6 PROJECT AIMS

To sum up, the main goal of this project is to uncover which pharmacological pathways are involved in the activity of the orphan receptor GPR150. The relevance of this research is given by the fundamental role played by GPCRs in any living organism, specifically in humans, where GPCRs constitute one third of the pharmacological targets of all marketed drugs. Of all GPCRs, roughly 15% are still orphans [36], and many severe pathologies may be caused by incorrect signaling or by misregulated expression of genes related to orphan receptors. Given the importance of GPCRs in the development of novel treatments for many wide-spread diseases, orphan GPCRs constitute a precious subject of investigation for the study of less known diseases and for the discovery of new therapeutic approaches to treat them.

The pharmacological pathways investigated in this research involve G proteins -Gs, Gi and Gq- and the protein β-arrestin2. In order to detect the interaction of GPR150 with these signal effectors, cell-based assays must be carried out. HEK cells will be transiently transfected with pEGFP-N1 plasmids containing the sequence of GPR150, both untagged and tagged with the GFP protein. These plasmids will be obtained from the molecular cloning of the gpr150 sequence from the Tango plasmid into the pEGFP-N1 plasmid. The first step of the cloning process starts with the polymerase chain reaction (PCR), to synthesize the sequence of gpr150 marked with two specific restriction sites at both ends of the double stranded DNA. For the cloning of these specific sequences, a forward and a reverse primer need to be designed. For the sequence of the untagged receptor, the reverse primer must contain a stop codon to avoid the transcription of the GFP protein sequence, contained in the pEGFP-N1 host plasmid. For the sequence of the receptor tagged with GFP, the reverse primer should not contain a stop codon and should not introduce any frameshift mutations, to allow correct transcription and successful expression of the GFP-tagged receptor. Restriction sites are short dsDNA sequences that are found in the multiple cloning site of the pEGFP-N1 plasmid, and each site is recognized and cleaved in a specific position by the correspondent restriction enzyme, during a reaction called “restriction digest”. This reaction causes cleavage of restriction sites in the host plasmid and in the cloned sequence of GPR150, leaving compatible sticky ends on both sequences and arranging the right conditions for the ligation of gpr150 into pEGFP-N1, operated by the ligase enzyme. Once obtained, these

plasmids will be transfected in HEK cells and GPR150, untagged and GFP-tagged, will be transiently expressed in the cells.

The expression of the GFP-tagged receptor will be confirmed by observation with fluorescence microscopy, while both plasmids will be analyzed with sequencing methods to confirm correct cloning of both.

The activation of different pathways will be tested upon stimulation with vasopressin, oxytocin and libraries of plant peptides. The chosen assay to observe any activation upon ligand binding is bioluminescence resonance energy transfer (BRET), that detects a change in relative light units (RLU) caused by the differential energy transfer that occurs between a fluorescent and a bioluminescent molecule. For this purpose, G protein biosensors and nano-luciferase tagged β-arrestin2 will be used to produce a detectable change in RLU.

To explore the hypothesis of constitutive activity of GPR150 in the Gi pathway, luciferase reporter gene assays will also be conducted on HEK cells transfected with GFP-tagged plasmids, to detect any change in luminescence caused by differential transcription of CRE elements that are under the control of the Gs and Gi pathways.

2. MATERIALS AND METHODS

2.1 BUFFERS AND MEDIA

The following chemicals were used for preparing buffers and media: ampicillin sodium salt (Sigma-Aldrich), Dulbecco's Modified Eagle Medium (DMEM, 1x) high glucose with HEPES (no phenol red, gibco), DMEM high glucose (4.5 g/L) with L-glutamine and phenol red sterile filtered (Capricorn Scientific), Fetal Bovine Serum (FBS, Capricorn Scientific), kanamycin sulfate (Sigma life science), LB-Agar (Lennox, Carl Roth), LB-broth base (Lennox, Invitrogen) MilliQ water, penicillin-streptomycin solution stabilized (10000 units penicillin and 10 mg/mL streptomycin, Sigma-Aldrich), potassium chloride (≥ 99%, Carl Roth GmbH + Co. KG) potassium phosphate monobasic (≥ 98%, Sigma life science), sodium chloride (≥ 99.5%, Carl Roth GmbH + Co. KG), sodium phosphate dibasic (≥ 99%, Sigma-Aldrich). Buffer Components: 10x PBS stock NaCl/KCl/KH2PO4/NaH2PO4, pH= 7.4, dissolved in MilliQ water. LB medium 20 g/l LB-broth base, dissolved in MilliQ water. LB-Agar selection plates 35 g/L LB-Agar with 0.1 % (v/v) and 50 mg/ml ampicillin or kanamycin, dissolved in MilliQ water. DMEM with phenol red, FBS/penicillin-streptomycin 500 ml DMEM high glucose with L-glutamine and phenol red, supplemented with 50 ml FBS and 5 ml penicillin-streptomycin solution. DMEM (1x) with FBS/penicillin-streptomycin 500 ml DMEM (1x) high glucose with HEPES, no phenol red, 50 ml FBS, and 5 ml penicillin-streptomycin solution.

2.2 SEQUENCE ALIGNMENT

The sequence of the receptor GPR150 was aligned to the sequence of oxytocin and vasopressin human receptors OXTR, V1aR, V1bR and V2R by using the GPCRdb sequence alignment tool, according to the most conserved fragments of GPCRs.

2.3 PCR CLONING

To perform the pharmacological characterization of the receptor GPR150 and discover which pathways are potentially involved in its signaling we must induce the expression of this receptor into competent cells to perform proper assays on. The cloning process starts from a plasmidic DNA sequence that contains the gpr150 gene. The plasmid

containing the human sequence of the receptor is the Tango plasmid. To transfect the plasmidic DNA into competent HEK cells, an adequate bacterial plasmidic vector must be used. In this project, Escherichia Coli was selected to produce recombinant plasmidic DNA, given its short culturing time, low-cost media and easy genetic manipulation. Further advantage is given by the presence of the coding sequence for GFP (green fluorescent protein) into the plasmidic DNA of the chosen vector pEGFP-N1, allowing to produce a recombinant receptor tagged by the fluorescent protein that is necessary when be useful when performing BRET assays to investigate recruitment of β-arrestin2. The method used for cloning gpr150 into the pEGFP-N1 plasmidic vector is PCR (polymerase chain reaction). PCR is an amplification technique for nucleic acids that allows to replicate specific DNA segments by using a DNA polymerase I enzyme and two short single-stranded DNA fragments that act as forward and reverse primers, specifically designed to be complementary to the sequence of the gene to be amplified. This technique is articulated in three steps: denaturation, annealing or hybridization and elongation. Each step is carried out at a specific temperature according to the features of the primers, to maximize the amplification outcome. The denaturation phase is carried out at 98°C, which is the optimal temperature that triggers the dissociation of the two strands of the template DNA by breaking the hydrogen bonds between complimentary base pairs. The annealing phase occurs when each primer binds to its complimentary sequence on the template DNA and it must be carried out at a precise optimal temperature to allow binding of both primers to their complimentary single-stranded DNA. When selecting the optimal temperature for the annealing of both primers, the following features need to be considered: the content in GC bases must be within the 40-60 % range, so that the annealing to the template strand is strong enough but still avoids the formation of a secondary structure within the primer itself. The melting temperature of both primers should be as close as possible and not differ for more than 5°C, to ensure the binding of both primers and grant a successful cloning reaction. Too many consecutive A and T bases can lead to low affinity to the template. Primers should contain a short pre-sequence of 5 or 6 bases on the 5’ end of the primer to assist the restriction enzyme during the digestion, the restriction site of 6-8 bases and the hybridizing sequence complimentary to the first and last bases of the open reading frame (ORF) of the gene of interest. The total length of the primer should be of 18-24

bases. If a stop codon is required to avoid translation of further proteins contained in the plasmidic sequence, the reverse primer should then contain a stop codon between the hybridizing sequence and the restriction site. After the annealing phase, the elongation step takes place at 72°C and many double-stranded DNA segments identical to the template are synthesized.

The following reagents were used: forward primer containing the restriction site cleaved by XhoI, 2 reverse primers coding for the restriction site cleaved by BamHI, one containing the stop codon (10 µM) and one without the stop codon (10 µM), nuclease free water (DEPC-H_2O) (Thermo Fisher Scientific), deoxyribonucleotide triphosphate mix (dNTP mix, 10 mM each) (Thermo Fisher Scientific), 5X Phusion HF buffer (Thermo Fisher Scientific), Phusion Hot Start Flex DNA Polymerase (2U/µl, Thermo Fisher Scientific) and gpr150-Tango plasmid as template (Addgene). The designed primers were provided by Sigma Aldrich.

Five reaction mixtures and one negative control were prepared into adequate PCR tubes. Each reaction mixture contained 2.5 µl of forward primer, 2.5 µl of reverse primer both at 10 µM concentration, 1 µl of dNTP mix, 4 µl of gpr150-Tango plasmid template and diluted at 25 ng/µl concentration (apart from the negative control), 10 µl of 5X Phusion HF Buffer, 0.5 µl of Phusion Hot Start Flex DNA Polymerase and 29.5 µl of DEPC H_2O. A Master Mix was prepared containing primers, buffer, dNTP mix and DEPC water for the negative control, the five reaction tubes and two more for pipetting error. The multiplier used for calculating the quantity of each reagent was therefore 8. Each PCR tube therefore contained 45.5 µl of Master Mix, 0.5 µl of polymerase and 4 µl of template DNA in each tube, apart from the negative control.

The polymerase chain reaction was conducted in the VWR UNO96 Gradient Thermocycler according to the following protocol: the initial denaturation phase was carried out at 98°C for 30 seconds, followed by a further denaturation phase at 98°C for 10 seconds. The annealing step was carried out for 30 seconds at 58°C for the reaction containing the reverse primer with the stop codon and at 56.7°C for the reaction mix containing the reverse primer without the stop codon, coding for the GFP-tagged GPR150 receptor. The annealing temperature was decided using the NEB Tm Calculator

online tool. The elongation phase was carried out at 72°C for 30 seconds. This cycle was repeated 35 times. The final elongation phase was carried out at 72°C for 10 minutes.

2.4 AGAROSE GEL ELECTROPHORESIS

The agarose gel electrophoresis technique is used to isolate the amplified DNA sample from the PCR reaction tubes. This method exploits the difference in size and relative charge of the different DNAs present in the reaction tube to separate them by applying a potential difference to the gel chamber that makes the negatively charged DNA strands migrate towards the positive cathode. According to the size of the DNA, smaller strands will migrate faster through the agarose gel meshes, therefore displaying a pattern of bands located at different heights beneath the gel pocket where the initial sample was pipetted.

For the 1% agarose gel preparation the following materials were used: 1.2 g of agarose for molecular biology (Sigma Aldrich), 120 ml of 1 X TAE buffer and 12 µl of SafeView classic nucleic acid dye (Applied Biological Materials Inc.). The agarose powder was completely dissolved into 120 ml of 1 X TAE buffer in the microwave for 3 minutes at 800 W. The agarose solution was then cooled down at 50°C and 12 µl of SafeView dye were added. A *12-well 1.4 ml* comb was used to create gel pockets.

The visual tracking of the migration of during electrophoresis was ensured by adding 8.33 µl of TriTrak loading dye (6x, Thermo Fisher Scientific) to each 50 µl PCR reaction tube and 46 µl of each dyed reaction mixture were pipetted into the gel pockets. The size of each band's DNA was assessed by using a 1 kb plus DNA Ladder (Thermo Fisher Scientific). Electrophoresis was performed at 150 V and 300 mA for 60 minutes.

The gel bands were visualized by using a PEQLAB Quantum-ST4 1100 – 26M (VWR). Gel excision was possible by visualization under UV light.

After excision with a scalpel, each band was purified by using the GeneJet PCR Purification kit (Thermo Fisher Scientific).

2.5 RESTRICTION DIGEST

Restriction digest is the reaction in which the selected restriction sites in the host vector and in DNA sequences amplified with PCR get cleaved by the corresponding restriction enzymes.

Materials used for this reaction were: 29 µl of gpr150 DNA insert sequence previously amplified with PCR, 10 µl of 2X Tango Buffer (Thermo Fisher Scientific), 1 µl of XhoI restriction enzyme (Thermo Fisher Scientific), 2 µl of BamHI restriction enzyme (Thermo Fisher Scientific), 3 µl of OTR GFP-tagged pEGFP plasmid at 500 ng/µl and DEPC-H_2O (Thermo Fisher Scientific) up to 50 µl for both 1.5 ml reaction tubes (Eppendorf). Two reaction tubes were prepared, one containing the gpr150 DNA template and the other containing the pEGFP host plasmid. The online DoubleDigest Calculator online tool was used to determine the optimal buffer for the restriction digest reaction and the adequate ratio between the two restriction enzymes, namely 1:2 for XhoI : BamHI. Both reaction tubes were incubated at 37°C for 1 hour and 30 minutes in the Thermomixer. Agarose gel electrophoresis (150 V, 300 mA for 60 minutes), gel excision and clean-up with GeneJet PCR Purification kit (Thermo Fisher Scientific) were performed immediately afterwards to isolate and purify the cleaved DNA products.

2.6 LIGATION

In this step, the ligase enzyme ligates the gpr150 DNA insert into the pEGFP plasmidic vector by linking the sticky ends of the restriction sites that were previously digested. Sticky ends are single stranded overhangs at both ends of the DNA insert and vector plasmid that are created when the restriction enzyme digests the DNA. Each sticky end of the insert DNA is complimentary to each sticky end on the host vector. The ligase enzyme contributes to the formation of phosphodiester bonds between bases of the insert DNA and the vector DNA, therefore creating a double stranded circular DNA composed of the plasmidic vector and the insert of interest. This reaction can be carried out by testing different ratios of insert DNA to vector DNA, such as 1:1, 2:1, 3:1, 5:1, 7:1 and 10:1.

The following reagents were used: 2 µl of 5X buffer (Thermo Scientific Fisher), 1 µl of T4 ligase (5 U/µl, Thermo Fisher Scientific), digested insert gpr150 DNA, pEGFP vector DNA

obtained from the restriction digest and DEPC-H_2O up to 30 µl. In this case, the 3:1 ratio and the 5:1 ratio were tested for the ligation reaction of the gpr150 DNA insert, both with and without stop codon.

Each ligation reaction was carried out in a 1.5 ml Eppendorf tube, in a 30 µl reaction volume. The 3:1 ligation involved 82.53 ng of insert DNA (both for gpr150 insert with and without stop codon) and 100 ng of pEGFP vector DNA. The 5:1 ligation involved 137.5 ng of insert DNA (both for gpr150 insert with and without stop codon) and 100 ng of pEGFP vector DNA. The volume of each reagent was assessed according to the concentration of digested DNA obtained from the previous restriction reaction. The mass of the insert DNA was calculated using the NEBio Calculator online tool, based on the number of base pairs of both the insert DNA (1302 bp) and vector DNA (4733 bp). Each reaction tube was incubated overnight at 16°C in the Thermomixer (Eppendorf).

2.7 BACTERIAL TRANSFORMATION

In this process, competent bacterial cells undergo heat shock to make their membrane more permeable and host the pEGFP plasmidic DNA containing the sequence for the receptor GPR150. The protocol consists of multiple steps: two 1.5 ml Eppendorfs containing 50 µl of frozen XL-10 *Escherichia Coli* Gold competent cells were taken out of the -80°C freezer and thawed on ice for 20 minutes. The entirety of the 3:1 and 5:1 30 µl ligation reactions were added to the two different Eppendorfs containing thawed competent cells and incubated for 20 minutes on ice. Afterwards, they were both incubated at 42°C for 45 seconds in the Thermomixer C for the heat shock step and permeation of the ligated plasmidic DNA into competent cells. Cells were consequently incubated for another 2 minutes on ice to help the transformation process. Subsequently, 250 µl of LB medium without antibiotic were added to each transformation tube and incubated at 37°C for 1 hour and 30 minutes at 400 rpm in the Thermomixer C (Eppendorf). In the meantime, 2 previously prepared agar plates were taken out of the 4°C fridge and warmed at room temperature. After incubation, the content of each transformation tube was added to agar selection plates with kanamycin as antibiotic, according to the kanamycin resistance gene carried in the pEGFP plasmid. Negative and positive control agar selection plates were also prepared. The thawed XL-

10 *E. Coli* competent cells were added to the negative control plate without any other addition. A further tube of XL-10 *E. Coli* competent cells was thawed and incubated with 2 µl of pEGFP plasmid at 500 ng/µl of concentration without the ligated gpr150 sequence but only with the kanamycin resistance gene. After incubation, the transformed cells were added to the positive control agar plate. Each plate was then incubated overnight at 37°C in the static incubator to let bacterial cultures grow.

