PhD Thesis

Trafficking and Signaling of the G protein-coupled receptor 55

submitted by

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Wer nichts weiß, muss alles glauben (Science Busters).

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Graz, 23.01.2012 …….. ……

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Content

Abstract

The endocannabinoid system consists of endogenous cannabinoids, two seven transmembrane spanning / G protein-coupled receptors (7TM/GPCRs), the cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors, as well as enzymes synthesizing and degrading endocannabinoids. Cannabinoids are compounds that can (i) be extracted form *Cannabis sativa*, (ii) are endogenously expressed mediators or (iii) are synthetic drugs, all of which display high affinity for cannabinoid receptors. Cannabinoids play an important role in medicine due to their psychoactive, analgesic and anti-inflammatory properties. Recently, the G protein-coupled receptor 55 (GPR55) was characterized as a novel receptor for synthetic cannabinoids and small lipid mediators. Besides being activated by the endogenous lipid ligand L-α-lysophosphatidylinositol (LPI), several synthetic CB1 inverse agonists/antagonists, such as AM251, AM281 and rimonabant (SR141716A), have been shown to activate GPR55. Rimonabant has further attracted attention since it was marketed to induce weight loss and reduce smoking. However, due to severe side effects, such as the development of anxiety and depression, rimonabant was withdrawn from the market.

In my thesis I elucidate the post-endocytic fate of GPR55 *in vitro* and *in vivo*. One regulatory mechanism to guarantee appropriate 7TM/GPCR expression levels in physiological conditions is that of downregulating 7TM/GPCRs via the GPCR-associated sorting protein 1 (GASP-1), thus leading to an attenuation of cellular signaling events. I provide evidence that GPR55 is targeted to the lysosomal/degradative pathway upon agonist stimulation and that this process involves a direct protein – protein interaction with GASP-1. Disrupting the GPR55-GASP-1 interaction prevents receptor degradation, and thereby allows receptor recycling. Further, I investigated downregulation of GPR55 and rimonabant induced side-effects in wild type (WT) C57BL/6 mice and GASP-1 knock-out (GASP-1 KO) mice. Repetitive rimonabant administration led to a significant downregulation of GPR55 in WT but not in GASP-1 KO mice. Interestingly, GASP-1 KO - rather than WT - mice show a higher degree of anxiety- and depression like behavior after chronic rimonabant administration. These data implicate GASP-1 as an important regulator of ligand mediated downregulation of GPR55 and provide tangible evidence that GPR55 – GASP-1 interactions play a key role in rimonabant induced side effects.

In addition, the concept of receptor homo- and heteromerization is well-established. Receptor homo- and heteromerization has been shown to impact the trafficking and signaling properties of the involved 7TM/GPCRs. I demonstrate that GPR55 can form heteromers with the cannabinoid 1 receptor. I show that the co-expression of CB_1 receptors and GPR55 specifically inhibits both, GPR55 mediated transcription factor and ERK1/2 MAP-Kinase activation. However, once the CB_1 receptor is activated, GPR55 mediated signaling is restored. In addition, I observe that the presence of GPR55 enhances CB_1R mediated ERK1/2 and transcription factor activation. These data provide first evidence that GPR55 can form heteromers with another 7TM/GPCR and that this interaction with the CB_1 receptor has functional consequences *in vitro*.

This work provides first insights into the cellular regulation of GPR55 by various processes, such as (i) the direct interaction with the sorting protein GASP-1, resulting in receptor degradation upon ligand stimulation and (ii) the heteromerization and cross-talk with the cannabinoid 1 receptor.

Keywords: 7TM/GPCR, CB_1 receptor, receptor degradation, GASP-1, GPR55, heteromerization, signal transduction

Zusammenfassung

Das Endocannabinoid-System umfasst zwei G-Protein gekoppelte Rezeptoren, die Cannabinoid-Rezeptoren 1 (CB_1R) und 2 (CB_2R), körpereigene Liganden (Endocannabinoide) und Enzyme, die für den Auf- und Abbau von Endocannabinoiden verantwortlich sind. Cannabinoide sind Wirkstoffe aus der Pflanze *Cannabis sativa* und werden seit Jahrhunderten in der Medizin wegen ihrer psychoaktiven, schmerzlindernden und entzündungshemmenden Wirkung eingesetzt. Weiters wurden auch körpereigene Moleküle (Enndocannabinoide) entdeckt und Wirkstoffe (synthetische Cannabinoide) mit Affinität zu den CB_1 und/oder CB_2 Rezeptoren entwickelt. Der kürzlich neu entdeckte G-Protein gekoppelte Rezeptor 55 (GPR55) wird sowohl von dem Lipid L-α-lysophosphatidylinositol (LPI), als auch von diversen CB_1R Inversen Agonisten/Antagonisten – zBsp. AM251, AM281 und Rimonabant (SR141716A) - aktiviert. Rimonabant wurde als Appetitzügler und zur Rauchentwöhnung entwickelt, musste jedoch aufgrund von vermehrt auftretenden Nebenwirkungen wie Angstzuständen und Depressionen vom Markt genommen werden.

Die vorliegende Arbeit beschäftigt sich hauptsächlich mit der zellulären Regulierung von GPR55. Die Rezeptor Expression an der Zelloberfläche spielt eine wichtige Rolle in vielen physiologischen Prozessen und unterliegt daher einem genau kontrollierten Mechanismus. Ein Protein, das in die Rezeptor-Regulierung involviert ist, heißt GPCR-associated sorting protein 1 (GASP-1). In meiner Dissertation kann ich erstmals zeigen, dass GPR55 mit GASP-1 interagiert und dadurch zu den Lysosomen transportiert wird, wodurch der Rezeptor im Endeffekt abgebaut wird. Wird diese Protein – Protein-Interaktion verhindert, wird der Rezeptorabbau blockiert und der Rücktransport (Recycling) zurück an die Oberfläche verstärkt. Weiters wurde der Zusammenhang von GPR55-Degradierung und den beschriebenen Nebenwirkungen von Rimonabant in einem Mausmodell (Wildtyp (WT) und GASP-1 Knock-Out (GASP-1 KO) Mäuse) getestet. Die wiederholte Verabreichung von Rimonabant führte zur Degradierung von GPR55 in WT, aber nicht in GASP-1-KO Mäusen. Außerdem konnte ich zeigen, dass GASP-1-KO Mäuse nach chronischer Rimonabant-Behandlung ängstlicheres - und depressiveres - Verhalten zeigen als WT-Mäuse. Ich zeige hier erstmals, dass GASP-1 ein wichtiges Regulatormolekül im

Abbauprozess von GPR55 *in vitro* und *in vivo* ist und dass die Interaktion von GASP-1 und GPR55 eine Rolle in den aufgetretenen Nebenwirkungen von Rimonabant spielt.

Rezeptor Heteromerisierung ist ein weiteres Konzept, die Aktivierung von Signalwegen und Rezeptorinternalisierung zu regulieren. Ich konnte zum ersten Mal zeigen, dass GPR55 und CB_1-Rezeptoren heteromerisieren und dadurch die Signaltransduktion der beiden Rezeptoren beeinflusst wird. Interessanterweise bewirkt die Anwesenheit des CB_1-Rezeptors eine reduzierte Transkriptionsfaktor- und ERK1/2 MAP-Kinase-Aktivierung über den GPR55 Rezeptor. Wird der CB_1-Rezeptor jedoch aktiviert, kann die GPR55-Aktivität wiederhergestellt werden. Im Gegensatz dazu konnte ich beobachten, dass die Aktivität des CB_1-Rezeptors in der Gegenwart von GPR55 verstärkt wird. Zusammenfassend konnte ich hier zum ersten Mal zeigen, dass der GPR55 Rezeptor mit anderen G-Protein gekoppelten Rezeptoren interagieren kann und dass die Interaktion mit dem Cannabinoid-Rezeptor 1 die Aktivierung von Signalwegen beeinflusst.

Zusammenfassend betrachtet konnte ich zeigen, dass zelluläre Prozesse von GPR55 durch mehrere Mechanismen reguliert werden können, wie (i) die Interaktion mit GASP-1 und der dadurch eingeleitete Abbau von GPR55 und (ii) die Heteromerisierung mit dem CB_1-Rezeptor – und in Folge auch die gegenseitige Beeinflussung der CB_1R- und GPR55-medierten Signalwege.