2.7.1 SMALL SCALE CULTURE

After overnight incubation, each single colony was picked from the agar plate and diluted in a Falcon tube containing 4 ml of LB medium and 4 µl of 50 mg/ml kanamycin and incubated overnight at 37°C and 200 rpm in the shaking incubator to let each colony replicate.

2.7.2 GLYCEROL STOCKS

After the overnight incubation, 700 µl of the replicated competent cells were drawn from each Falcon tube and added to a 2 ml Cryo tube containing 700 µl of 50% glycerol solution. Cryo tubes were stored overnight in the -80°C freezer.

2.7.3 PLASMID ISOLATION AND PURIFICATION

The pEGFP plasmid containing the gpr150 sequence was amplified in a small-scale *E. Coli* XL-10 Gold culture. With a sterile pipette tip, the top of the glycerol stock containing frozen cells was scraped and the pipette tip was inserted into a flask containing 250 ml of LB medium and 250 µl of kanamycin. The flask was then incubated overnight at 37°C and 200 rpm in the shaking incubator. After incubation, the bacterial culture was centrifuged at 6000 rpm for 15 minutes at 4°C to pellet the cells. The plasmidic DNA was then isolated by following the manufacturer protocol provided with the GeneJET Plasmid Midi-Prep purification kit (Thermo Fisher Scientific). After purification, the plasmid concentration was measured using NanoDrop™. The purified plasmids were then stored at -20°C.

2.8 CELL CULTURE

2.8.1 CELL CULTURE

All cell culture working steps, including passaging, transfection, and seeding were performed under aseptic conditions in a laminar flow hood. Adherent HEK293 cells from passages 29 to 40 were used as an expression system for G proteins, ß-arrestins, and

receptors. They were grown in DMEM with phenol red, FBS/penicillin-streptomycin in 10 cm adhesion plates at 5% CO2 and 37°C. For detaching the cells, a trypsin-EDTA solution (0.5 g porcine trypsin, 0.2 g EDTA, 4 Na/L Hanks´ Balanced Salt Solution, Sigma-Aldrich) was used.

2.8.2 CELL PASSAGING AND MAINTENANCE

The following reagents were used for passaging HEK293 cells: DMEM with phenol red, FBS/penicillin-streptomycin, 1x PBS (see Buffers and media), and trypsin-EDTA solution. The HEK293 cells were cultured at 5% CO2 and 37 °C until they reached a confluence of 70-80% before being passaged. This was performed by removing DMEM with phenol red, FBS/penicillin-streptomycin, washing with 1 ml of 1x PBS, and detaching the adherent cells with 1 ml trypsin-EDTA solution. The cells were washed off the Petri dish with two times 5 ml DMEM with phenol red, FBS/penicillin-streptomycin. After centrifugation at room temperature and 1000 rpm for 3 minutes, the supernatant was discarded, and the cell pellet was resuspended using 5 ml DMEM with phenol red, FBS/penicillin-streptomycin. The cell suspension was then diluted to the desired concentration using DMEM with phenol red, FBS/penicillin-streptomycin in 10 cm Petri dishes (10 ml in total) or in 6-well plates (2 ml) if the cells were supposed to be transfected the next day.

2.9 BIOLUMINESCENCE RESONANCE ENERGY TRANSFER ASSAY

Bioluminescence resonance energy transfer (BRET) is the method that allows us to characterize the pharmacological pathway behind the signaling of a receptor. This method is based on the increase or decrease in energy transfer between a bioluminescent energy donor and a fluorescent energy acceptor according to the distance between each other. This measuring technique is useful in showing the activation of different signaling pathways upon ligand binding and can reveal which signal transducers are involved. When investigating the signaling pathway underlying an orphan GPCR such as GPR150, both G protein and β-arrestin need to be inquired as possible transducers of the message brought by a ligand. By tagging the molecular components responsible for the signal transduction of GPCRs, it is possible to observe a decrease or increase in energy transfer according to the pathway activated upon ligand binding. The activation of the pathway of G proteins can be observed by transfecting the

cells with G protein biosensors where the α subunit is labeled with a bioluminescent molecule as energy donor, nano-luciferase, and γ subunit is labeled with a yellow fluorescent protein as energy acceptor, cpVenus. When the G protein is inactive, the proximity between the Gα and the Gβγ dimer will trigger an energy transfer from the Gα to the Gγ subunit. Upon ligand binding and activation, the Gα subunit will dissociate from the Gβγ dimer and the increase in distance will cause a decrease in energy transfer, therefore revealing the activation triggered by the binding of the ligand. When investigating β-arrestin2 recruitment upon ligand binding, cells are transfected with a bioluminescent β-arrestin2 tagged with nano-luciferase as energy donor and with a plasmid coding for the GFP (green fluorescent protein) tagged receptor of interest (i.e.: GPR150), that will act as energy acceptor. If the binding of the ligand activates the β-arrestin2 pathway, an increase in energy transfer will be detected, revealing the recruitment of β-arrestin2 to the GFP-tagged C-terminus of the receptor, where phosphorylation occurs. The activation of the energy emission of the bioluminescent nano-luciferase is possible thanks to a furimazine or hikarazine molecule that acts as substrate.

2.9.1 MATERIALS

For BRET assays, the following reagents were applied: DMEM with phenol red, FBS/penicillin-streptomycin, DMEM (1x) with FBS/penicillin-streptomycin, DMEM (1x), arginine-vasopressin trifluoroacetate salt (Bachem), dimethyl sulfoxide (DMSO, ≥99.5%, Carl Roth®), ethanol (≥99.5%, Carl Roth®), Hanks' Balanced Salt Solution (HBSS, 1x, gibco), hydrochloric acid (HCl, ≥37%, Sigma-Aldrich), jetPRIME transfection buffer (polyplus-sartorius), jetPRIME® transfection reagent (polyplus-sartorius), Nano-Glo luciferase assay substrate (hikarazine, Promega), oxytocin acetate salt (Bachem), vasopressin (Bachem), plant extracts of *S.nigra, B. alba, C. limon, V. odorata, C. ipecacuanha, D. moroides, V. tricolor, M. charantia* and *P. poeppigiana*.

2.9.2 TRANSIENT TRANSFECTION OF HEK293 CELLS

HEK293 cells were grown in 6 well plates in 2 ml DMEM with phenol red, FBS/penicillin-streptomycin until they reached a confluence of about 70-80% before they were transiently transfected. For G protein transfection, 2 µg of G protein biosensor and 2 µg of receptor were added to 200 µL jetPRIME buffer (polyplus-sartorius). After mixing, 8 µL of jetPRIME transfection reagent were added. For β-arrestin2 recruitment

measurements, 200 µL jetPRIME buffer, 200 ng of β-arrestin, 1800 ng of the receptor, and 4 µL of jetPRIME transfection reagent were used. The mixtures were incubated for 10 minutes at room temperature before being pipetted onto the desired well of the 6-well plate containing HEK293 cells. The cells were then incubated at 5% CO2 and 37°C for 4-6 hours. The BRET based G protein biosensors were obtained from the group of Hannes Schihada, subsequently transformed into competent E. coli cells, and stored at -80°C. After plasmid purification, the biosensors were ready to use.

2.9.3 CELL SEEDING

Within 4-6 hours after transfection, cells were ready to be seeded into a 96-well plate. DMEM High Glucose with FBS/Penicillin-Streptomycin was removed from the transfected well of the 6-well plate, the cells were washed with 1x PBS and detached using 200 µl trypsin-EDTA solution. After 1 minute incubation at 5% CO2 and 37°C, cells were washed off using 2 ml DMEM (1x) with FBS/penicillin-streptomycin. The cells were then centrifuged at room temperature and 1000 rpm for 3 minutes, and the supernatant was removed. The cell pellet was then resuspended in DMEM (1x) with FBS/penicillin-streptomycin. Subsequently, 50000 cells per well in 100 µl of this suspension were seeded into a 96-well white/clear bottom plate. The plates were measured after 16-24 hours of incubation at 5% CO2 and 37°C. The prepared cells were starved 1 hour before the measurements by removing DMEM (1x) with FBS/penicillin-streptomycin and adding 100 µl DMEM (1x) without supplements.

2.9.4 TIME DEPENDENT MEASUREMENTS

To achieve a final concentration of 10 µM per well on the 96-well plate, ligand stocks were prepared at 40 µM in 1x HBSS. 100 µ of 1x HBSS (negative control), AVP and OXT were pipetted onto a ligand plate. Plant extract ligand stocks were prepared at 400 µg/ml in 1x HBSS to achieve a final concentration of 100 µg/ml per well in the 96-well plate and were also pipetted onto a ligand plate. A hikarazine-108 solution was prepared by dissolving 1 mg of solid Hikarazine-108 in 200 µl of DMSO and 300 µl of acidic ethanol. Acid ethanol was prepared by mixing 12 ml of ethanol with 100 µl of HCl. The 4.22 mM hikarazine-108 stock solution was then diluted 1:50 and 1:100 for the assays. 5 minutes before measurements, 50 µl of 1:50 or 1:100 diluted hikarazine-108 stock solution was added to the starved cells. BRET measurements were conducted using a FlexStation 3 multi-mode microplate reader. For G protein recruitment, the emission was measured

at 460 nm for the nluc-tag and 530 nm for the CpVenus-tag. β-arrestin2 recruitment assays were measured at 460 nm for the nluc-tag and 510 nm for the GFP-tag. The BRET signal was measured at 37°C for 25 minutes in total. Each experiment required starvation of cells in DMEM (1X) media for 1 hour prior to the assay and incubation in the dark with 50 µl of forskolin (FSK) for 5 minutes before the measurement. After incubation of the substrate FSK in the dark, the measurement started and ligands were added to the cells after five minutes by the FlexStation. Five minutes after starting the measurement, 50 µL negative control (1x HBSS) or ligand solutions with the desired concentrations were automatically pipetted by the Flex Station from the ligand plate into the 96-well plate. All samples were measured as technical duplicates. The raw data was obtained as the ratio between acceptor emission and donor emission (BRET ratio).

2.9.5 DATA EVALUATION

Data evaluation and visualization were performed using GraphPad PRISM. BRET ratio values were processed by calculating the mean value of the technical duplicates for each time point.

2.10 LUCIFERASE REPORTER GENE ASSAY

Given the hypothesis, based on existing evidence, that the receptor GPR150 may be intrinsically active, this method helps to verify whether any constitutive activity can be detected. The principle on which this method is based exploits the co-transfection of the plasmid containing the sequence of the receptor to be investigated with a plasmid containing a reporter gene (in this case the gene codifies for luciferase), together with the cAMP response element sequence (CRE). The CRE containing plasmid tagged with nano-luciferase will only undergo transcription when an increase in cAMP levels activates PKA, which phosphorylates the CRE binding elements that enter the nucleus and promote the replication of the CRE sequence. This method can detect the intrinsic activity of a receptor because it directly measures the expression of the downstream effector cAMP, which can increase or decrease regardless of the binding of a ligand to the receptor. Compared to the BRET method, this procedure can detect the activity of the receptor even when it is unbound, whereas the energy transfer detected in BRET is only appreciated when G proteins or β-arrestin2 are respectively activated or recruited to the receptor upon ligand binding. Constitutive activity, however, can be appreciated

by comparing the luminescence signal produced by luciferase between cells transfected with both the receptor-containing plasmid and the CRE-plasmid, and cells only transfected with the CRE plasmid. An increase or decrease in luciferase signal in cells expressing the receptor will reveal possible constitutive recruitment of a Gs or Gi protein and the related increase or decrease in cAMP production, leading to the consequent variation of transcription of the CRE sequence that will result in a higher or lower luminescence signal compared to CRE-only control cells. In this specific experiment, the constitutive activity of GPR150 was tested based on the hypothesis that it may be intrinsically active in the Gi-coupled pathway. Cells were stimulated with forskolin (FSK, 100 μM) to promote the activity of adenylyl cyclase. An increase in luciferase signal is expected compared to the negative control cells transfected with the GPR150-containing plasmid and the CRE-containing plasmid but not stimulated by FSK, given by the increase in cAMP levels and the increased transcription of the CRE plasmid. A further sample of cells were further stimulated both with FSK 100 μM and pertussis toxin (PTX, 500 ng/ml). Pertussis toxin is known to inhibit the activity of the Gi protein, so if the hypothesis that the GPR150 receptor constitutively couples to Gi is true, a further increase in luciferase signal is expected compared to both the negative control cells and the cells only stimulated with FSK. Cells were also stimulated with vasopressin and oxytocin, both in presence and absence of FSK, to detect any agonistic or inverse agonistic behavior that might decrease or enhance the coupling to the Gi protein even beyond the level of constitutive activity. A positive control sample of cells were co-transfected with the kappa-opioid receptor (KOR) plasmid and the CRE plasmid to observe how the stimulation with FSK and dynorphin A (DYN) would affect the luciferase signal and to compare it with cells expressing GPR150. For the normalization of luciferase signal to the magnitude of expression of the receptor, both the control KOR plasmid and the GPR150 plasmid used for transfection were also tagged with GFP to normalize luminescence intensity for the fluorescence intensity of GPF, which is directly proportional to the expression of the receptor.

2.10.1 MATERIALS

The following reagents were used: DMEM with phenol red, FBS/penicillin-streptomycin, DMEM (1x) with FBS/penicillin-streptomycin, DMEM (1x), jetPRIME transfection buffer (polyplus-sartorius), jetPRIME transfection reagent (polyplus-sartorius), forskolin (100

µM), cell culture lysis 5X reagent (Promega, Madison, USA), luciferase assay substrate (Promega, Madison, USA), luciferase assay buffer (Promega, Madison, USA), firefly luciferase plasmid pGL4.29 luc2P (Promega, Madison, USA), oxytocin acetate salt (Bachem), arginine-vasopressin trifluoroacetate salt (Bachem), pertussis toxin (500 ng/ml), dynorphin A (1-13) (Bachem).

2.10.2 TRANSIENT TRANSFECTION OF HEK293 CELLS

HEK293 cells were grown in 6 well plates in 2 ml DMEM with phenol red, FBS/penicillin-streptomycin until they reached a confluence of about 70-80% before they were transiently co-transfected with 2 µg of firefly luciferase plasmid pGL4.29 luc2P (CRE plasmid) and 2 µg of receptor were added to 200 µL jetPRIME buffer (polyplus-sartorius). After mixing, 8 µl of jetPRIME transfection reagent were added. The mixtures were incubated for 10 minutes at room temperature before being pipetted onto the desired well of the 6-well plate containing HEK293 cells. The cells were then incubated at 5% CO2 and 37°C for 8-10 hours.

2.10.3 CELL SEEDING

Within 8-10 hours after transfection, the cells were ready to be seeded into a 96-well plate. DMEM High Glucose with FBS/Penicillin-Streptomycin was removed from the transfected well of the 6-well plate, the cells were washed with 1x PBS and detached using 200 µl trypsin-EDTA solution. After 1 minute incubation at 5% CO2 and 37°C, cells were washed off using 2 ml DMEM (1x) with FBS/penicillin-streptomycin. The cells were then centrifuged at room temperature and 1000 rpm for 3 minutes, and the supernatant was removed. The cell pellet was then resuspended in DMEM (1x) with FBS/penicillin-streptomycin. Subsequently, 40000 cells per well in 100 µl of this suspension were seeded into a 96-well white/clear bottom plate. The plates were then incubated for 16-24 hours at 5% CO2 and 37°C.

2.10.4 CELL STIMULATION

On the following day, cells were stimulated with 100 µl of different combinations of ligands in a solution of DMEM (1X) with and without forskolin for 4 hours at 5% CO2 and 37°C. Following the incubation, the cell medium was replaced with 20 µl of lysis buffer and cells were lysed by shaking the plate at 300 rpm for 20 minutes at room temperature on the Thermomixer C (Eppendorf).

2.10.5 FLUORESCENCE AND LUMINESCENCE MEASUREMENTS

The fluorescence intensity of the GFP protein used to normalize luminescence data was measured at 510 nm, with excitation at 475 nm. The luciferase intensity was measured as relative light units (RLU) by detecting luminescence after adding 50 µl of luciferase substrate luciferin to each well. Both fluorescence and luminescence data were measured using a Synergy H4 plate reader (Agilent, Santa Clara, USA). Each measurement was acquired as the mean value of technical triplicates and normalization of luminescence data was obtained by dividing it by the fluorescence data.

2.12 SITE DIRECTED MUTAGENESIS

This method was used to correct the plasmid DNA sequence of the GFP-tagged GPR150 receptor, which originally presented a stop codon between the GFP protein sequence and the sequence of the receptor that was previously inserted in the pEGFP-N1 plasmid. This method allowed to change one base pair in the stop codon and transform it into another codon that codified for a leucine amino acid, which allowed the expression of the receptor together with GFP.

2.12.1 MATERIALS AND PROTOCOL

The mutagenesis reaction was performed using the PCR method and it included 10x reaction buffer (2.5 µl), dNTP mix (1 µl), Quik solution reagent (1.5 µl), *PfuTurbo* DNA polymerase (0.5 µl), forward primer (5'-ccggtggatccaagaaagctgactcacaggaa-3', 100 ng), reverse primer (5'-ttcctgtgagtcagctttcttggatccaccgg-3', 100 ng), dsDNA template containing the original GPR150-GFP sequence to be mutated (100 ng) and ddH_2O water to a final volume of 50 µl. Forward and reverse primer were designed with the QuikChange Primer Design Program online tool and obtained from Sigma-Aldrich. Buffer and reagents were provided with the QuikChange Site-Directed Mutagenesis Kit (Agilent). PCR was conducted with the VWR UNO96 Gradient Thermocycler using the following cycling parameters: a first segment consisting of one single cycle was carried out at 95°C for 30 seconds and a second segment of 16 cycles divided in three phases: 30 seconds at 95°C, 1 minute at 55°C and 7 minutes at 68°C. After PCR, 10 µL of reaction product were incubated with 1 µl of DpnI restriction enzyme provided with the QuikChange Site-Directed Mutagenesis Kit for 1 hour at 37°C in the Thermomixer to

digest the parental, non mutated plasmid. The restriction enzyme DpnI selectively digests only the methylated parental DNA, whereas the mutated DNA is left intact.

After the restriction digest reaction, a bacterial transformation was performed using XL-10 *E. Coli* competent cells according to the protocol described at paragraph 2.7. The sequence of the plasmidic DNA obtained after MIDI prep purification was then verified with sequencing performed by LGC Genomics Biosearch Technologies.

3. RESULTS

3.1 SEQUENCE ALIGNMENT AND ANALYSIS OF BINDING POCKETS OF V1Ar, V1bR, V2R, OXTR AND GPR150

Based on phylogenetic analysis that correlates the orphan receptor GPR150 to the cognate receptors of oxytocin and vasopressin, this research project started from the alignment of the sequences of these receptors. In **Figure 13**, the sequences for the three vasopressin receptors and the oxytocin receptor are aligned and compared to the GPR150 sequence. The similarity score and the overall consensus of the corresponding residues for each receptor is shown. This research focuses on the alignment of a set of 43 residues that were shown to be the most conserved and essential for ligand recognition and activation of oxytocin and vasopressin receptors. A research from 2017 studied the residues involved in the interaction with the putative ligands and the activation of these receptors. These residues are the most conserved among vertebrate

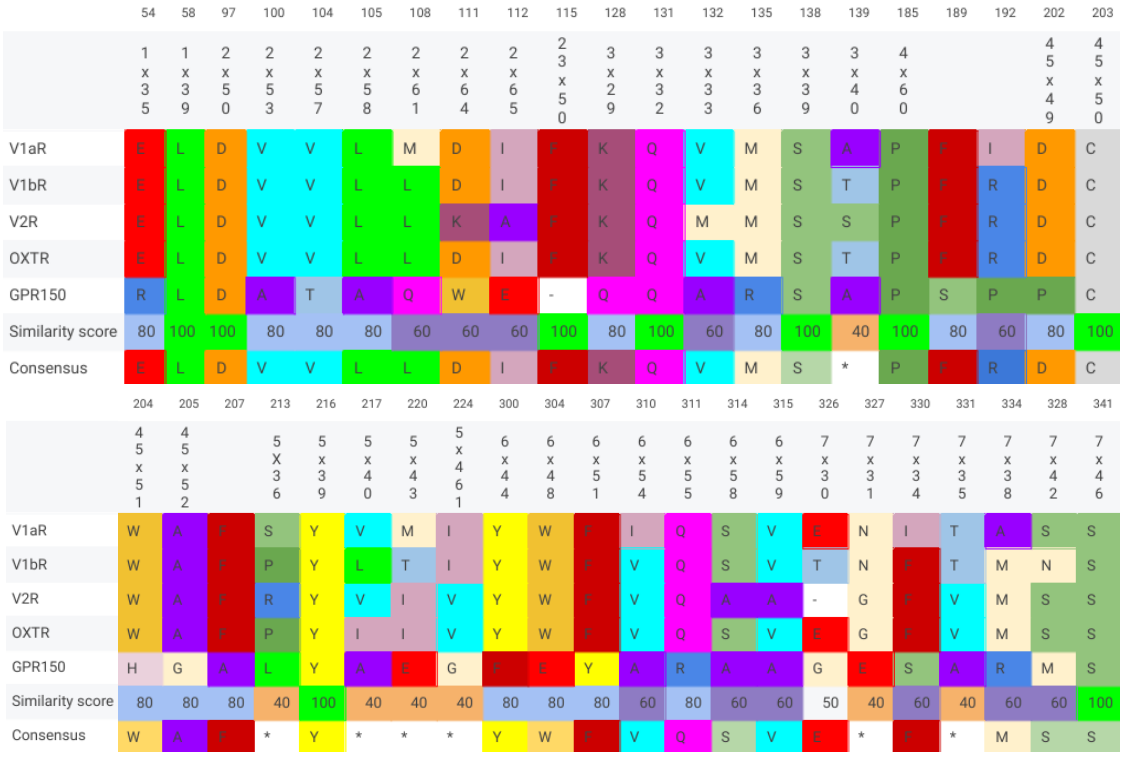

***Figure 13**: Sequence alignment of the most conserved residues of V1aR, V1bR, V2R, OXTR with the corresponding residues in the sequence of GPR150. Similarity score is indicated in percentage values and consensus indicates the prevalent amino acid for each residue of the five receptors, with * indicating no prevalent amino acid for that specific residue. Each residue is indicated by its numeric position both in the amino acid chain and with the Ballesteros Weinstein nomenclature.*

and invertebrate species, and their alignment might reveal which structural properties are responsible for the selectivity of the receptors for determinate ligands over other

peptides [73]. Each residue is indicated both with its relative number in the amino acidic chain and with the Ballesteros Weinstein nomenclature.

3.2 CLONING PROCESS AND PREPARATION OF PLASMIDS EXPRESSING GPR150

The cloning process was repeated multiple times for the testing of different reverse primers at different annealing temperatures to produce the genomic sequence of GPR150, both with and without stop codon. Forward primers were designed after choosing adequate restriction sites in the multiple cloning site (MCS) in the pEGFP-N1 plasmidic sequence found in the SnapGene database and after comparing the sequence to the gpr150 sequence provided by AddGene to find the correspondent restriction sites. The suitability of features of these primers was checked with the Sigma Aldrich's OligoEvaluator online tool.

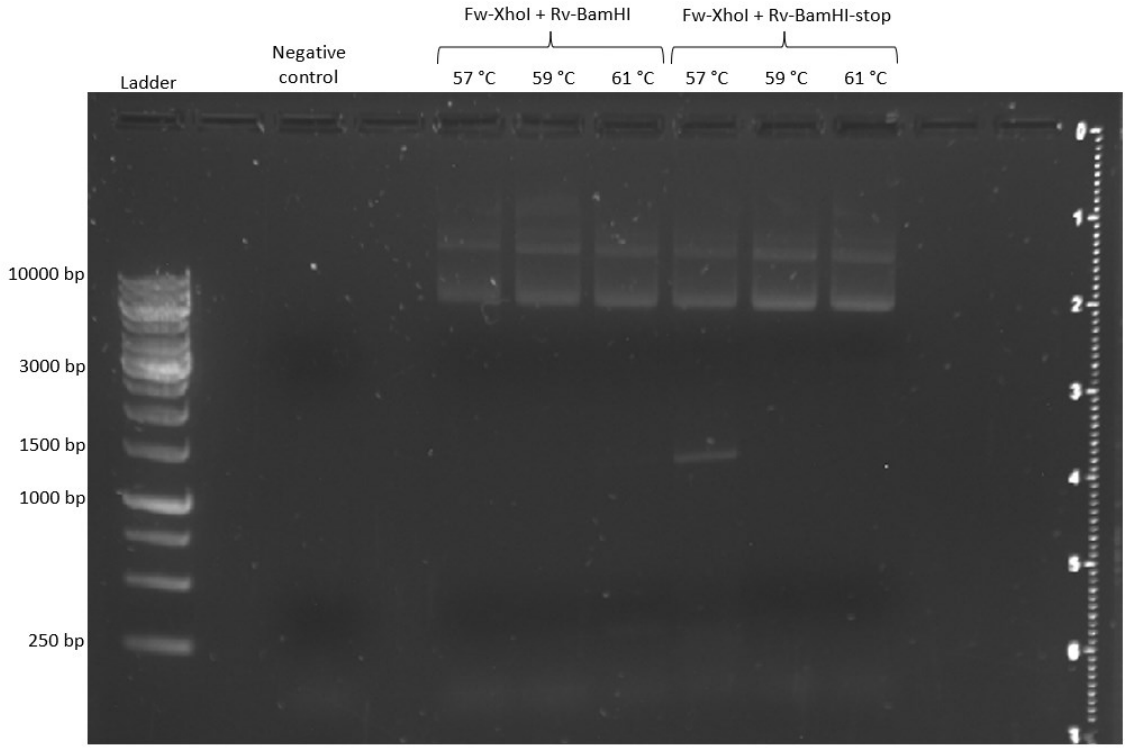

***Figure 14**: six PCR reactions (45 µl) containing primer couples Fw-XhoI+Rv-BamHI and Fw-XhoI+Rv-BamHI-stop at three annealing temperatures: 57, 59 and 61 °C were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. The gpr150 insert is 1302 bp long and the plasmidic vector Tango without insert is 6632 bp long. A 10 kb plus ladder (Thermo Scientific) was used.*

Forward primers must contain the sequences of restriction sites cleaved by the correspondent restriction enzymes. The upstream restriction sites chosen for the design of two different forward primers correspond to restriction enzymes XhoI and BglII. The downstream restriction sites are cleaved by enzymes BamHI and PstI. The following primers were designed (pre sequence-restriction site-stop codon-hybridizing sequence):

Forward primer-XhoI: TAAGCACTCGAGATGGAGGATCTGTTCTCTC

Reverse primer-BamHI without stop codon: TGCTTAGGATCCGAAAGCTGACTCACAGGA

Reverse primer-BamHI with stop codon: TGCTTAGGATCCTTAGAAAGCTGACTCACAGGA

Forward primer-BglII: TAAGCAAGATCTATGGAGGATCTGTTCTCTC

Reverse primer-PstI without stop codon: TGCTTACTGCAGGAAAGCTGACTCACAGGA

Reverse primer-PstI with stop codon: TGCTTACTGCAGTTAGAAAGCTGACTCACAGGA

Fw-XhoI was coupled to Rv-BamHI and Rv-BamHI-stop in six different PCR reactions, both couples at the annealing temperatures of 57, 59 and 61°C. Only one reaction showed binding of both primers to the gpr150 sequence, containing primers Fw-XhoI and Rv-BamHI-stop (**Figure 14**).

In the second attempt, Fw-XhoI was coupled respectively with Rv-BamHI and Rv-BamHI-stop in three PCR reactions respectively for each couple to test primers binding at three

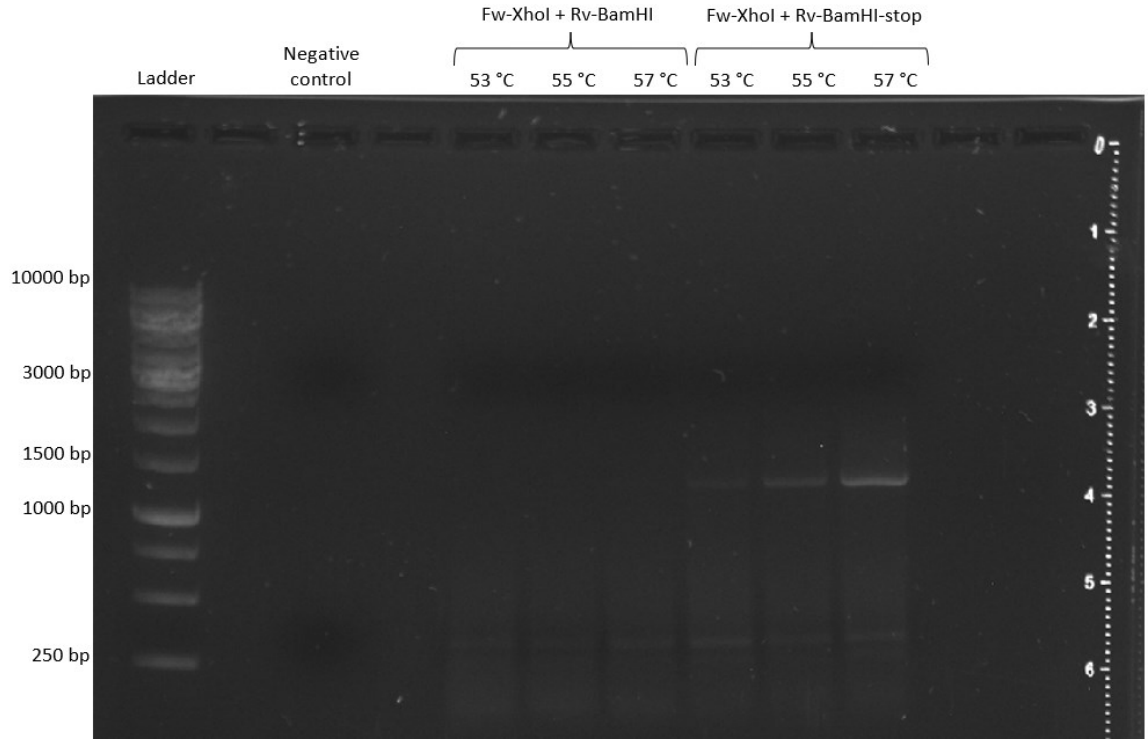

***Figure 15**: six PCR reactions (45 µl) containing primer couples Fw-XhoI+Rv-BamHI and Fw-XhoI+Rv-BamHI-stop at three annealing temperatures: 53, 55 and 57°C were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. The gpr150 insert is 1302 bp long and the plasmidic vector Tango without insert is 6632 bp long. A 10 kb plus ladder (Thermo Scientific) was used.*

different annealing temperatures: 53°C, 55°C and 57°C, for a total of six PCR reactions. The results reconfirmed the outcome of the first PCR attempt, showing successful

binding of the couple Fw-XhoI and Rv-BamHI-stop with different intensities according to the tested annealing T, while primer Rv-BamHI showed no binding (**Figure 15**).

Six further reverse primers without stop codon were designed for the cloning of the gpr150 sequence tagged with GFP to test their binding during PCR.

Reverse primer-BamHI 2: TGCTTAGGATCCATAGAAAGCTGACTCACAGGA

Reverse primer-BamHI 3: TGCTTAGGATCCATAGAAAGCTGACTCACATGA

Reverse primer-BamHI 4: TGCTTAGGATCCGTAGAAAGCTGACTCACAGGA

Reverse primer-BamHI 5: TGCTTAGGATCCAAAAGCTGACTCACAGGAA

Reverse primer-BamHI 6: TGCTTAGGATCCAAATGCTGATTCACATGAAC

Reverse primer-BamHI 7: TGCTTAGGATCCAAATGCTGATTCACATGAA

Each of these primers was tested in a PCR reaction coupled to Fw-XhoI and each couple was tested at the annealing temperature of 58°C. The results showed successful binding of primers Rv-BamHI2, Rv-BamHI3 and Rv-BamHI4 at 58°C and weak binding of Rv-BamHI6 at 58°C (**Figure 16**).