Abbreviations

AM251	[1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide]
AM281	[1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide]
AP-2	Adaptor protein-2
AT1	Angiotensin 1 receptor
AEA	Anandamide
β2-AR	Beta 2 adrenergic receptor
B2R	Bradykinin 2 receptor
BRET	Bioluminescence resonance energy transfer
DAG	Diacylglycerol
DNA	Desoxyribonucleic acid
CBD	Cannabidiol
CB_1R	Cannabinoid 1 Receptor
CB_2R	Cannabinoid 2 Receptor
cGASP-1	C-terminally truncated GPCR associated sorting protein 1
CP55940	[2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol],
CREB	cAMP Response Element Binding protein
D_2R	Dopamine D_2 receptor
DOP	Delta opioid receptor
ECS	Endocannabinoid System
e.g.	Exempli gratia
EGFP	Enhanced green fluorescent protein
ERK	Extracellular Regulated Kinase
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal Bovine Serum
fMLP	*N*-Formylmethionyl-leucyl-phenylalanine
FRET	Fluorescence resonance energy transfer
GASP-1	G protein-coupled receptor associated sorting protein 1

GDP	Guanine-nucleotide Diphosphate
GEF	Guanine-nucleotide Exchange Factor
GPCR	G Protein-coupled Receptor
GPR55	G Protein-coupled Receptor 55
GPR55-HEK	Human Embryonic Kidney Cells stable expressing FLAG-GPR55
GRK	G protein-coupled receptor kinase
GTP	Guanine-nucleotide Triphosphate
H1R	Histamine 1 receptor
HEK293 cells	Human Embryonic Kidney Cells
HEK-GPR55	HEK293 cells stable expressing HA-GPR55
HEK-CB1	HEK293 cells stable expressing FLAG-CB_1R
HEK-GPR55+CB1	HEK293 cells stable expressing HA-GPR55 + FLAG-CB1
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
i.e.	id est
IP3	Inositol triphosphate
KO	Knock out
LAMP	Lysosomal associated membrane protein
LPI	L-α-LysoPhosphatidylInositol
MAPK	Mitogen Activated Protein Kinase
MCF-7	Human breast cancer cell line
MOP	Mu-opioid receptor
MVB	Multivesicular bodies
NFAT	Nuclear Factor of Activated T cells
NF-kB	Nuclear Factor κ B
PDZ	Postsynaptic density 95/disc large/zonula occludens-1
PKC	Proteinkinase C
PLC	Phospholipase C
RGS	Regulator of G-protein Signaling
2-AG	2-Arachidonoyl Glycerol
7TM	Seven Transmembrane spanning receptors
shGASP-1	Short hairpin RNA targeting GASP-1
shRNA	Small hairpin RNA
shScr	Short hairpin RNA scrambled

SNX-1	Sorting nexin -1
SR141716A	5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
SRF	Serum response factor
Δ^9 THC	Δ9–tetrahydrocannabinol
T1R1	Taste receptor type 1
TSG101	Tumor suppressor gene 101
U2OS	Human osteosarcoma cell line
UEV	Ubiquitin E2 variant
US28	Unique short 28
WIN55-212-2	(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
WT	Wild type

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PART I GENERAL INTRODUCTION

1 7 transmembrane spanning (7TM)/G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are also referred to as seven transmembrane (7TM) spanning receptors because of their heptahelical structure (1). 7TM/GPCRs act at the interface between the extracellular and the intracellular environment of a cell and are critically involved in many physiological and pathophysiological processes. Their capacity to mediate extracellular stimuli resulting in intracellular responses renders them potent drug targets. More than 30% of all marketed drugs target 7TM/GPCRs (2). These receptors represent the largest protein superfamily comprising more that 800 human genes and 1000 functional receptors in mammals. Sequence analysis of the 7TM core region, in which these receptors share the highest similarity, in combination with pharmacological properties allowed the classification in five subfamilies (3-5), including the rhodopsin-like receptors (family A), the secretin-like receptors (family B), the glutamate-like receptors (family C) (6) as well as the adhesion and frizzeled/ taste families (7;8).

7TM/GPCRs can be activated by diverse ligands, ranging from small endogenous molecules, such as biogenic amines, lipids, proteins, amino acids, hormones, nucleotides, chemokines, ions, neurotransmitters and enzymes like proteases, to exogenous stimuli like light, smell and taste (7;9;10). Although a wide range of 7TM/GPCRs have been identified and characterised, there are still many ‘orphan’ receptors with unknown pharmacology and function.

In general, 7TM/GPCRs signal via G protein dependent and/or independent pathways. Transmitting extracellular stimuli into intracellular responses typically coincides with a conformational change of the receptor in a ligand-specific manner. The change from an ‘inactive’ to ‘active’ receptor conformation is a crucial step in 7TM/GPCR mediated signaling (11;12). For instance, studies on the interactions of full and partial agonists with the β2-adrenergic receptor (β2-AR) led to (i) the discovery of distinguishable ‘active’ conformations of the receptor and, in turn, (ii) diverse signaling pathways via this receptor (13;14). In contrast, inverse agonists have been demonstrated to change the receptor conformation into a more ‘inactive’ state. This mechanism can ‘silence’ constitutively

active 7TM/GPCRs, i.e. receptors that signal in the absence of any agonist. Receptor antagonists likewise stabilize receptors in an 'inactive conformation' and thereby prevent the binding of agonists to the receptor binding pocket (11;12).

The rhodopsin-like (family A) receptors form the largest and most diverse 7TM/GPCR subfamily. They share a structural signature, including the seven transmembrane α-helices (TMs) core region, connected by three intracellular and three extracellular loops. Each transmembrane domain consists of 20 to 27 amino acids (15). The N-terminus and the extracellular loops are highly diverse to augment the ligand binding variability. In contrast, the c-terminus and intracellular loops are important for the binding of heteromeric G proteins. The structure of 7TM/GPCRs have been intensively studied in the past few decades and conserved motifs were identified, such as the E/DRY motif (glutamic acid/aspartic acid – arginine – tyrosine) on TM 3 or NPxxY (asparagine – proline – x – x – tyrosine; x stands for any amino acid) at the end of TM 7. The E/DRY motif is important for the coupling and activation of G proteins. Importantly, mutations of this motif frequently either (i) result in complete inactivation or (ii) induce constitutive activity of the receptor. The NPxxY motif at the junction between the end of TM7 and the beginning of the c-terminus is part of the helix 8. This helix 8, together with typically numerous phosphorylation sites on the remainder of the c-terminus, is involved in the beta-arrestin-clathrin-mediated endocytosis of 7TM/GPCRs (7;16;17). A breakthrough in 7TM/GPCR structure biology was the description of the first three-dimensional crystal structure of rhodopsin (18) and the human β2-adrenergic receptor (19). To date, several other 7TM/GPCR crystal structures have been described, such as the avian β1-adrenergic receptor (β_1-AR) (20), the human A_{2A} adenosine receptor (21), the human histamine H1 receptor (22), the CXCR4 chemokine receptor (23), as well as the crystal structure of the active form of β2-AR in complex with the Gαs heterotrimer (24).

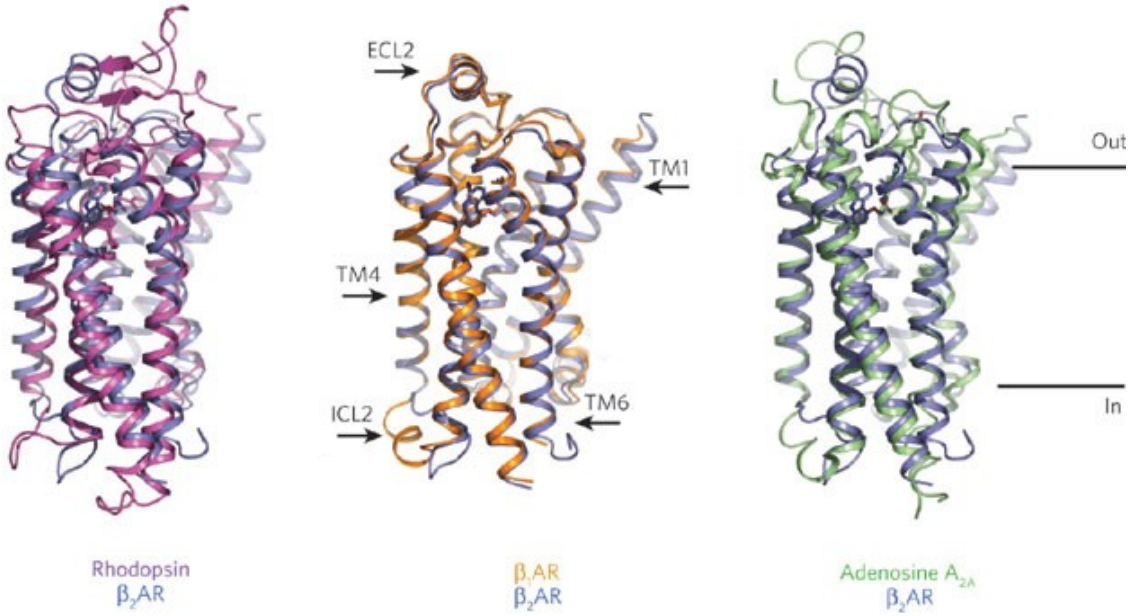

FIGURE 1. Bovine rhodopsin, avian β_1-AR and human adenosine A_{2A} receptor are superimposed on the human β_2-AR crystal structures. The extracellular ends of the transmembranes as well as the extracellular connecting loops show highest diversity in their structure. Extracellular loop 2 (ECL2), transmembrane 1, 4 and 6 (TM1, TM4, TM6) and the intracellulare loop 2 (ICL2) are indicated. (adapted from (12)).