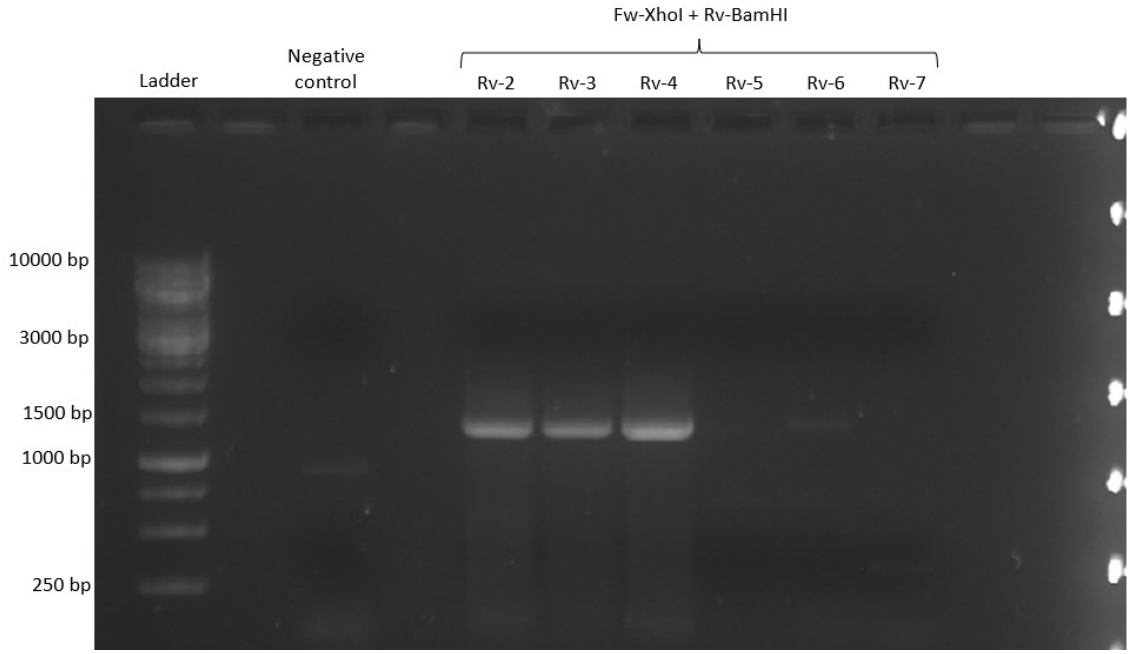

***Figure 16**: six PCR reactions (45 µl) containing primer couples of Fw-XhoI and reverse primers Rv-BamHI 2,3,4,5,6 and 7 at the annealing temperature of 58°C were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. The gpr150 insert is 1302 bp long and the plasmidic vector Tango without insert is 6632 bp long. A 10 kb plus ladder (Thermo Scientific) was used.*

Three PCR reactions with primers Fw-XhoI and Rv-BamHI4 were then performed at the annealing temperature of 58°C to obtain quantitative amounts of amplified gpr150 sequence for the following restriction digest step.

After observation of HEK293 cells with fluorescence microscopy following transfection with the GPR150-GFP tagged plasmid, a lack of fluorescence was appreciated, leading to the discovery of a frameshift in the sequence. The frameshift was caused by the introduction of one base pair between the receptor and the GFP sequence, given by the incorrect design of the reverse primer Rv-BamHI4. The primer was therefore redesigned by deleting the base pair that caused the frameshift, and the reverse primer Rv-BamHI8 was obtained. An additional PCR with primers Fw-XhoI and Rv-BamHI8 was run at four different annealing temperatures to clone the correct plasmid (**Figure 17**).

Reverse primer-BamHI 8: TGCTTAGGATCCTAGAAAGCTGACTCACAGG

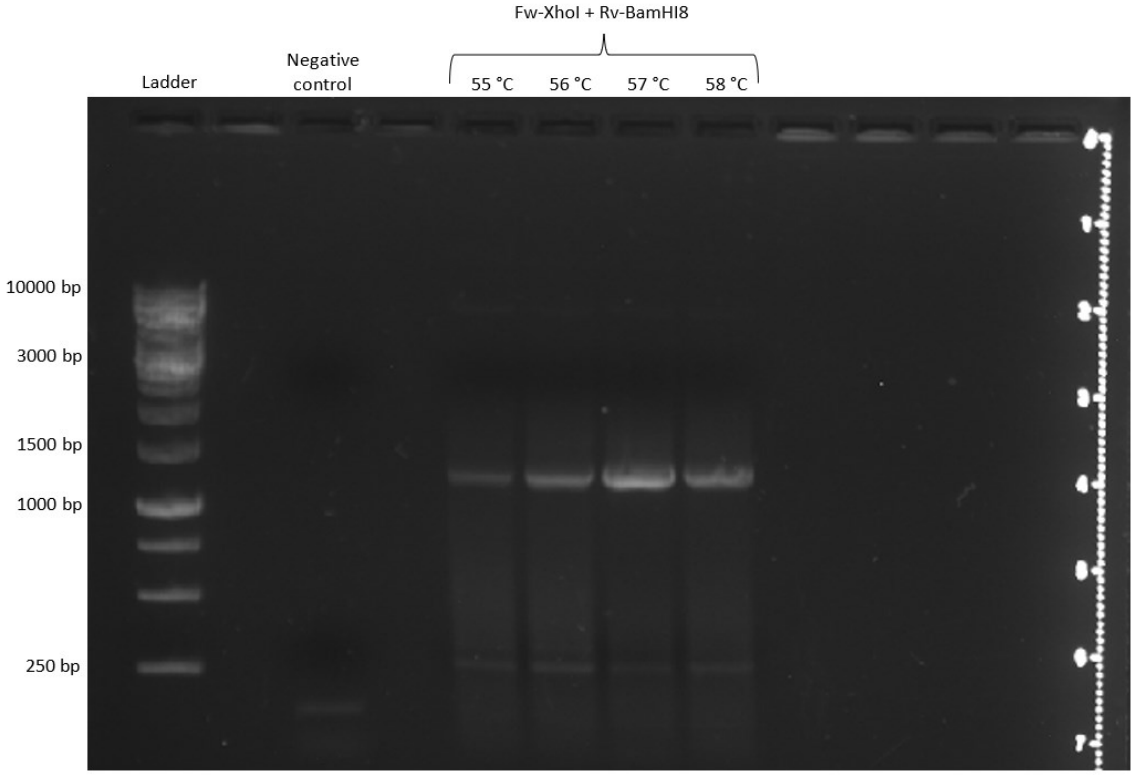

***Figure 17**: four PCR reactions (45 µl) containing primers Fw-XhoI and Rv-BamHI8 at the annealing temperatures of 55, 56, 57, 58°C were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. The gpr150 insert is 1302 bp long and the plasmidic vector Tango without insert is 6632 bp long. A 10 kb plus ladder (Thermo Scientific) was used.*

As shown in the following pages, the deletion of one base pair in Rv-BamHI4 to create Rv-BamHI8 led to the introduction of an undesired TAG stop codon in the cloned sequence, hence leading to a missense mutation and the consequent creation of an untagged receptor plasmid. The plasmid was therefore modified with site-directed

mutagenesis to delete the stop codon TAG and resolve the missense mutation. Subsequently, the plasmid coding for the GFP-tagged receptor GPR150 was created.

The restriction digest reaction was conducted with the cloned gpr150 sequence obtained from previous PCR reactions and purified from agarose gel, which was digested with restriction enzymes XhoI and BamHI. The host vector pEGFP-N1 was also digested with the same enzymes. Each reaction mixture was run on agarose gel to isolate the digested DNA. This step was repeated twice due to the presence of undigested pEGFP-N1 plasmid that, in the bacterial transformation step, led to the growth of colonies containing DNA coding for the empty plasmid without the GPR150 insert.

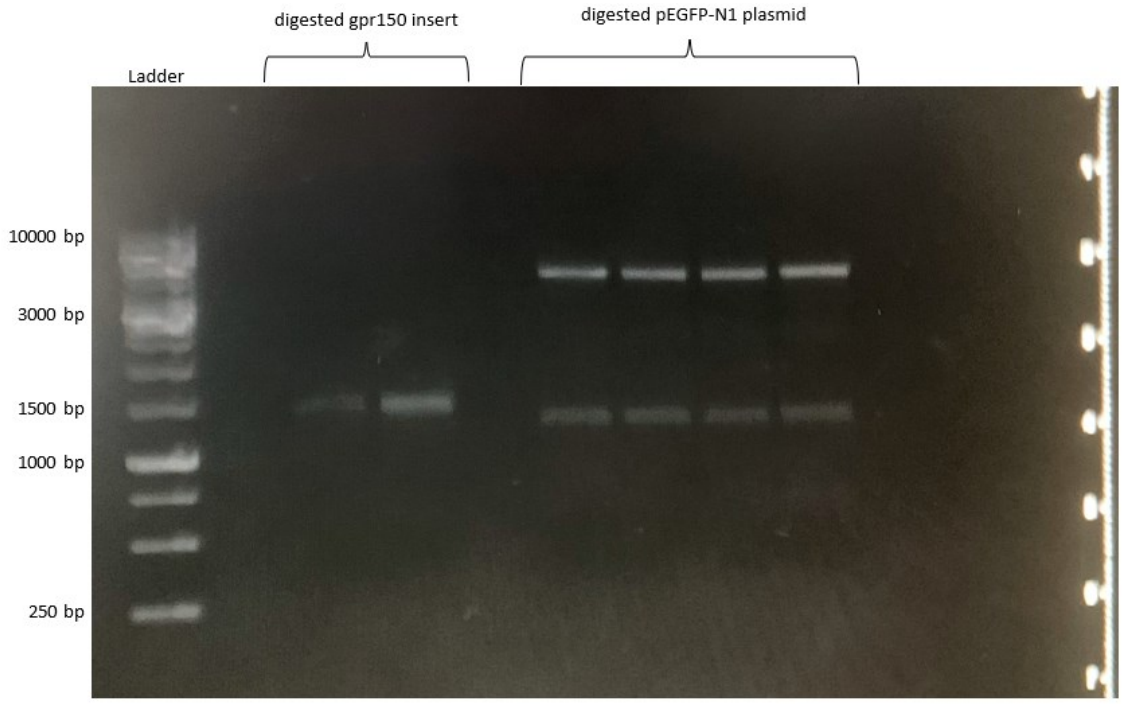

***Figure 18**: gel visualization of digested gpr150 insert without stop codon cloned with PCR (1302 bp) and of the digested pEGFP-N1 host vector (4692 bp) with restriction enzymes XhoI and BamHI. The restriction digest products (45 µl) were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. A 10 kb plus ladder (Thermo Scientific) was used.*

The band corresponding to undigested host vector has the same molecular weight as the undigested plasmid, their separation with electrophoresis on agarose gel is therefore inefficient because their correspondent bands co-migrate towards the positively charged cathode. This difficulty was overcome by lowering the starting quantity of pEGFP-N1 plasmid added to the restriction digest reaction (**Figures 18-19**).

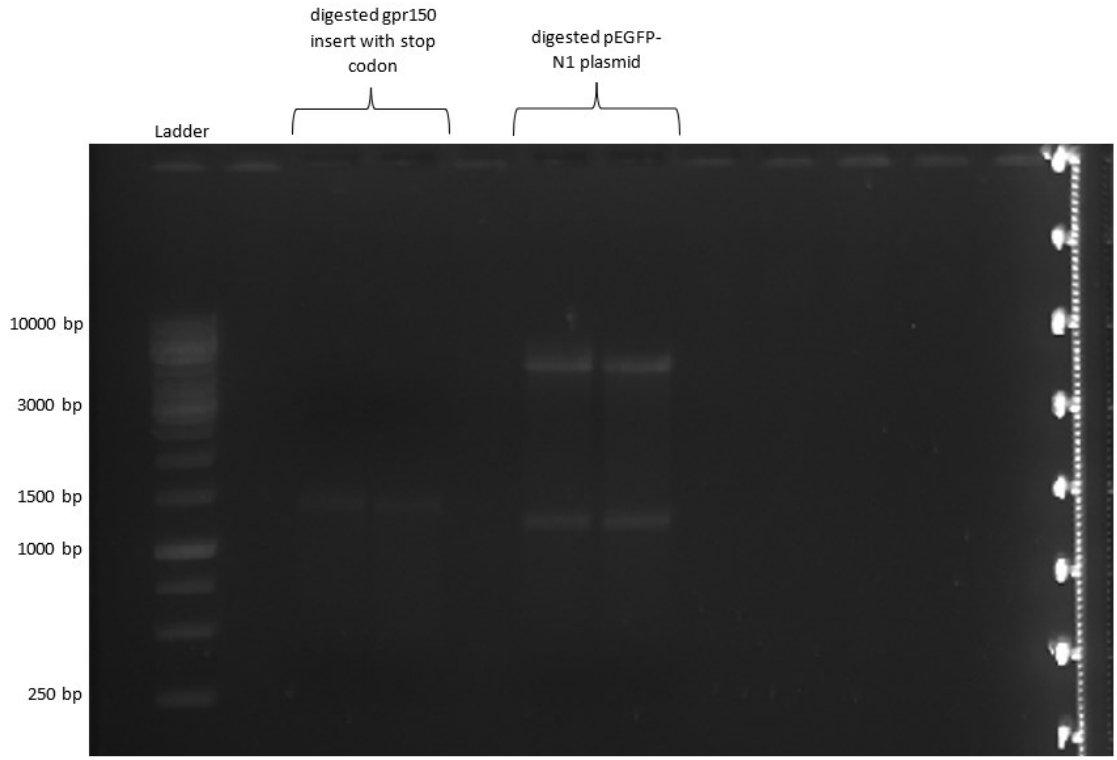

***Figure 19**: gel visualization of digested gpr150 insert with stop codon cloned with PCR (1305 bp) and of the digested pEGFP-N1 host vector (4692 bp) with restriction enzymes XhoI and BamHI. The restriction digest products (45 µl) were loaded onto a 1% agarose gel with 0.005% SafeView™ Classic DNA stain and run at 120 V and 150 mA for one hour. A 10 kb plus ladder (Thermo Scientific) was used.*

3.3 CONFIRMATION OF EXPRESSION OF GFP-TAGGED GPR150

The confirmation of successful cloning and expression of the receptor GPR150, both untagged and tagged with GFP, were verified with sequencing analyses provided by LGC Genomics. Expression of the GFP-tagged receptor was also confirmed after transfection in HEK cells and observation with fluorescence microscopy. This technique highlighted the absence of fluorescence after transfection in HEK293 cells caused by incorrect cloning of the first and second GPR150-GFP plasmids, caused respectively by a frameshift mutation and by a missense mutation in the sequence of the GFP-tagged plasmid. Cells transfected with the plasmid containing the frameshift mutation showed no fluorescence, while cells transfected with the missense-mutated plasmid showed poor fluorescence (**Figures 21 a-b**), which was very weak in comparison to control cells transfected with the VR2-GFP tagged plasmid (**Figure 20**). After site-directed mutagenesis performed on the incorrect plasmid containing the missense mutation, the stop codon was successfully deleted and cells revealed the expected fluorescence signal, confirming successful expression of the GFP-tagged receptor GPR150 (**Figures 21 c-d**).

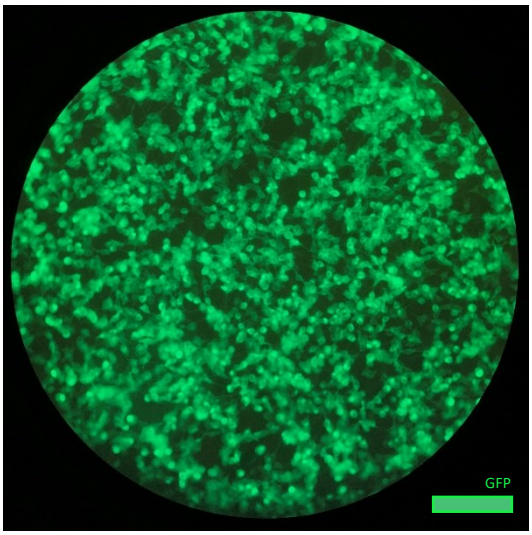

***Figure 20**: control HEK293 cells transfected with 2000 ng of V2R-GFP tagged receptor plasmid and incubated for 24 hours at 37°C and 5% CO_2. Fluorescence of the GFP-tagged V2R receptor was visualized with fluorescence microscopy.*

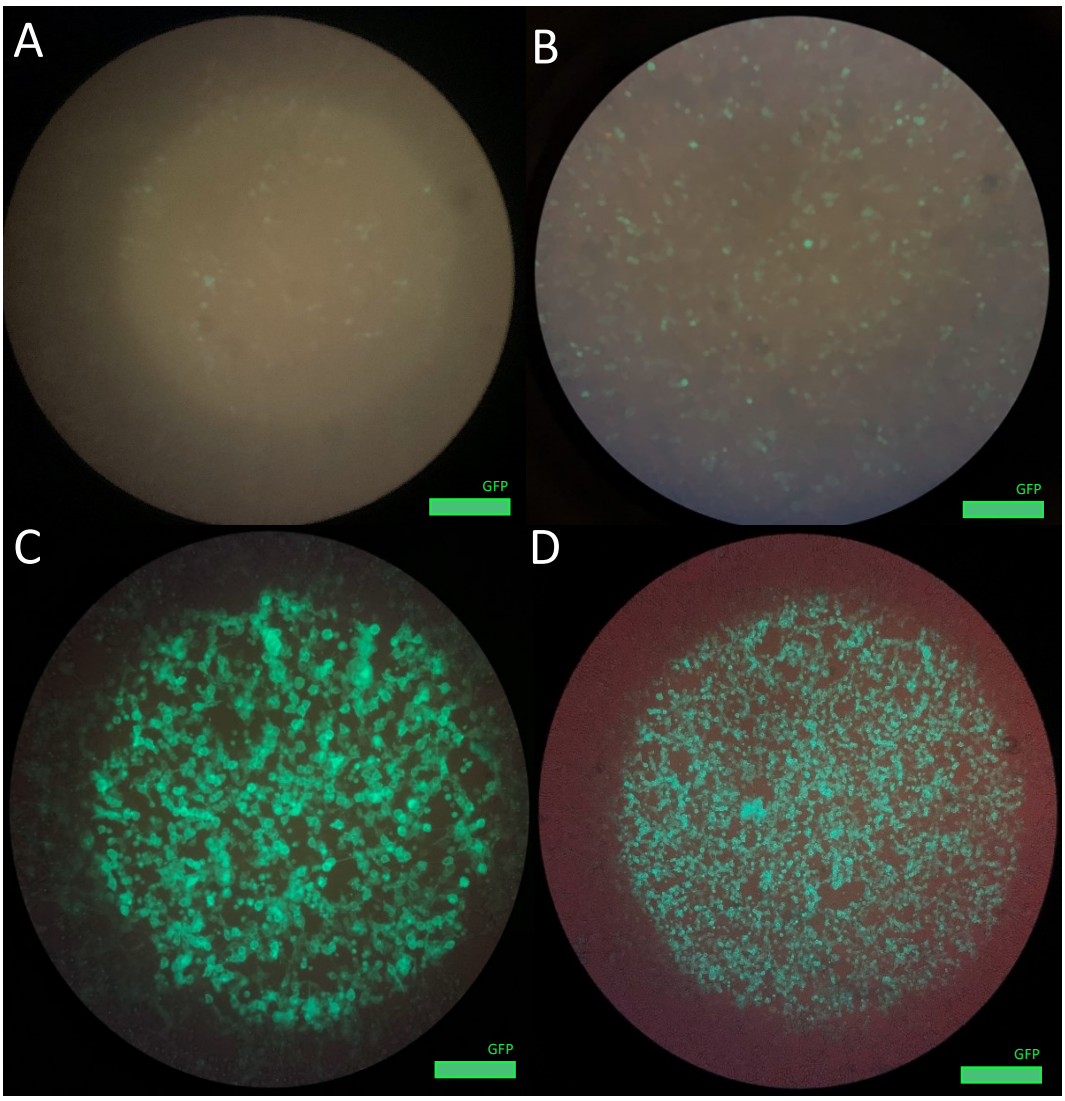

***Figure 21**: HEK293 cells transfected with 2000 ng of GPR150-GFP plasmid and incubated at 37°C and 5% CO_2 for 24 hours. Three different GPR150-GFP tagged plasmids were cloned, transfected into HEK cells and visualized with fluorescence microscopy to observe intensity of the GFP fluorescence and verify the expression of the GFP-tagged receptor. **Figure 21a:** HEK cells transfected with the frameshift-mutated GPR150-GFP tagged plasmid obtained from cloning with primers Fw-XhoI and Rv-BamHI4. **Figure 21b**: HEK cells transfected with the missense-mutated GPR150-GFP tagged plasmid obtained from cloning with primers Fw-XhoI and Rv-BamHI8. **Figures 21 c-d**: HEK cells transfected with the correct GPR150-GFP tagged plasmid obtained after site directed mutagenesis of the missense-mutated GPR150-GFP tagged plasmid.*

3.4 ACTIVATION OF G PROTEIN PATHWAYS AND RECRUITMENT OF β-ARRESTIN2 IN BRET ASSAYS

HEK293 cells were co-transfected with GPR150-containing plasmids, both GFP-tagged and untagged, and with G protein biosensors (Gs, Gi or Gq) or β-arrestin2 to highlight their possible recruitment to the receptor upon binding of different peptides, ranging from oxytocin, to vasopressin, to libraries of peptides derived from plant extracts. Each measurement is compared to cells treated with HBSS only. All experiments were performed in technical duplicates (n=2) or technical triplicates (n=3) and data is presented as mean values ± SD.

3.4.1 GPR150 UNTAGGED AND Gs, Gi AND Gq BIOSENSORS

The positive control for this experiment were HEK cells co-transfected with 2000 ng of V2R-untagged receptor and 2000 ng of Gs protein biosensor, recruited upon stimulation with AVP 10 µM, which confirmed activation of the receptor by revealing a decrease of about 0.05 RLU in BRET signal caused by dissociation of the Gα subunit from the Gβγ dimer (**Figure 22**). The recruitment of Gs, Gi and Gq proteins was investigated after transfection of equal amounts (2 µg) of GPR150-untagged receptor and G protein biosensors.

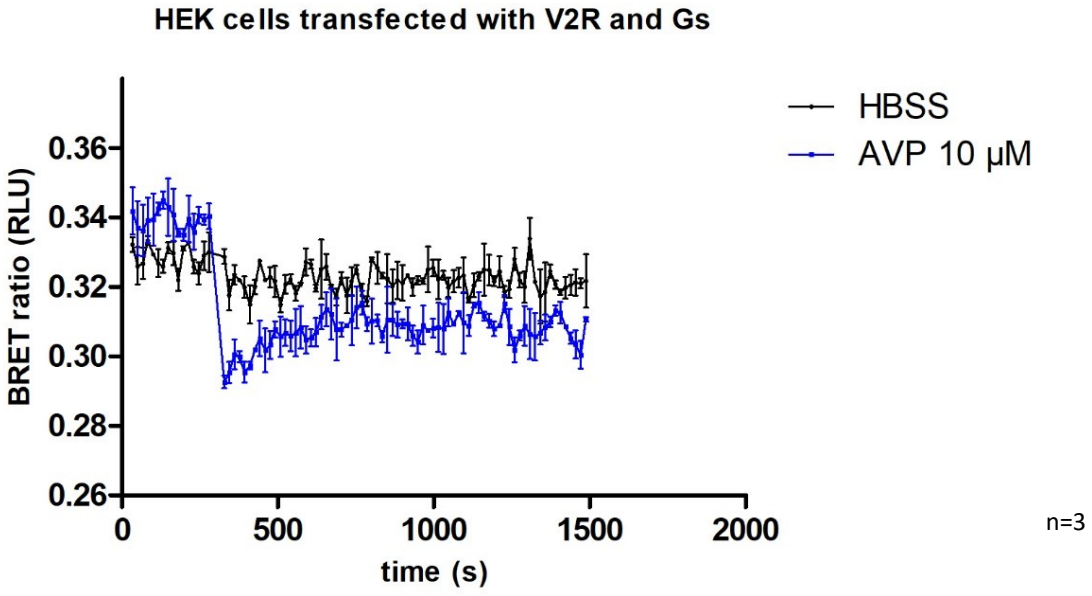

***Figure 22**: control HEK293 cells were co-transfected with 2000 ng of V2R-untagged receptor plasmid and 2000 ng of Gs biosensor. BRET assays were performed to measure the dissociation of the Gs trimer upon binding of the endogenous ligand AVP 10 µM. n=3.*

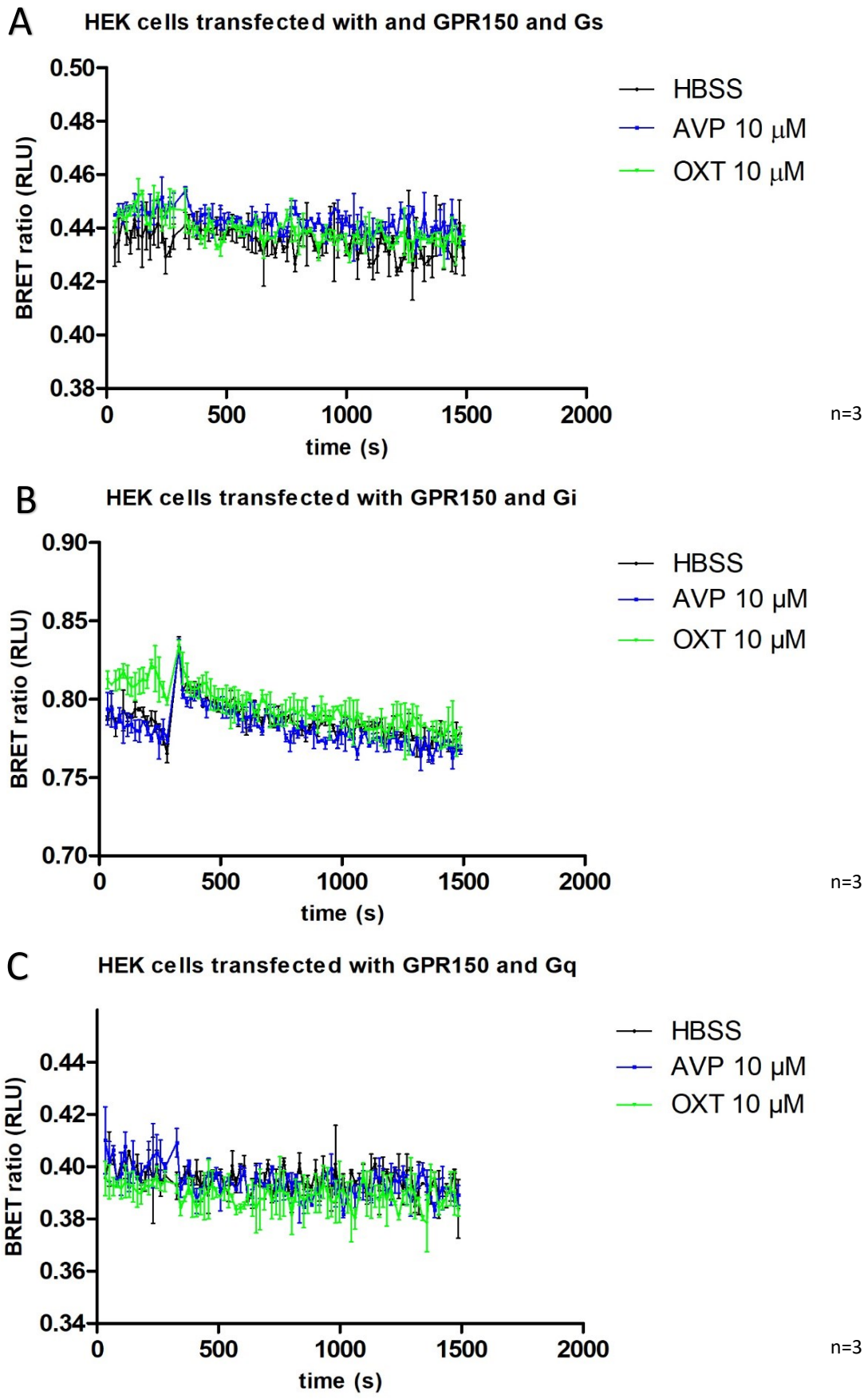

***Figure 23**: HEK293 cells were co-transfected with 2000 ng of GPR150 untagged pEGFP-N1 plasmid and 2000 ng of G protein biosensors. Graphics show BRET ratio variations linked to the dissociation of Gs (**A**), Gi (**B**) and Gq (**C**) proteins upon binding of AVP and OXT in the concentration of 10 µM in comparison to cells only treated with HBSS buffer. n=3.*

Cells were stimulated with AVP 10 µM and OXT 10 µM. Results showed no significant decrease in BRET ratio in comparison to cells stimulated with HBSS only (**Figure 23**).

3.4.2 GPR150-GFP TAGGED AND β-ARRESTIN2

The positive control for this experiment were HEK cells co-transfected with 1800 ng of OXTR-GFP tagged receptor and 200 ng of β-arrestin2, recruited upon stimulation with OXT 10 µM (full agonist) and AVP 10 µM (partial agonist). The experiment confirmed activation of the receptor by revealing a marked increase in BRET signal caused by the highly specific interaction between OXTR and its endogenous ligand OXT, causing recruitment of the bioluminescent β-arrestin2 tagged with nano-luciferase and the consequent energy transfer with the fluorescent GFP-tagged receptor (**Figure 24**). Vasopressin also activates OXTR with a less specific interaction compared to oxytocin. The BRET ratio is in fact lower than the one triggered by interaction with OXT, even though a strong increase is still recognizable. The increase in RLU produced by the physiological interaction of OXT and AVP with OXTR has a magnitude of 0.1 RLU.

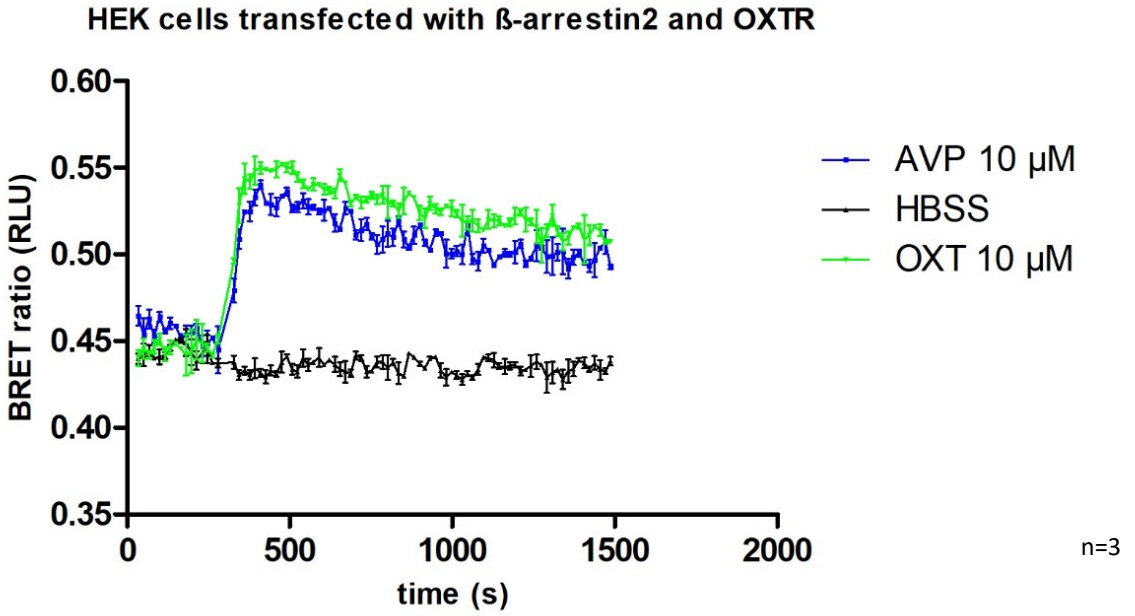

***Figure 24**: control HEK293 cells were co-transfected with 1800 ng of OXTR-GFP tagged receptor plasmid and 200 ng of ß-arrestin2 plasmid. BRET assays were performed to measure the recruitment of ß-arrestin2 upon binding of the endogenous ligand OXT and of the partial agonist AVP in the concentration of 10 µM. n=3.*

Subsequently, 1800 ng of GPR150-GFP tagged plasmid were co-transfected with 200 ng of β-arrestin2 in HEK293 cells. Stimulation was carried out with AVP 10 µM and OXT 10 µM, however no significant increase in BRET ratio was appreciated when compared to cells exposed to HBSS only (**Figure 25**).