2 Heterotrimeric G proteins and G protein mediated signaling

Extracellular ligand binding is the initial step of 7TM/GPCR activation. This leads to a conformational change of the receptor and thereby allows the coupling to different heterotrimeric G proteins. G proteins act as guanine-nucleotide exchange factors and consist of three subunits, the GTP binding α-subunit, as well as the complex of β- and γ-subunits (25-27). 7TM/GPCR activation, G protein coupling and the activation of effectors is a highly dynamic process and undergoes an activation – inactivation cycle (Fig. 2).

The Gα-subunit bound to GDP is associated with the β- and γ- subunit complex in the basal state. Upon ligand binding and subsequent changes of the receptor conformation, the GDP bound G protein couples to the receptor and causes the G protein α-subunit to exchange guanosine diphosphate (GDP) to guanosine triphosphate (GTP) (28). Subsequently, the GTP-bound form of the α-subunit dissociates from the heterotrimeric G protein and releases the stable βγ-dimer. Both subunits dissociate from the receptor and can modulate downstream effectors, such as adenylyl cyclases, phospholipases, protein kinases,

potassium and calcium channels (25). The hydrolysis of GTP to GDP by specific GTPases inherent to the Gα-subunits terminates the G protein mediated signaling. Additionally, 'regulators of G protein signaling' (RGS proteins) were identified and have the ability to regulate GTPase activity of Gα-subunits, thereby promoting the termination of G protein signaling (29-31). The last step of the cycle, the reassembly of the three subunits, allows a new activation round. (Fig. 2) (32).

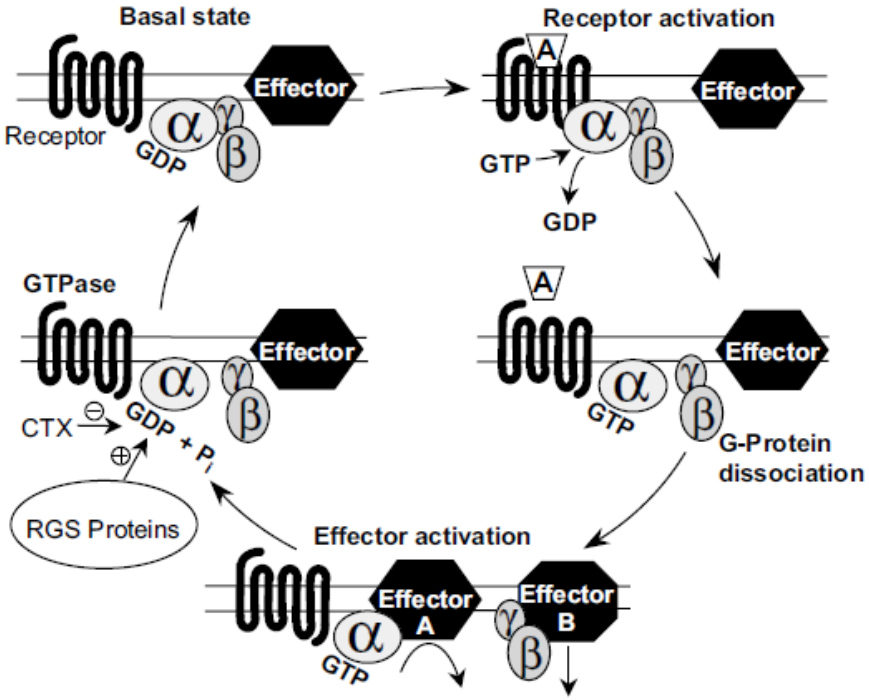

FIGURE 2. Scheme of the G protein activation – inactivation cycle. 7TM/GPCR interaction with an agonist (A) promotes the exchange of GDP to GTP at the α-subunit of the heterotrimeric G protein. Gα-subunit bound to GTP allows the dissociation of the Gβγ-subunit, resulting in the ability to modulate effector function. Spontaneous GTP to GDP hydrolysis is enhanced by several effectors as well as by regulators of G protein signaling (RGS) proteins, leading to the reassociation of the Gαβγ-complex. (adapted from (33)).

2.1 Gα-subunits

The Gα-subunit can be classified in four main groups on account of structural and functional similarities; Gαs, Gαi/o, Gαq/11 and Gα12/13 (34), each consisting of numerous subtypes. In general, different G protein subunits can couple to the same receptor, resulting in the activation of distinct signaling pathways mediated via diverse Gα-subunits, as well as the dissociated βγ-complexes. The G protein coupling is receptor as well as tissue and/or

cell type dependent. Taken together, some 7TM/GPCRs interact with one specific G protein, whereas others have the ability to couple to different subunits (32).

2.1.1 Gαs

Gαs proteins activate adenylyl cyclase upon receptor coupling and subsequently lead to the accumulation of intracellular cyclic AMP (cAMP, cyclic adenosine 3'-, 5'-monophosphate) (35). Moreover, increased cAMP levels convert the enzyme protein kinase A (PKA) into its active form, whereby the complex dissociates into its four subunits and translocates to the nucleus. There, PKA can phosphorylate gene regulatory proteins, like the cAMP response element-binding (CREB) protein (36). Cholera toxin (CTX) from *Vibrio cholerae* is widely used to study Gαs signaling, since it is able to ADP ribosylate the Gαs subunit, thereby constitutively activating adenylyl cyclase and elevating cAMP levels (37); (for review (38)).

2.1.2 Gαi/o

The Gαi/o family members are widely expressed among several tissues and their expression levels are relatively high. It is believed that activated receptors coupled to Gαi/o proteins mediate the inhibition of several adenylyl cyclase subtypes (39). Pertussis toxin (PTX) from *Clostridium botulinum* adds an ADP-ribose moiety close to the carboxyl-terminus of the Gαi/o subunit and thereby inhibits its coupling to a receptor. Therefore, PTX can be used to study Gαi/o dependent signaling pathways (32).

2.1.3 Gαq/11

The initial step of the Gαq/11 mediated signaling cascade is the stimulation of phospholipase C-β subunits (PLC-β), resulting in elevated inositol triphosphat (IP3) and diacylglycerol (DAG) levels. IP3 triggers the release of calcium from intracellular stores, while DAG activates protein kinase C (PKC) and recruits it to the cell membrane (40). Gαq and Gα11 proteins are ubiquitously expressed, in contrast to other family members, such as

Gα14 and Gα15/16 which are expressed in more specific patterns, for example in some hematopoietic cells (32).