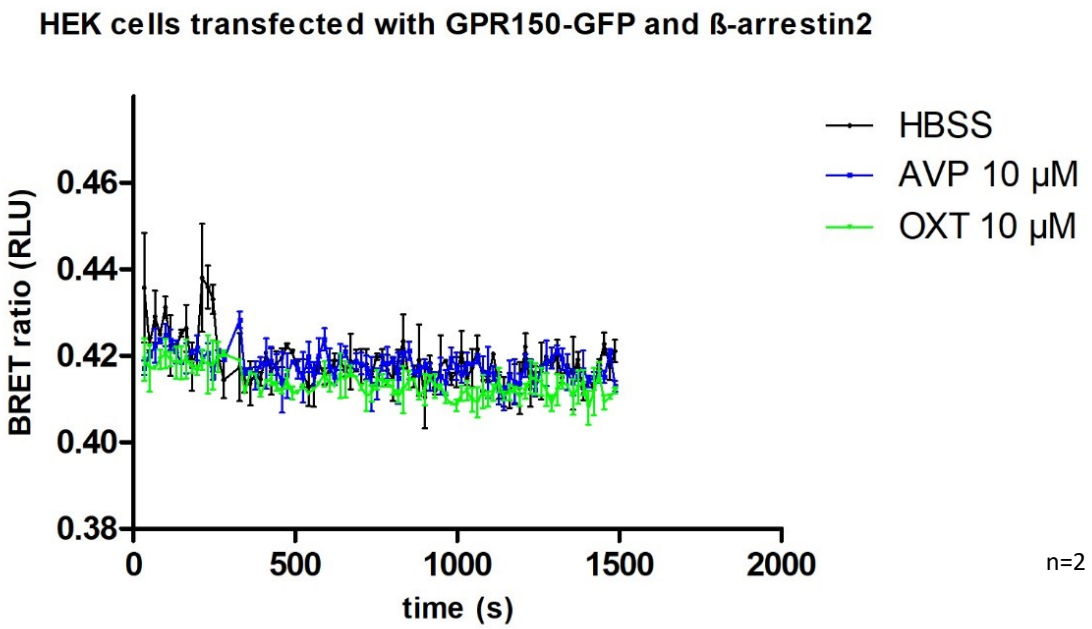

***Figure 25**: HEK293 cells were co-transfected with 1800 ng of GPR150-GFP tagged pEGFP-N1 plasmid and 200 ng of ß-arrestin2 plasmid. Graphics show BRET ratio variations linked to recruitment of ß-arrestin2 upon binding of AVP and OXT in the concentration of 10 µM in comparison to cells only treated with HBSS buffer. n=2.*

3.4.3 GPR150 AND PEPTIDE LIBRARIES FROM PLANT EXTRACTS

HEK cells were transfected with 1800 ng of GPR150-GFP tagged or 2000 ng GPR150 untagged receptor plasmids to test recruitment of β-arrestin2 or activation of Gs, Gi and Gq proteins, respectively. The activation of these pathways was investigated after stimulation with peptide libraries from *S. nigra, C. limon, B. alba, V. odorata, C. ipecacuanha, D. moroides, V. tricolor, M. charantia* and *P. poeppigiana* extracts, used as probes to test the activation of pharmacological pathways related to GPR150. Results showed no noteworthy variation of BRET ratio in comparison to cells only treated with HBSS, both for G protein dissociation and β-arrestin2 recruitment (**Figures 26, 27, 28, 29**). However, it can be observed that stimulation with peptide libraries from *V. tricolor, M. charantia* and *P. poeppigiana* extracts triggered a weak recruitment of β-arrestin (**Figure 29C**), with an increase in BRET ratio of about 0.02 RLU.

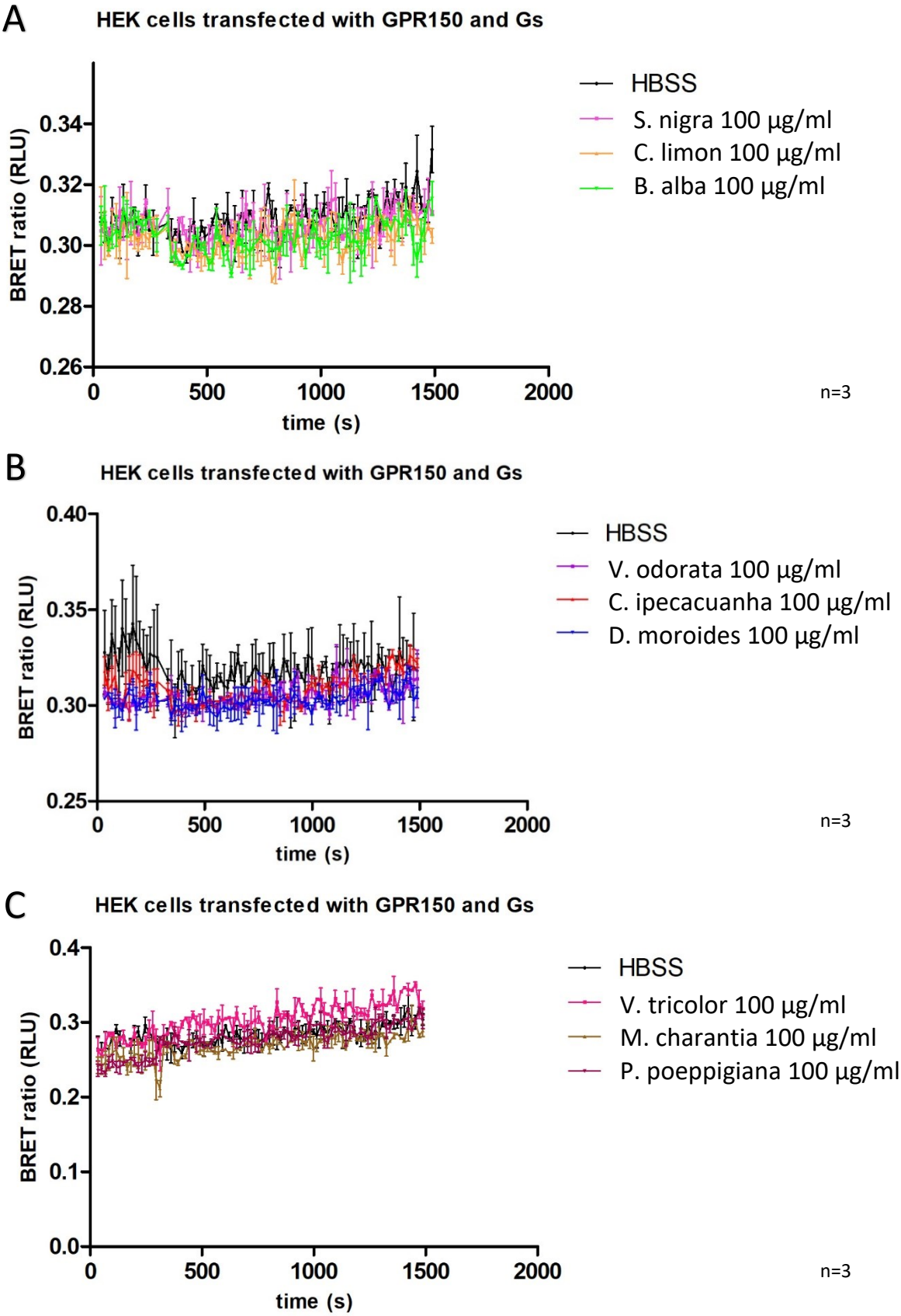

***Figure 26**: HEK293 cells were co-transfected with 2000 ng of GPR150 untagged pEGFP-N1 plasmid and 2000 ng of Gs biosensor. Graphics show BRET ratio variations linked to dissociation of the Gs trimer upon binding of peptides from different plant extracts in comparison to cells only treated with HBSS buffer. Plant extracts used to investigate activation of the Gs pathway are S. nigra, C. limon, B. alba (**A**), V. odorata, C. ipecacuanha, D. moroides (**B**), V. tricolor, M. charantia and P. poeppigiana (**C**) at a concentration of 100 µg/ml. n=3.*

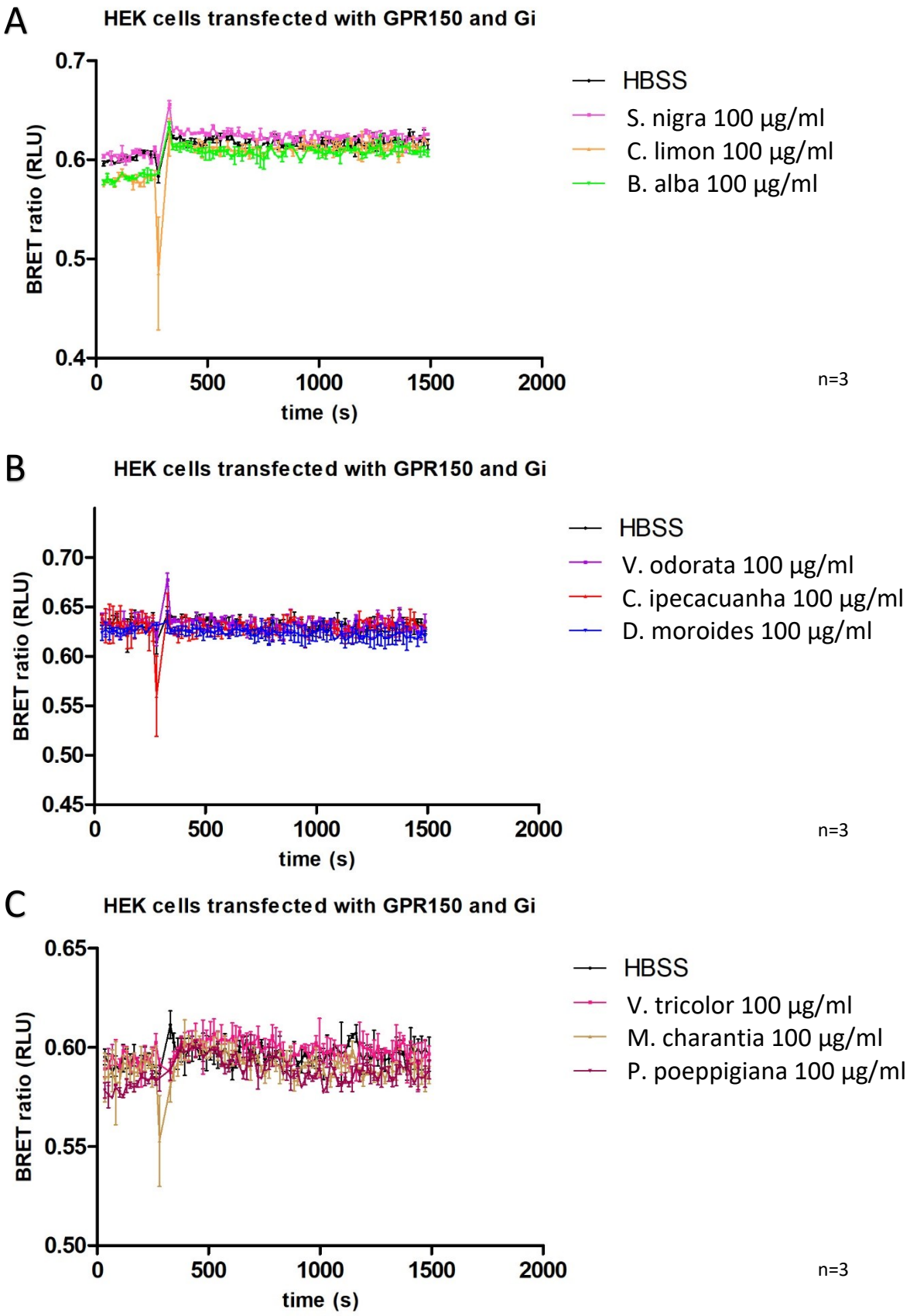

***Figure 27**: HEK293 cells were co-transfected with 2000 ng of GPR150 untagged pEGFP-N1 plasmid and 2000 ng of Gi biosensor. Graphics show BRET ratio variations linked to dissociation of the Gi trimer upon binding of peptides from different plant extracts in comparison to cells only treated with HBSS buffer. Plant extracts used to investigate activation of the Gi pathway are S. nigra, C. limon, B. alba (**A**), V. odorata, C. ipecacuanha, D. moroides (**B**), V. tricolor, M. charantia and P. poeppigiana (**C**) at a concentration of 100 µg/ml. n=3.*

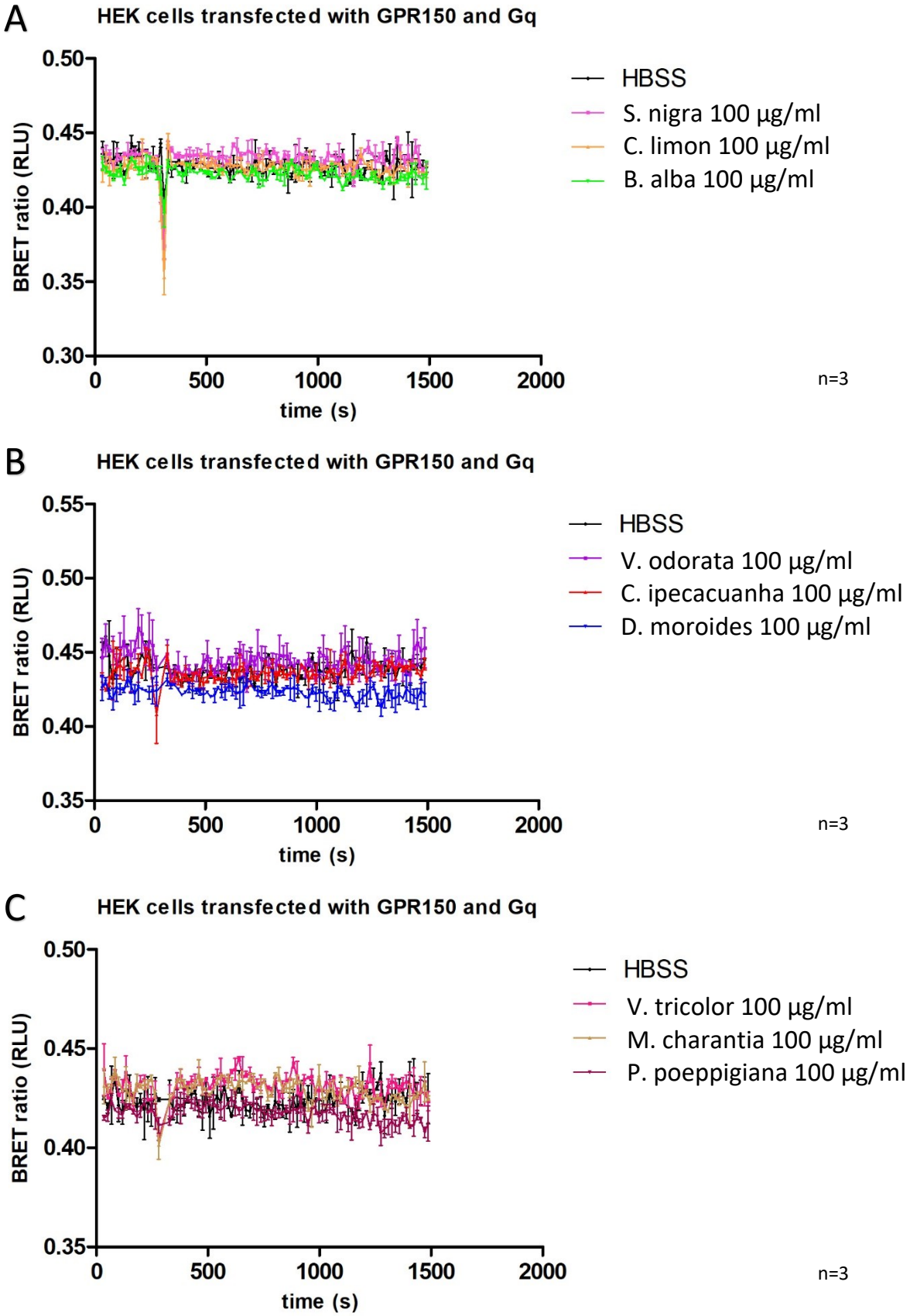

*Figure 29: HEK293 cells were co-transfected with 2000 ng of GPR150 untagged pEGFP-N1 plasmid and 2000 ng of Gq biosensor. Graphics show BRET ratio variations linked to dissociation of the Gq trimer upon binding of peptides from different plant extracts in comparison to cells only treated with HBSS buffer. Plant extracts used to investigate activation of the Gq pathway are S. nigra, C. limon, B. alba (**A**), V. odorata, C. ipecacuanha, D. moroides (**B**), V. tricolor, M.charantia and P. poeppigiana (**C**) at a concentration of 100 µg/ml. n=3.*