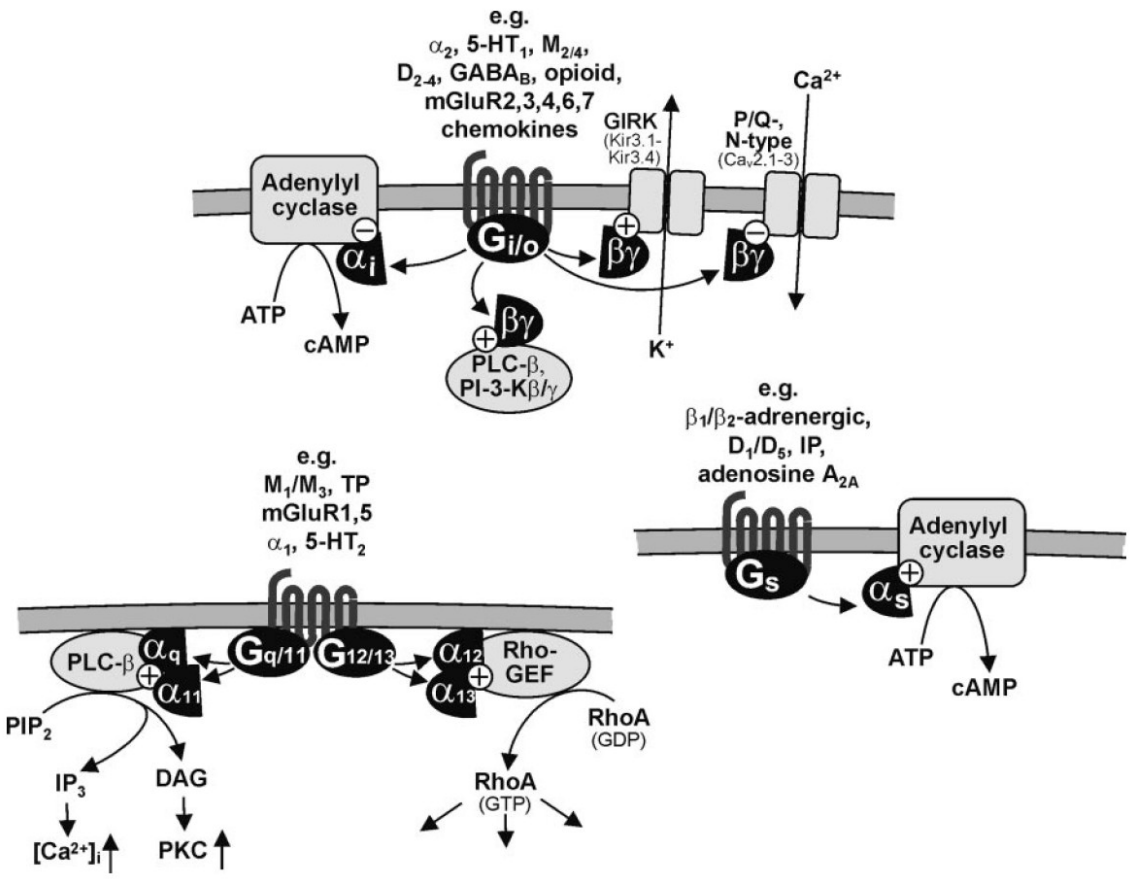

FIGURE 3. Classical signaling patterns for Gαi/o, Gαs, Gαq/11 and Gα12/13 receptor coupling as well as βγ-subunit signaling. α2, α2-adrenergic receptor; $D_{1–5}$, dopamine receptor subtypes 1 to 5; GIRK, G protein-regulated inward rectifier potassium channel; $5\text{-HT}_{1,2}$, serotonin receptor subtypes 1 and 2; $M_{1–5}$, muscarinic acetylcholine receptor subtypes 1 to 5; $mGluR_{1–7}$, metabotropic glutamate receptor subtypes 1 to 7; PLC-β, phospholipase C-β; PI-3-K, phosphoinositide-3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; Rho-GEF, Rho-guanine nucleotide exchange factor; TP, thromboxane A2 receptor; IP, prostacyclin receptor. (taken from (32)).

2.1.4 Gα12/13

The function and signaling cascades of the Gα12 and Gα13 protein subunits are less well understood. Gα12/13 proteins were first described in the early 90s and have been shown to be conserved among many species (41;42). Early studies in *Drosophila melanogaster* indicated the involvement of these Gα proteins in cell morphology and migration (43).

Gα12/13 is ubiquitously expressed. Most receptors which couple to Gα12/13-subunits also interact with Gαq/11 proteins, and, in rare cases, with Gαi proteins (41;44;45). Gα12/13 proteins directly activate and regulate the small GTPase RhoA (46) and Rho guanine nucleotide exchange factors (RhoGEFs). RhoGEFs exhibit a RGS (regulator of G-protein signaling) domain, which can bind to activated Gα-subunits and comprises several regulatory proteins, including PDZ-RhoGEF, leukaemia-associated RhoGEF (LARG) and p115-RhoGEF (lymphoid blast crisis-like-2, Lsc). In addition, Gα12/13 proteins can interact with cadherins and thereby trigger the release of β-catenin (47;48).

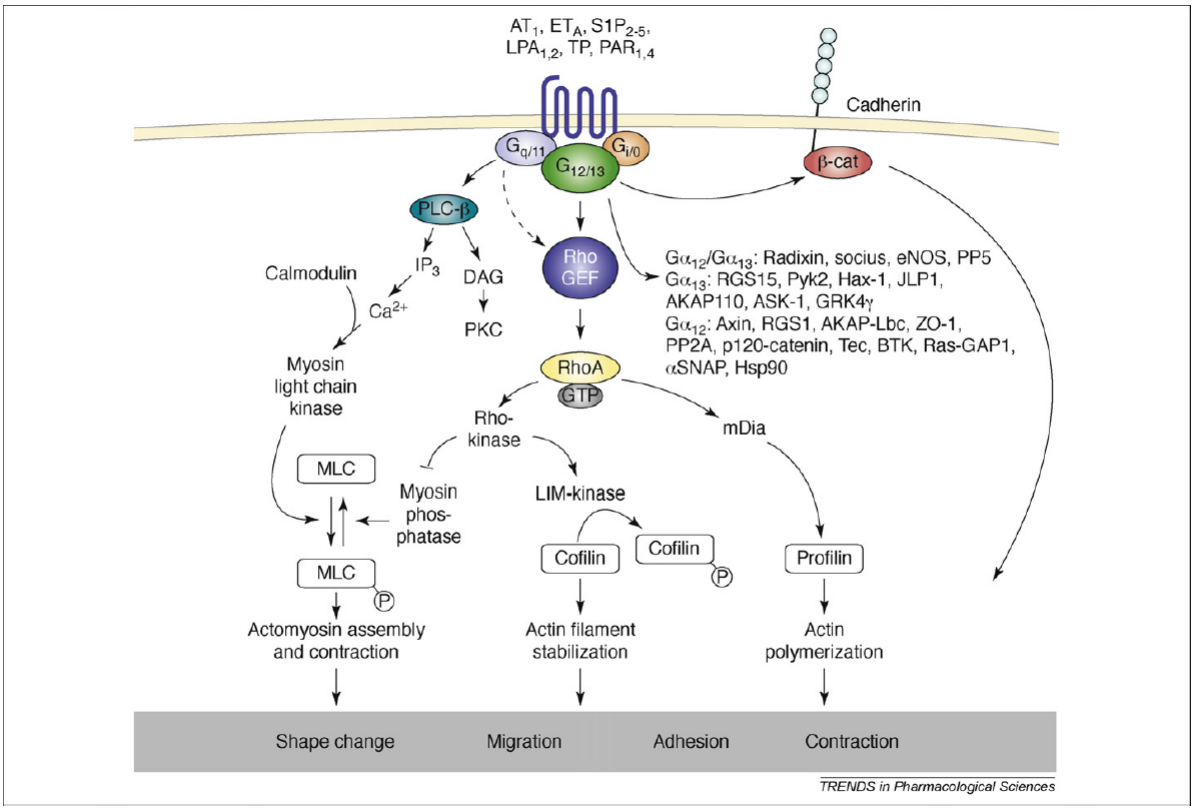

FIGURE 4. Signaling pathways and cellular functions mediated via Gα12/13 proteins: RhoA activation, shape change, cell migration, adhesion and actin polymerization. The angiotensin II (AT_1), endothelin (ET_A), sphingosine-1-phosphate ($S1P_{2-5}$), lysophosphatidic acid ($LPA_{1,2}$), thromboxane A_2 (TP), thrombin ($PAR_{1,4}$) and GPR55 (49) receptors were described to couple to Gα12/13 (taken from (50)).

Gα12/13 signaling plays a crucial role in a variety of physiological processes, such as cell polarity, migration (51) and cell proliferation (52). Additionally, Gα12/13 signaling cascades are involved in angiogenesis (53), immune responses (54) and apoptosis (55). Further, tumor formation and metastasis (56), cell transformation (57) as well as leukemia (58) are related to dysfunctional Gα12/13 signaling pathways. The formation of stress

fibers, the activation of transcription factors, such as SRF (45) and proper cell growth are under control of Gα12/13 mediated RhoA signaling (46;59).