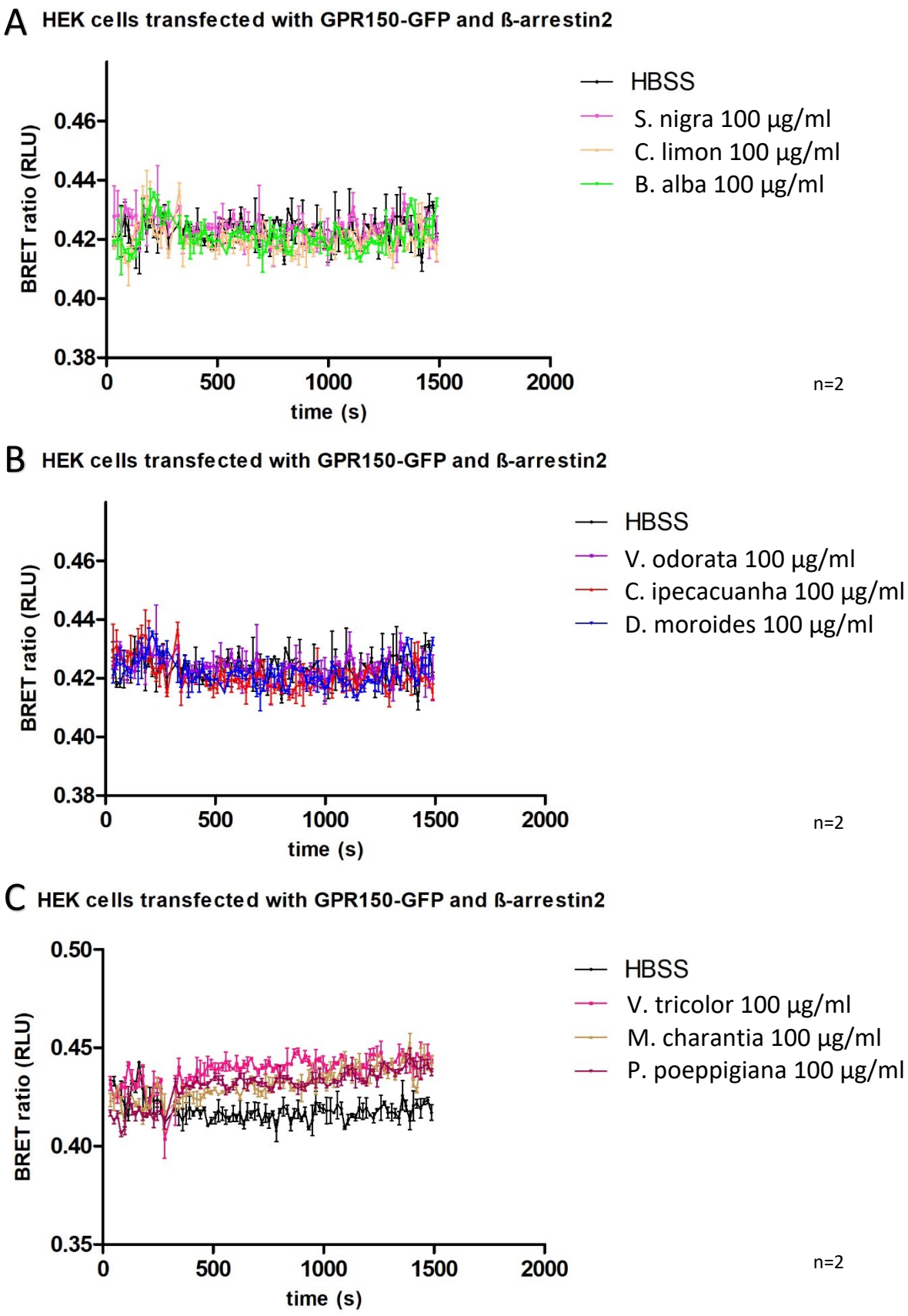

***Figure 29**: HEK293 cells were co-transfected with 1800 ng of GPR150-GFP tagged pEGFP-N1 plasmid and 200 ng of ß-arrestin2 plasmid. Graphics show BRET ratio variations linked to recruitment of ß-arrestin2 upon binding of peptides from different plant extracts in comparison to cells only treated with HBSS buffer. Plant extracts used to investigate activation of the ß-arrestin2 pathway are S. nigra, C. limon, B. alba (**A**), V. odorata, C. ipecacuanha, D. moroides (**B**), V. tricolor, M. charantia and P. poeppigiana (**C**) at a concentration of 100 µg/ml. n=2.*

3.5 CONSTITUTIVE ACTIVITY OF GPR150 WITH LUCIFERASE REPORTER GENE ASSAYS

The intrinsic activity of the receptor GPR150 within the Gi pathway was investigated by comparing luminescence produced by luciferase in cells stimulated with forskolin (FSK) 100 µM, which activates adenylate cyclase bypassing G protein dissociation, and cells stimulated with a mixture of FSK and pertussis toxin (PTX) 500 ng/ml, which inhibits Gi activity and could reveal an increase in luminescence in case of constitutive activity within the Gi pathway. A mixture of FSK and AVP or OXT in presence and absence of PTX was also tested to detect the possible partial or inverse agonism of these ligands when interacting with GPR150. For this experiment, HEK293 cells were co-transfected with equal amounts (2000 ng) of CRE plasmid tagged with nano-luciferase (nluc) and GPR150-GFP tagged plasmid. Considering the high amount of aspecific effects influencing this assay, fluorescence intensity of the transfected GFP-tagged receptor was used as input for the normalization of luminescence results according to the actual expression magnitude of the receptor. As positive control, HEK cells were co-transfected with 2000 ng of KOR-GFP tagged receptor and 2000 ng of CRE plasmid and stimulated with different mixtures of FSK containing the peptide dynorphin or PTX, or a combination of both, or none of them (**Figure 30**). Cells were lysed for 20 minutes before measurements to allow interaction between luciferase and its substrate luciferin. Luminescence data was normalized by fluorescence values and all experiments were performed in technical

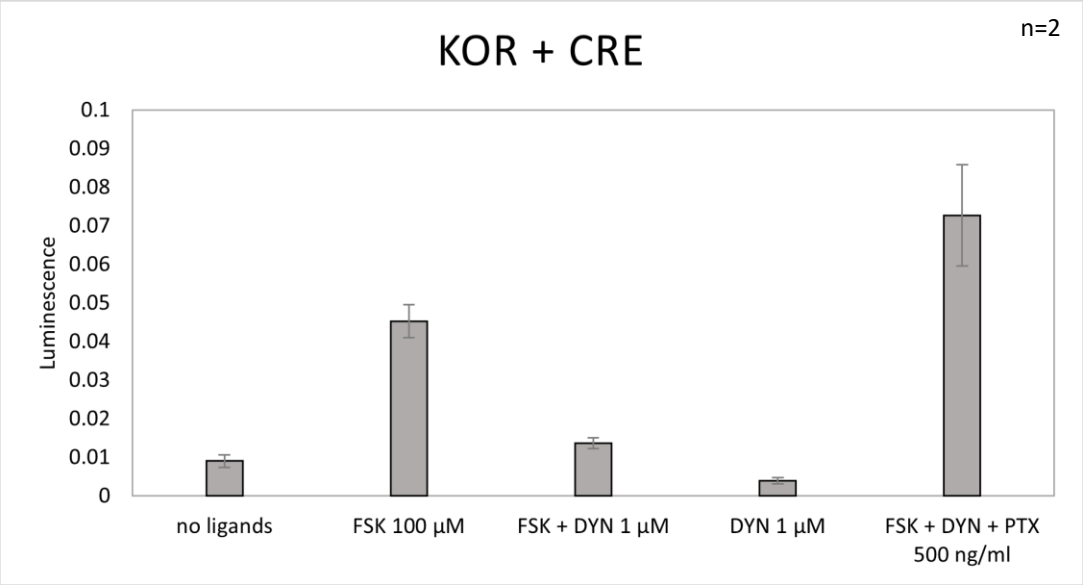

***Figure 30**: HEK cells were co-transfected with the KOR-GFP tagged plasmid and the nluc-CRE plasmid and stimulated with five different combinations of molecules: 1) unstimulated, 2) FSK 100 µM, 3) FSK + DYN 1 µM, 4) DYN 1 µM, 5) FSK + DYN +PTX 500 ng/ml. After stimulation luminescence was measured and compared between each condition. These measurements were used as positive control to compare luminescence values produced in the GPR150 signaling. n=2.*

duplicates (n=2). Data is presented as mean values ± SD. Control cells reconfirmed the expected behavior and expression of luciferase upon stimulation with different combinations: stimulation with FSK causes an increase in luminescence of 0.04 units and the addition of DYN activates the Gi pathway, causing decreased adenylate cyclase activity and lower luminescence (decrease of 0.035 units). After adding PTX, an even further increase of 0.03 units of luminescence can be noticed, compared to cells only treated with FSK.

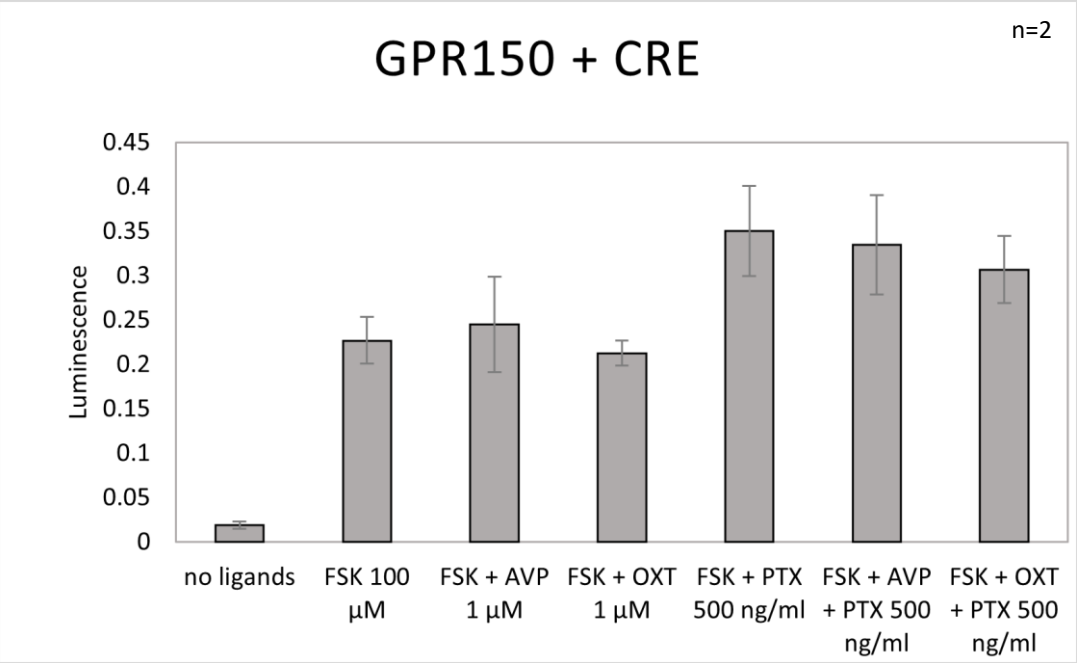

Figure 31: *HEK cells were co-transfected with the GPR150-GFP tagged plasmid and the nluc-CRE plasmid and stimulated with seven different combinations of molecules: 1) unstimulated, 2) FSK 100 µM, 3) FSK + AVP 1 µM, 4) FSK + OXT 1M, 5) FSK + PTX 500 ng/ml, 6) FSK + AVP + PTX 500 ng/ml, 7) FSK + OXT + PTX 500 ng/ml. After stimulation luminescence was measured and compared between each condition to evaluate possible constitutive activity and partial or inverse agonism of AVP and OXT. n=2.*

For cells transfected with GPR150 and CRE, results showed an increase of 0.125 units of luminescence in cells stimulated both with FSK and PTX, compared to cells only stimulated with FSK. This might indicate inhibition of the Gi protein caused by PTX, which would lead to an increase in CRE replication and subsequent increase in luminescence.

Cells stimulated with AVP and OXT in solution with FSK produced luminescence which intensity is comparable in cells only stimulated with FSK, while cells stimulated with FSK, AVP or OXT and PTX generally show higher luminescence. However, there's no indicative behavior of AVP or OXT as partial or inverse agonists, as no relevant difference can be

appreciated between cells treated only with FSK and cells treated with a mixture of FSK and AVP or OXT.

Comparison between cells only stimulated with FSK and PTX and cells stimulated with FSK, PTX and AVP or OXT show a slight decrease in luminescence, however this cannot be easily reconduced to a partial or inverse agonistic behavior of AVP and OXT on GPR150 (**Figure 31**).

4. DISCUSSION

The aim of this study was to explore the pharmacological features of the orphan receptor GPR150 to uncover more information about the signaling pathways that contribute to its activity. This was achieved by cloning plasmids with the sequence of GPR150 to be transfected in HEK cells and by conducting proper cell-based assays in order to highlight the behavior of this receptor after stimulation with different peptides, from vasopressin, to oxytocin, to libraries of peptides derived from plant extracts. Results showed no noteworthy activation of any of the investigated pathways among assays conducted with Gs, Gi and Gq biosensors and β-arrestin2, although BRET ratios detected after stimulation of GPR150 with peptide libraries from *V. tricolor, M. charantia and P. poeppigiana* extracts suggested that there might be low recruitment of β-arrestin2 triggered by their non-specific interaction with the receptor. The absence of activation of GPR150 upon treatment with different peptides pushed this research towards the hypothesis of constitutive activity of GPR150, based on previous studies that proved GPR150 to be intrinsically active in the Gi pathway. This was examined through luciferase reporter gene assays, and results reconfirmed the possibility that GPR150 might be constitutively active.

The starting point of this project was the alignment between amino acid sequences of GPR150 and V1aR, V1bR, V2R and OXTR. A study from 2017 identified 43 residues directly involved in the activation and interaction of AVP and OXT receptors with their putative ligands [73]. The alignment carried out in the present study showed complete homology for 55% of residues, while alignment of the same residues between GPR150 and OXT and AVP receptors only revealed homology of 21%, which is in line with results of the full sequence alignment obtained in previous phylogenetic research conducted on orphan GPCRs [42]. Although this finding supports the classification of GPR150 as part of the vasopressin-like subfamily of GPCRs [38], the degree of identity between residues involved in the interaction with their putative ligands suggests that OXT and AVP might not be the endogenous ligands that physiologically bind to this receptor. The physico-chemical properties linked to the interaction of peptide and receptor are directly related to the sidechains of amino acids that must occupy specific positions in the seven transmembrane helices in order to exert their role in the activation of the receptor [74].

This principle is particularly relevant, especially when considering some residues identified as essential for high-affinity agonist binding and receptor activation, which proved to be highly conserved throughout the neurohypophyseal hormone receptor subfamily of GPCRs. These residues are 45 x 49 and 45 x 50, and are located in the second extracellular loop of GPCRs (ECL2) [75]. These two residues are the most conserved in ECL2 among different species and they all show complete homology between vasopressin and oxytocin receptors. When compared to GPR150, residue 45 x 49 presents a proline molecule, whereas V1aR, V1bR, V2R and OXTR show an aspartate residue. Residue 45 x 50, however, presents a cysteine molecule in all receptors. 45 x 50 is in fact, by definition of the Ballesteros Weinstein nomenclature, the most conserved residue in ECL2 among different species and is almost always a cysteine residue, even in functionally different receptors (e.g. small molecule GPCRs as β-ARs and peptide GPCRs as OXTR) [76]. This suggests that GPR150 does not present sufficient homology to confidently suggest GPR150 as putative receptor for vasopressin or oxytocin, at least from the alignment viewpoint. Keeping these observations into account, the following step was to verify whether AVP or OXT would trigger any activation of GPR150, even through non-specific interactions, despite the fact that low homology of the orphan receptor with vasopressin and oytocin receptors suggested equally low chances of affinity between their putative ligands and GPR150.

All assays of this research project were performed on HEK293 cells transfected with plasmidic DNA containing the sequence of the receptor GPR150. This DNA was obtained after molecular cloning of the sequence from the Tango plasmid into the pEGFP-N1 plasmid. This step requires the design of proper cloning primers for the PCR cloning reaction. A forward primer and two different reverse primers were designed for the cloning of two different DNA sequences: one coding for the GPR150 receptor tagged with the GFP protein and one for the untagged receptor. The forward primer was the same for both sequences, while reverse primers differed for a three base pair stop codon, which was only contained in the reverse primer designed for the cloning of the receptor without the GFP protein. Different attempts were made to obtain correctly functioning plasmids, especially for the plasmid coding for the GFP-tagged receptor. The untagged receptor plasmid was obtained after various PCR attempts at different annealing temperatures, to which the binding of both primers to their complementary

sequence is very sensitive. Some difficulties in the cloning process were also encountered in the restriction digest step, where different quantities of the pEGFP-N1 plasmid were tested to avoid undigested pEGFP-N1 plasmid, which, in the first attempts, resulted in the growth of colonies containing the pEGFP-N1 plasmid without the GPR150 insert. For this reason, different quantities of pEGFP-N1 host vector were tested before successful transformation of XL-10 *E. Coli* competent cells containing the correct plasmid. The plasmid containing the GPR150 sequence tagged with GFP (derived from the cloning reaction with the reverse primer without stop codon) was obtained after several attempts of primer design and PCR cloning. While the first designed forward primer showed successful binding since the first PCR attempts, many reverse primers were designed and tested to accomplish successful binding to the sequence of gpr150. After purification of cloned plasmids, their sequence was verified with DNA plasmid sequencing to validate the outcome of the cloning procedure. Results of sequencing analyses conducted on plasmids obtained by cloning with two different reverse primers without stop codon revealed in one case a frameshift mutation caused by the insertion of one base pair between the GPR150 sequence and the GFP sequence, introduced after incorrect design of the reverse primer. In the second case, the first reverse primer was modified by deleting one base pair to avoid the previously introduced frameshift. The deletion of this one base pair from the reverse primer led to the creation of an unwanted stop codon between the insert and the GFP sequence, thus introducing a missense mutation and leading to the creation of a plasmid coding for the untagged receptor. The cloning of incorrect plasmids was also acknowledged after transfection of these incorrectly cloned plasmids into HEK293 cells and visualization with fluorescence microscopy, which showed no expression of GFP in cells transfected with the plasmid containing the frameshift mutation, while cells transfected with the untagged plasmid generated by the missense mutation showed a weak fluorescence signal in comparison to control cells, which were transfected with a GFP-tagged V2R plasmid. The correct DNA sequence was obtained by performing site directed mutagenesis as a PCR method to mutate one base pair in the stop codon between the insert and GFP, using the missense-mutated plasmid as template DNA. Expression of the GFP-tagged receptor was confirmed with sequencing, transfection in HEK293 cells and visualization with fluorescence microscopy. Different attempts at PCR, restriction digest and bacterial

transformation significantly delayed the experiments scheduled in the workflow of this project, leading to multiple repetitions of assays that were first conducted on cells transfected with incorrect plasmids, which produced invalid results. After completion of the molecular cloning process and successful transient expression of GPR150, cells were finally ready to be stimulated with different ligands to test the activation of different signaling pathways.

BRET assays were performed to verify whether stimulation with vasopressin, oxytocin and libraries of peptides derived from different plant extracts was able to exert any activation of GPR150 expressed in HEK cells. The signaling pathways investigated were Gs, Gi, Gq, and β-arrestin2, and each of these were tested upon stimulation with the abovementioned peptides. Overall, results showed no significant difference in BRET signal between cells treated with ligands and unstimulated cells. When testing activation of G protein pathways, a decrease in BRET ratio with respect to cells only treated with HBSS was expected upon binding with the peptide. On the contrary, recruitment of β-arrestin2 would show an increase in BRET ratio, as seen in the positive control represented by cells transfected with OXTR-GFP and activated with the endogenous ligands OXT (full agonist) and AVP (partial agonist). In quantitative terms, physiological interaction between OXTR and OXT or AVP specifically triggered the recruitment of β-arrestin2, translating into a BRET ratio increase of 0.1 RLU in comparison to cells treated with HBSS only. In general, cells stimulated with peptides showed BRET ratios comparable to the signal of cells only treated with HBSS, suggesting no peculiar interaction between GPR150 and OXT and AVP in any of the investigated pathways. When testing recruitment of β-arrestin2 upon screening with peptides derived from *V. tricolor, M. charantia* and *P. poeppigiana* extracts, however, a weak increase in BRET ratio was appreciated in comparison to signal of cells treated only with HBSS. In contrast to control cells expressing OXTR, the recruitment of β-arrestin2 produced an increase in BRET ratio of about 0.02 RLU in cells expressing GPR150. Such a mild increase in comparison to positive control cells cannot be considered as indicative of a specific interaction between peptides from the abovementioned plant libraries and GPR150, and it should only be reconducted to a low non-specific recruitment of β-arrestin2. A verification of this non-specific recruitment should be further investigated in future research on the pharmacology of GPR150. A possible approach would be to

repeat these assays to reconfirm the signal increase and to define the precise variation of BRET ratio produced by stimulation with each plant extract. Upon reaffirmation of activation, isolation of single peptides from each plant extract would be beneficial for the repetition of these BRET assays to attribute the recruitment of β-arrestin2 to precise compounds from the screened libraries. Successful isolation would allow us to study the specific structure of each peptide and recognize which features of these compounds establish an interaction with GPR150. This would help in the study of the binding pockets involved in the interaction with the isolated peptides.

When observing BRET signal curves produced by G protein biosensors, a marked background noise makes interpretation of results slightly more difficult, especially in the case of the Gs biosensor. For this reason, HEK cells were transfected with the V2R untagged plasmid and the Gs biosensor and stimulated with AVP to act as positive control in the comparison with results obtained from Gs biosensor assays. Although signal from positive control presents high background noise as well, a decrease in BRET ratio of about 0.05 RLU can still be clearly distinguished upon activation with the endogenous ligand AVP. This acts as a useful basis for comparison when observing BRET signals produced by Gs dissociation, which, despite high background noise, don't show any difference from HBSS only signals. Overall, Gi and Gq biosensors showed the most reliable results in terms of background noise, which was much lower than that produced in Gs assays. Considering these limitations, a higher number of measurements for each assay would have been desirable to confidently confirm that none of the investigated pathways are activated upon stimulation with AVP and OXT, even though present results don't suggest the opposite. The starting question of this research was based on the classification of the orphan receptor GPR150 as part of the vasopressin-like subfamily of GPCRs, and BRET assays were the first method chosen to answer this question. As results did not show any activation of GPR150-linked pathways upon stimulation with OXT, AVP and screening with peptide libraries, a further hypothesis was formulated, suggested by already existing literature that proved GPR150 to be constitutively active in the Gi pathway. This evidence was therefore explored through luciferase reporter gene assays.

Reporter gene assays were performed on HEK293 cells transfected with the GPR150-GFP plasmid and the nluc-CRE plasmid. A study from 2015 investigated constitutive activity of orphan GPCRs and revealed spontaneous dissociation of the Gi protein in CHO cells, where expression of GPR150 was induced [47]. This led us to set up an experiment to verify this finding by reproducing this luciferase reporter gene assay on HEK cells. In order to detect any intrinsic activity of Gi linked to GPR150, we had to consider the downstream effects of the activation of this pathway. Both Gi and Gs signaling begin with the dissociation of Gα from the Gβγ dimer. Gαi then inhibits adenylyl cyclase, which role is to promote cyclization from ATP into cAMP. The decrease of cytosolic cAMP levels lowers the activity of PKA, and the phosphorylation of CREB catalytic units decreases. The dissociation of CREB triggered by phosphorylation would cause migration into the nucleus and transcription of CRE sequences contained in the DNA. To sum up, Gi dissociation causes a decrease in the transcription of CRE sequences. This downstream effect is the ideal readout to detect any constitutive activation of the Gi pathway linked to GPR150. By transfecting cells with a plasmid containing CRE sequences tagged with nano-luciferase, it was possible to notice an increase or decrease of CRE transcription by detecting the respective increase or decrease in luminescence signal produced by the translated nano-luciferase. Constitutive activity, however, is always present in the cell and it was necessary to find an appropriate term of comparison to detect it. This was possible by using forskolin (FSK), which is a molecule that can activate adenylyl cyclase even without Gs or Gi dissociation. The independent activation of adenylyl cyclase triggered the downstream transcription of the CRE sequence and nano-luciferase, leading to an increase in luminescence. To create a comparison, we exploited the activity of pertussis toxin (PTX), which inhibits Gi activity. If Gi is intrinsically active, cells stimulated only with FSK should show less luminescence in comparison to cells treated with a mixture of FSK and PTX, since inhibition of Gi would enhance adenylyl cyclase's activity even more, in addition to activity already induced by FSK. As observed in bar diagrams showed at point 3.6, this expectation was reconfirmed, as cells stimulated only with FSK showed less luminescence than cells treated with both FSK and PTX. This suggests that PTX inhibited Gi, causing annulation of inhibition of adenylyl cyclase and leading to increased transcription of CRE and nano-luciferase sequences. In addition, cells were also treated with AVP and OXT in solution with FSK and with PTX. These

measurements were carried out to detect whether these peptides exerted any effect on the Gi pathway as partial agonists or inverse agonists. Luminescence was compared between cells treated with FSK + peptide and cells treated with FSK + peptide + PTX, and results showed a mild increase in luminescence after treatment with both AVP and OXT. This can be attributed to inhibitory activity of PTX on Gi and reinforces the hypothesis of constitutive activity, regardless of stimulation with AVP and OXT. A comparison between cells treated with FSK only and cells treated with AVP or OXT in solution with FSK, however, showed variation of luminescence, which could highlight the possible modulation of constitutive activity triggered by binding of these peptides. Cells treated with FSK + AVP manifested an increase in luminescence, while cells treated with FSK + OXT showed a decrease. This weak difference, however, doesn't necessarily indicate interaction between peptides and GPR150, as standard deviation values for each measurement make luminescence signals closely comparable to one another. For this reason, these results cannot confirm the hypothesis of AVP or OXT as partial or inverse agonists for GPR150.

A positive control was set up for this assay to establish a term of comparison with the variation of luminescence in GPR150 assays. HEK cells were transfected with a KOR-GFP plasmid and with a nluc-CRE plasmid. Cells were stimulated with FSK, FSK + DYN, DYN and FSK + DYN + PTX. The endogenous ligand of KOR is dynorphin, which physiologically triggers dissociation of the Gi protein and activation of the relative pathway. As expected, cells treated with FSK and DYN showed a decrease in luminescence given by dissociation of Gi and decrease of adenylyl cyclase activity. Cells treated with FSK + DYN + PTX showed higher luminescence than cells treated with FSK only, confirming the expected inhibition of Gi caused by PTX.

The expression of receptors was induced in HEK cells after transfection with GFP tagged plasmids. GFP fluorescence was measured in each sample after stimulation with different combinations of ligands, and data was used to normalize luminescence values for the degree of expression of the receptors. This was done to minimize incidence of aspecific effects on results, as this assay is subjected to multiple factors that can influence measurements. The difference in absolute luminescence values between positive control and cells transfected with GPR150 is therefore connected to different

GFP intensities in HEK cells transfected with KOR or GPR150 plasmids, as expression of different receptors directly depends on efficacy of transfection, which can vary from one plasmid to another.

All in all, a definite increase in luminescence can be distinguished after the treatment of cells with PTX in comparison to cells only treated with FSK, leading to clear evidence of constitutive activity of Gi in relation to the receptor GPR150.

Some technical shortcomings of this project were caused by delays in the workflow, given by the difficulties encountered in the molecular cloning process, due to multiple failed attempts at the correct reproduction of GPR150-containing plasmids. This represented a significant setback for the completion of BRET and reporter gene assays, which would have required more repetitions of measurements for an adequate interpretation of results and to give a clear statement regarding the main question of this research, given the impact of high variability between each repetition of same measurements. Such complications also caused us to give up further experiments, which would have contributed to the definition of the pharmacology of GPR150. These results, however, should be considered as a starting point to address further research on the characterization of GPR150. There are a few strategies that could be exploited for the study of GPR150 which were successfully used in past research for the deorphanization of orphan GPCRs [36]. Some of these strategies are not applicable for GPR150, i.e. the function similarity strategy that led to deorphanization of LGR4 and LGR5 receptors [77], since the function of GPR150 is still unknown. A valid approach for the aim of characterizing GPR150 is based on the "reverse pharmacology". This strategy is based on the stimulation with different peptides of tissue extracts that are known to certainly express GPR150. The recognition of activation patterns would indicate affinity between the receptor and a specific ligand, giving useful insight into which features characterize the signaling of the orphan receptor in question. This strategy led to the deorphanization of many hormone receptors, from orexin, to apelin, to nociptin receptors [78] [79] [80], and it constitutes a promising step towards an accurate characterization of GPR150. Finally, a valuable tool for the study of GPR150 is presented by *in-silico* methods and AI-supported systems (e.g. AlphaFold) that can help with predictions of the receptor's structure and with simulations of its interaction with different candidate ligands [81].

5. CONCLUSION

The aim of this research was to uncover the pharmacological pathways involved in the signaling of the orphan receptor GPR150. BRET assays were performed to investigate the activation of Gs, Gi, Gq and β-arrestin2 pathways upon stimulation with vasopressin and oxytocin, but no significant activity was observed. Peptide libraries derived from plant extracts were also screened against GPR150, and exposure to peptides from *V. tricolor, M. charantia* and *P. poeppigiana* extracts showed weak recruitment of β-arrestin2, probably triggered by non-specific interactions. Reporter gene assays were then carried out to detect possible intrinsic activity of the receptor linked to the Gi pathway, based on findings from existing literature. Results reported higher luminescence in cells stimulated with PTX, which inhibits Gi protein. This suggests that GPR150 has a high basal activity and interacts with Gi to suppress expression of CRE genes, which leads to the confirmation of constitutive activity of GPR150.

Starting from evidence that shows the silencing of the gpr150 gene as a contributing factor in the progression of ovarian cancer, we can suggest that basal activity of Gi linked to GPR150 might be crucial to the suppression of pro-carcinogenic sequences contained in the DNA. Therefore, further steps need to be taken towards a more certain identification of the mechanisms involved in the signaling of GPR150, in order to define the exact role of this receptor. Furthermore, a precise analysis of the expression patterns of GPR150 among different cells and tissues is paramount to the correct evaluation of its possible involvement in regulatory mechanisms of carcinogenic genes, as it would lead to fundamental progress in the development of new therapeutics against pathologies caused by impaired functioning of GPR150. The “reverse pharmacology” strategy constitutes a valid method for the deorphanization of GPR150, and a further step towards the identification of its endogenous ligand, given that there is one, would profit from *in-silico* instruments that could predict the structural features of GPR150 and its probable interactions with candidate ligands. These strategies altogether represent a strong approach towards a more accurate characterization of GPR150, which is worth studying in future research.

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