TABLE 1. Expression and effectors of mammalian Gα protein subtypes. AC, adenylyl cyclase; GIRK, G-protein-regulated inward-rectifier potassium channel; PDE, phosphodiesterase; PDZ, PSD95–Disc-large–ZO-1; PLC, phospholipase C; RhoGEF, Rho guanine nucleotide exchange factor; VDCC, The G-protein cycle. voltage-dependent Ca2+-channel. (taken from (50))

Name	Expression	Effector
$G\alpha_s$ class		
$G\alpha_s$	Ubiquitous	AC ↑
$G\alpha_{olf}$	Olfactory epithelium, brain	AC ↑
$G\alpha_i/G\alpha_o$ class		
$G\alpha_{i1}$	Widely distributed	AC ↓ (directly regulated)
$G\alpha_{i2}$	Ubiquitous	Various other effectors are
$G\alpha_{i3}$	Widely distributed	regulated via Gβγ released from activated G_{i1-3}
$G\alpha_o$	Neuronal, neuroendocrine	VDCC ↓, GIRK ↑ (via Gβγ)
$G\alpha_z$	Neuronal, platelets	AC ↓ (directly regulated); Rap1GAP
$G\alpha_{gust}$	Taste cells, brush cells	Unknown
$G\alpha_{t-r}$	Retinal rods, taste cells	PDE ↑
$G\alpha_{t-c}$	Retinal cones	PDE ↑
$G\alpha_q/G\alpha_{11}$ class		
$G\alpha_q$	Ubiquitous	PLC-β ↑
$G\alpha_{11}$	Almost ubiquitous	
$G\alpha_{14}$	Kidney, lung, spleen	
$G\alpha_{15/16}$	Haematopoietic cells	
$G\alpha_{12}/G\alpha_{13}$ class		
$G\alpha_{12}$	Ubiquitous	PDZ-RhoGEF/LARG, others
$G\alpha_{13}$	Ubiquitous	Lsc, PDZ-RhoGEF/LARG, others

2.2 βγ-subunits

To date, 5 β-subunits and 12 γ-subunits have been described. Thus, βγ-complexes have different properties, depending on the composition of the subunit partners. For example, the β5-subunit forms weak complexes with members of the γ-subunit, whereby β1 to β4 interact strongly with γ-subunits (60;61). Gβγ-subunit mediated signaling is rather homogenous among the different subunit-complexes (62). The regulation of ion channels (63), adenylyl cyclase, phospholipse C (39;64) and phosphoinositide-3-kinase isoforms (65) are the most prominent effector molecules of the βγ-subunits (66).

3 Homo- and heteromerization of 7TM/GPCRs

For many years it was thought that 7TM/GPCRs exist and signal as monomes. However, within the last decade, the concept of hetero-oligmerization of 7TM/GPCRs has been widely recognized (67;68). Heteromerization of 7TM/GPCRs has been shown to be a prerequisite for the proper function of certain receptors, such as for example for the $GABA_B$ receptor family (69-71) or the taste receptor type 1 (T1R1) and taste receptor type 3 (T1R3) (72). The heteromers of T1R1 – T1R3 are also referred to as sweet receptors (73). Receptor oligomers can either be composed of identical (homomers) or diverse (heteromer) 7TM/GPCRs.

Western blot and co-immunoprecipitation experiments gave first evidence that 7TM/GPCRs can form homo- and heteromers. Lately, biophysical techniques such as Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) allow the examination of receptor-receptor interactions in real-time (74).

The conformation of 7TM/GPCRs can be modulated by the formation of homomers (75-78) or heteromers (67;68;79-81). These receptor complexes can display a distinct pharmacology, signaling patterns as well as trafficking properties (74;82). Many functionally relevant 7TM/GPCR heteromers have been described in the past years; a few examples are i.e. the dopamine D_1 and D_2 (83-85), the mu- and delta-opioid (MOP and DOP) (86-89) and the CRTH2 and DP receptor heteromers (90).

The existence of heteromers with distinct signaling pathways necessitates heteromer selective ligands. One of the first heteromer-selective ligand described was 6`-guanidinoaltrindole (6'-GNTI), a compound that selectively activates the delta opioid receptor (DOP)/kappa opioid receptor (KOP) heteromer both *in vitro* and *in vivo* (91).

In addition, receptor homo- and heteromerization has been shown to be crucial for the correct maturation and folding of several 7TM/GPCRs. For example, the vasopressin V1a, V2 and the oxytocin receptor form homo- and heteromers at an early stage of maturation in the ER and those dimers remain stable during the activation process (92). Incorrect receptor maturation can result in disease development and/or progression. For example, the retention of the mutant Ca^{2+}sensing receptor in the endosplasmatic reticulum (ER) is associated with familial hypercalcaemia and neonatal hyperthyroidism (76;93).

4 Trafficking and post-endocytic sorting of 7TM/GPCRs

4.1 Endocytosis

7TM/GPCR-mediated signaling is extensively regulated to guarantee an appropriate cell surface receptor density in a given physiological setting. One of these regulatory mechanisms is that of endocytosis (another common term used is 'internalization'). The rapid removal of agonist-activated receptors from the cell surface terminates and reversibly silences receptor mediated signaling (94). Upon agonist stimulation, G protein coupling and activation of downstream effectors, 7TM/GPCRs are generally desensitized by the phosphorylation of the carboxyl terminus of the receptor via 7TM/GPCR kinases (GRKs) and the subsequent interaction with β-arrestins. In general, 7TM/GPCRs can be internalized via either (i) clathrin-coated pits, (ii) caveolae or (iii) uncoated pits (95). The invagination and pit asssembly is further mediated by clathrin and adaptor proteins, *e.g.* adaptor protein 2 (96;97).

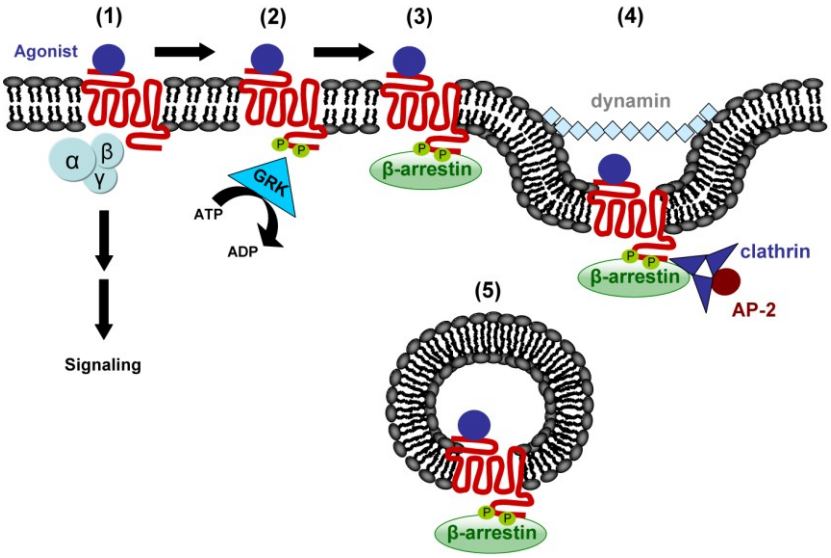

FIGURE 5. Principles of β-arrestin/clathrin dependent endocytosis of 7TM/GPCRs. (1) Agonist mediated intracellular activation of signaling pathways. (2) The phosphorylation of the c-termini of activated 7TM/GPCRs via GRKs leads to the desensitization of the receptor and (3) the recruitment of β-arrestin. (4) Receptor endocytosis is facilitated by clathrin and AP-2 and (5) dynamin is necessary for vesicle abscission (from (98)).

4.2 Post-endocytic sorting of G protein-coupled receptors

Post-endocytic sorting of 7TM/GPCRs is also referred to as 'receptor trafficking'. Trafficking is the process of sorting endocytosed receptors between recycling and degradative pathways (99). Trafficking of internalized 7TM/GPCRs by a rapid recycling pathway restores the complement of functional receptors in the plasma membrane and promotes resensitization of receptor-mediated signal transduction. In contrast, the sorting of internalized 7TM/GPCRs to lysosomes promotes proteolytic downregulation of receptors, leading to a prolonged attenuation of cellular signal transduction (100). The endocytic and post-endocytic fate of a receptor is typically already determined at the cell surface, i.e. by receptor phosphorylation and/or ubiquitination. Carboxyl-terminal phosphorylation of 7TM/GPCRs is one of the initial steps of endocytosis (101-103). Indeed, the phosphorylation state of an internalized receptor additionally influences the post-endocytic fate, by regulating interactions with sorting proteins or inducing posttranslational modifications (104;105). For instance, the covalent attachment of ubiquitin to c-terminal lysine residues acts as a 'sorting signal' that promotes endocytosis and/or targeting to the degradative pathway/lysosomes (106-108). Additionally, several proteins have been identified that specifically target 7TM/GPCRs to either recycling (104;109-112) or the degradative pathways (98;113-115).

4.2.1 Lysosomal sorting of 7TM/GPCRs

Targeting 7TM/GPCRs to the degradative/lysosomal pathway is highly regulated by interactions with sorting proteins or posttranslational modifications. To date, two major sorting processes have been described for mammalian 7TM/GPCRs, (i) the ubiquitination-dependent lysosomal sorting or (ii) the inhibition of recycling facilitated by the interaction with sorting proteins, such as the GPCR-associated sorting protein 1 (GASP-1) or the sorting nexin 1 (SNX-1) (98;116).

ESCRT - The highly conserved endosomal sorting complex required for transport (ESCRT) machinery recognizes and sorts ubiquitinated proteins via multivesicular bodies for degradation (117). The sorting complex comprises four cytosolic protein complexes also referred to as ESCRT-0 to ESCRT-III. The ESCRT-0 complex involves a hepatocyte

growth factor regulated tyrosine kinase substrate (Hrs) and the signal transduction adapter molecule (STAM). The recognition of ubiquitinated proteins with their ubiquitin-interaction motifs (UIMs) is the initial step in the unbiquitination-dependent lysosomal sorting process. Subsequently, Hrs interacts with the ubiquitin E2 variant (UEV) domain of the tumor suppressor gene-101 (TSG101) and is necessary for ESCRT-I complex binding. ESCRT-I and –II complexes are proposed to cause stable inward budding of the membrane, followed by interaction with ESCRT-III. The ESCRT-III protein shuttles the cargo to the lysosomes for degradation (118-120). In addition, it was reported that the ESCRT machinery is necessary for non-ubiquitinated proteins, such as the delta opioid receptor (DOP). DOP is endocytosed and targeted to the degradative pathway in an ubiquitinated as well as non-ubiquitinated state (121). This indicates that the ESCRT machinery can target non-ubiquitinated proteins for degradation.

SNX-1 - Sorting nexin-1 (SNX-1) is a sorting protein that targets membrane proteins to the lysosomal pathway independent of the ESCRT machinery. Originally, SNX-1 was described to bind Hrs and target the epidermal growth factor (EGF) receptor to the degradative pathway (122). However, it was shown that SNX-1 can interact with several 7TM/GPCR c-termini *in vitro*, such as DOP, the viral chemokine receptor US28 and the glucagon-like peptide 1 receptor (GLP1) (123). In addition, the downregulation of the protease-activated receptor-1 (PAR1) receptor has been reported to be SNX-1 dependent (124). Intriguingly, both muscarinic receptors M1 and M4 interact with SNX-1, but are efficiently recycled back to the cell surface upon agonist stimulation (125;126). Hence, the detailed mechanism by which SNX-1 targets 7TM/GPCRs to the degradative pathway still remains elusive.

Dysbindin - The cytoplasmic protein dysbindin was recently reported to be involved in the post-endocytic sorting of the DOP and the dopamine D_2 receptors (127).

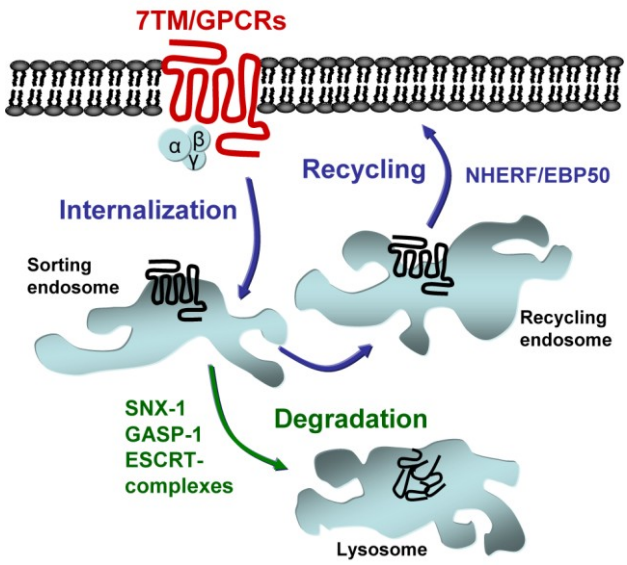

FIGURE 6. Scheme of the post-endocytic sorting of 7TM/GPCRs. Receptors are either targeted for degradation or quickly recycled back to the cell surface following agonist-induced receptor internalization. This process is highly regulated and involves receptor ubiquitination or interactions with sorting proteins (from (98)).

4.2.2 The G protein-coupled receptor associated sorting proteins – GASPs

As briefly described above, the sorting of individual 7TM/GPCRs between recycling ('resensitization') and degradation is a fundamental mechanism that controls the signaling capacity of these receptors and thus needs to be highly regulated. Several proteins have recently been identified that specifically target 7TM/GPCRs, typically by interacting with their carboxyl terminal domain, to either the recycling or the degradative pathways (104;115).

The G protein-coupled receptor associated sorting protein 1 (GASP-1) was identified in a yeast two-hybrid screen and was originally found to target DOPs to lysosomes and hence a degradative pathway (115). GASP-1 has since been shown to interact with the carboxyl-termini of many 7TM/GPCRs (115;123;128-134) and to regulate the trafficking properties of many of these, both *in vitro* and *in vivo* (98;135).

GASP-1 is a large acidic protein of 1395 amino acids that is highly expressed in most brain regions and in a few additional peripheral tissues ((136) and see http://www.kazusa.or.jp/huge/gfpage/KIAA0443) and is encoded by a gene located on chromosome X in human and mice (137).

A family of nine additional GASP homologes was suggested (137), however, except for GASP-2 (~ 150kDa protein, 68% identity with the carboxy-terminal sequence of GASP-1) none of the other family members has more than 20% homology to GASP-1. Moreover, to date no functional data have been shown for any of these GASP-homologues. GASP-2, the closest homologue to GASP-2, has been suggested to interact with the D_1 dopamine receptor (D_1R) and the β_2AR (132), the viral chemokine receptor US28 (134) as well as huntingtin (htt), a protein playing a role in the neurodegenerative disorder Huntington´s disease (HD) (138). However, to date no functional consequences have been shown for 7TM/GPCR – GASP-2 interactions. GASP-2 shows a 35 % amino acid similarity to GASP-1. Interestingly, the COOH terminal 497 amino acids of GASP-1 and of GASP-2 share a 62% sequence identity. Further, a 250 amino acid domain was described at the carboxyl terminal end of GASP-1 with a sequence identity of GASP-2 to GASP-9 ranking from 77% to 25% respectively (98).

4.2.2.1 GASP-1 dependent sorting of 7TM/GPCRs in vitro

Several 7TM/GPCRs that interact with GASP-1 are targeted to the degradative pathway, whereas the inhibition of this interaction results in the recycling of the receptor back to the cell surface (131;134). Interestingly, GASP-1 seems to specifically interact with individual members of 7TM/GPCR families. For instance, GASP-1 plays a pivotal role in downregulating the DOP, bradykinin 1 receptor (B_1R) and D_2 dopamine receptor (D_2R) after agonist stimulation. In contrast, their family members mu opioid receptor (MOP), bradykinin 2 receptor (B_2R) and D_1R do not bind to GASP-1 and are rapidly recycled back to the cell surface (98;115;129).

Thompson et al. (132) suggested that different sorting proteins can interact with an indiviual receptor and that the relative affinity of each sorting protein can regulate the post-endocytic sorting of this receptor. For instance, the β_2-AR directly interacts with GASP-1 in a GST-pull down assay, however, no interaction between the receptor and GASP-1 was observed in HEK293 cells, hence resulting in β_2-AR recycling. It was suggested that GASP-1 can compete with the recycling proteins Ezrin-Radixin-Moesin-binding Phosphoprotein-50/Na^+/H^+ Exchanger Regulatory Factor (NHERF/EBP50) and N-ethylmaleimide-sensitive factor (NSF) for c-terminal β_2-AR binding and it appears that the

recycling proteins 'win' in this case. In fact, disrupting the PDZ domain of the receptor inhibits the interaction with these recycling proteins and subsequently the β_2-AR mutant is targeted for degradation via GASP-1 (132). In another example, the truncation of the last 17 amino acids of the recycling MOP receptor allows GASP-1 binding, resulting in lysosomal degradation (132). In contrast, recycling of the D_1R was blocked by the deletion of a recycling signal within the carboxyl-tail of the receptor, but no GASP-1 – receptor interaction was detected. The D_1R mutant neither recycled nor degraded after agonist-induced endocytosis (132). Taken together, blocking recycling proteins from interacting with the cytoplasmic-tail of individual 7TM/GPCRs alone does not automatically result in receptor downregulation.

4.2.2.2 GASP-1 dependent sorting of 7TM/GPCRs in vivo

The post-endocytic fate of 7TM/GPCRs plays an important role in long-term drug treatment and thus the development of tolerance. However, to date little is known about how trafficking of 7TM/GPCRs alters their responsiveness to e.g. neuropsychiatric drugs. Experiments examining the function of the dopamine D_2 receptor (D_2R) revealed that GASP-1 plays a crucial role in regulating agonist responses via this receptor both *in vitro* and *in vivo* (128;129;133). Studies on rat ventral tegmental area (VTA) brain slices pre-treated with the D_2R agonist quinpirole showed that the receptors failed to recover from desensitization, which was consistent with the ability of the D_2Rs to degrade after endocytosis. However, using an inhibitory antibody and thereby blocking the D_2R – GASP-1 interaction restored D_2R function in this brain area (129). Along these lines, repeated treatment of wild type - but not GASP-1 knock-out mice with cocaine lead to a downregulation of D_2Rs in mouse striatum (133). In addition, GASP-1 knock out mice showed reduced locomotor sensitization to cocaine (133) and reduced acquisition of cocaine self administration (128). Further it was demonstrated that prolonged morphine stimulation of MOP leads to the development of adverse events *in vitro* (139) and *in vivo* (140).

Cannabinoids are used in the clinics as i.e. analgesics and appetite enhancers, but as reported for other drugs, long-term use is problematic and limited by the development of tolerance. The CB_1R is sorted to the lysosomes via GASP-1 after agonist treatment *in vitro*

(130). Further *in vivo* studies showed that the post-endocytic sorting of the CB_1R is involved in the development of analgesic tolerance. Mice received either control adenovirus or an adenovirus expressing a dominant negative version of GASP-1 – i.e. cGASP-1 (115;131) - and cannabinoid drugs were administrated chronically. Mice treated with control adenovirus developed tolerance to the analgesic effects of the cannabinoids. In contrast, mice lacking functional GASP-1 – receptor interaction showed a reduced development of tolerance (131). Moreover, long-term treatment of wildtype (WT) and GASP-1 knock-out (GASP-1 KO) mice with the CB_1R agonist WIN55,212-2 resulted in a significant loss of receptor levels in spinal cord and cerebellum membranes in WT, but not in GASP-1 KOs. In addition, a development of tolerance to the effects of WIN55,212-2 on antinociception, motor incoordination and locomoter hyperactivity were observed in WT, but not in GASP-1 KO mice (141).

Taken together, these early studies suggested that GASP-1 dependent sorting of 7TM/GPCRs was crucial in the development of tolerance and the responsiveness to cannabinoid drugs *in vivo*.

4.2.3 The recycling pathway

Many 7TM/GPCRs recycle from endosomal compartments back to the cell surface after agonist-mediated endocytosis. Typically, this process occurs by default (142). However, for some 7TM/GPCRs, specific recycling sequences at the far end of the carboxyl termini have been identified (109;117;121;143). For instance, efficient recycling of the β_2-AR requires the distal consensus (postsynaptic density 95/disc large/zonula occludens-1) PDZ motif in the c-terminus of the receptor (104). When this motif is fused to e.g. the carboxyl terminus of the DOP (a typically degrading receptor), the DOPs recycle (109). In addition, the β_2-AR interacts with PDZ domain-containing proteins of the NHERF/EBP50 family members, which results in receptor recycling (144).

5 The endocannabinoid system

Extracts from *Cannabis sativa* have been used for medicinal and recreational purposes for at least 4000 years. More than 60 different pharmacologically active components have been described, including the most prominent and psychoactive plant derivative Δ^9 – tetrahydrocannabinol (Δ^9-THC) (145-147). The endocannabinoid system consists of endogenous cannabinoids, two well-defined 7TM/GPCRs, the cannabinoid 1 (CB_1R) and cannabinoid 2 (CB_2) receptors (148), as well as enzymes synthesizing and degrading endocannabinoids (149;150).

In 1990 the first molecular target for phytocannabinoids was discovered. Δ^9 –THC showed effects on an orphan 7TM/GPCR from rat cerebral cortex cDNA library, now referred to as the CB_1 receptor (151). Three years later, the CB_2 receptor was discovered and cloned from human promyelocytic leukemic cells (HL60) (152;153). Both cannabinoid receptors belong to the rhodopsin-like 7TM/GPCR family A. CB_1 and CB_2 receptors predominantly couple to Gαi/o proteins, leading to the inhibition of adenylyl cyclase, activation of mitogen-activated protein kinases (MAPK) and further regulating transcription factor activation. However, depending on the tissue and the ligand involved, Gαs and Gαq/11 proteins can also be activated via CB_1 and CB_2 receptors. In addition, several potassium channels were described to be activated by CB_1 and CB_2 receptors (147;154). CB_1 receptors were described to be constitutively active, i.e. the receptors can both signal and internalize in the absence of any ligand (130;155).

The CB_1 receptor is one of the most abundant 7TM/GPCRs in the CNS and is involved in many physiological processes, such as cognition and memory, control of motor function and analgesia (156-158). For instance, the inhibition of neurotransmitter release at the termini of central and peripheral neurons is mediated by CB_1 receptors (147;159;160). In contrast, CB_2 receptors are mainly expressed on immune cells (152;161), *e.g.* neutrophils, macrophages, T cells and B cells (148;154;162-165) and play a role in cytokine release and immune cell migration (166). However, peripheral CB_1 receptors were detected on non-neuronal cells, such as adipocytes, liver, pancreas (167-169) and skeletal muscles (170) and CB_2 receptors were found on neurons (171-178).

The endocannabinoids arachidonoyl ethanolamide (anandaminde, AEA) and 2-arachidonoyl glycerol (2-AG) were identified as the first endogenous agonists for both the CB_1 and CB_2 receptor (147). Endocannabinoids are synthesised 'on demand' as shown in Figure 7 (149;179).

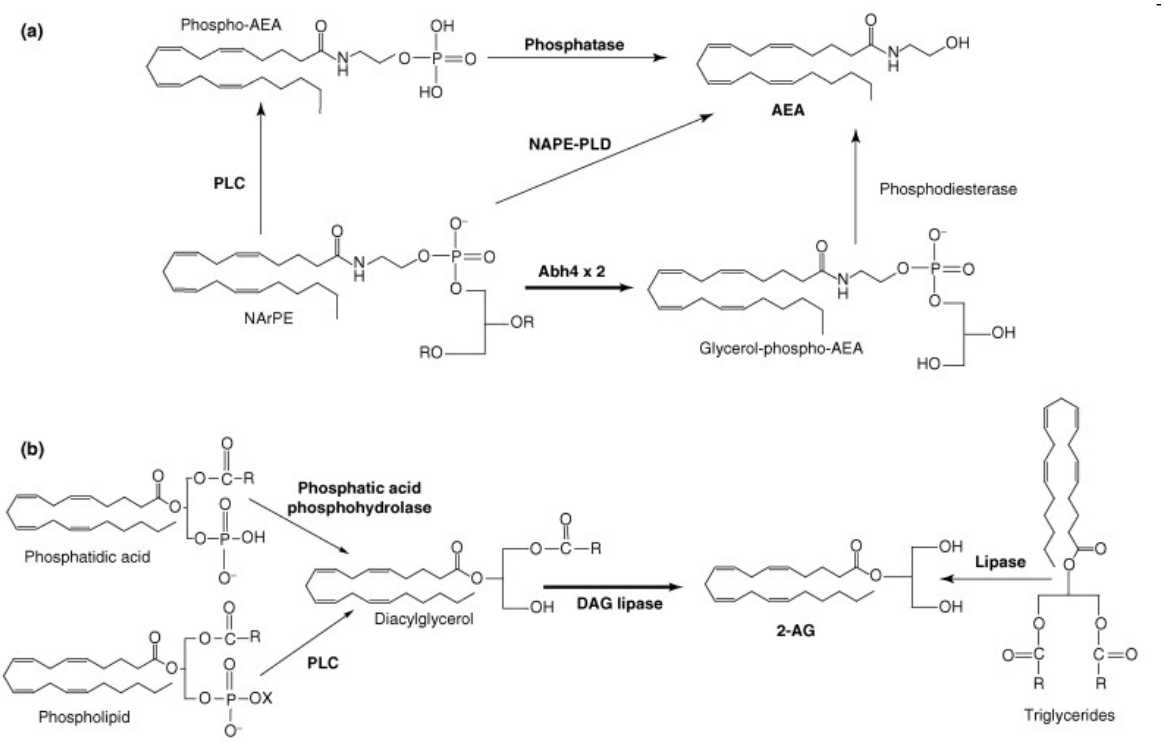

FIGURE 7. Scheme of endocannabinoid synthesis. (a) N-arachidonoyl-phosphatidyl-ethanolamine (NArPE) is converted into AEA mainly by NAPE-PLD. (b) The formation of 2-AG in the brain occurs mainly through the hydrolysis of triglycerides or phosphatidic acid (adapted from (149)).

CB_1 and CB_2 receptors can be activated by numerous ligands, such as the psychoactive phytocannabinoid Δ^9-THC and synthetic compounds such as WIN55,212–2 or CP55940 (148;180). In addition, numerous synthetic CB_1 receptor inverse agonists/antagonists, including AM251, AM281, SR141716A (Rimonabant), were described (181;182), among which SR141716A recently gained a lot of attention as a promising treatment for obesity and smoking cessation (148;180;183). AM630 and SR144528 are potent CB_2 receptor antagonists (180).

Cannabinoid CB_1 receptor homomers (78) and heteromers have been described. For instance, the G protein coupling is altered in $CB_1R - D_2$ heteromers (184) and the CB_1R – orexin-1 receptor heteromer shows altered trafficking and signaling properties (185;186).

Likewise, the CB_1R – A_{2A} receptor heteromer induces different signaling patterns than the respective receptors alone (180;187).

Several studies claim that both cannabinoid receptors play an anti-tumorigenic role. Overexpression of CB_1 and CB_2 receptors as well as activation of both receptors prolongate colon (188), prostate (189;190) and breast cancer growth *in vitro* and *in vivo* (191-193).

6 The G protein-coupled receptor 55 (GPR55)

Cannabinoids bind to and modulate the signaling via the CB_1 and CB_2 receptors. Nevertheless, studies on cannabinoid knock out mice suggested additional cannabinoid-sensitive targets (194;195). Several receptors were described to be activated by cannabinoid ligands, such as the G protein-coupled receptor 55 (GPR55), GPR18, GPR119 and others, but their function as novel cannabinoid receptors is still under debate (see review (180)).

In 2006, AstraZeneca and GlaxoSmithKline suggested GPR55 to be a novel cannabinoid receptor ((196) see patent references). This receptor is highly expressed in the CNS, *e.g.* putamen, striatum, hippocampus, as well as in intestine, bone marrow, spleen, immune and endothelial cells (Fig. 8) (197-202). Moreover, GPR55 was detected in cancer tissues and cancer cell lines (203-206).

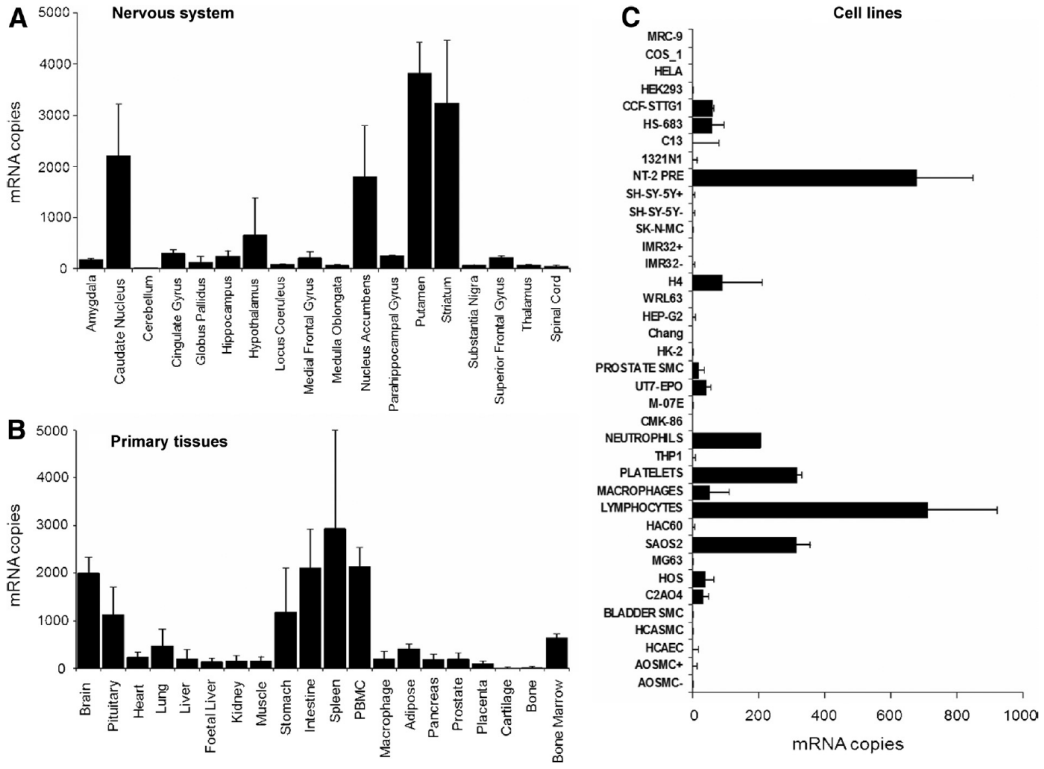

FIGURE 8. GPR55 mRNA expression in human tissue and cells. TaqMan quantitative RT-PCR analysis of GPR55 in human tissues and cells was conducted. The level of mRNA expression from (A) 18 regions of the brain and nervous system and(B) 20 primary peripheral tissues were determined. Data show the mean ±SD copies of mRNA detected in samples from three or four individuals per tissue. In addition, (C) mRNA levels in 37 distinct human cell lines and primary cells were measured. Data show mean ±SD copies per nanogram mRNA detected in duplicate from a single batch of cells (from (198)).

6.1 Structure of GPR55

The human GPR55 (hGPR55, NM_005683.3) is a 319 amino acid protein and a member of the rhodopsin-like 7TM/GPCR family. hGPR55 was first isolated and cloned in 1999 and mapped to the human chromosome 2q37 (200). Different sequences have been reported for hGPR55, most likely due to polymorphisms or technical errors during cloning or sequencing. So far, orthologues of GPR55 have been identified in the genomes of rat, mouse, dog, cow, chimpanzee, zebrafish, pufferfish and humans (196;201;207). The closest homologues to GPR55, based on its amino acid sequence, are the purinergic receptor P2Y5 (29%, NM_005767.4), the purinoceptor-like orphan receptors GPR23 (30%, NM_005296.2) and GPR35 (27% NM_005301.2) and the chemokine receptor CCR4 (23%, NM_005508.4) (200). Interestingly, GPR55 shows low sequence identity with the

two traditional cannabinoid receptors CB_1 (13.5%) and CB_2R (14.4%). Sequence alignment analysis with rhodopsin shows conserved patterns in TM1, 2, 4 and 5, but instead of the highly conserved DRY motif (16) in TM3, GPR55 exhibits a DRF motif. GPR55 contains a conserved proline in TM5, which is absent in CB_1R and CB_2R. Furthermore, the highly conserved NPxxY motif found in TM7 of rhodopsin, CB_1R and CB_2R sequences is altered in the GPR55 structure (208;209). GPR55 comprises several putative protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites as well as conserved patterns for glycosylation, and conserved cysteines are located in extracellular loop one and two (200). Although several cannabinoid ligands can activate GPR55, it lacks the classical "cannabinoid binding pocket" (210). Using sequence to structure alignments with the well-known β_2-adrenergic receptor (PDB: 2RH1) structure (211), a homology model of GPR55 in both its active and inactive state was constructed (209). These models were used to examine the ligand binding pocket of GPR55 and identify important amino acid interactions for ligand binding.

6.2 GPR55 signaling pathways

Several endogenous GPR55 signaling pathways have been described to date (197;208;212-214). One consensus within several groups is that GPR55 couples to Gα13 and/or Gαq proteins in recombinant HEK 293 cells transiently or stably expressing GPR55 (49;201;208;213;215;216). In addition, GPR55 has been reported to activate small GTPases (49;197;201;216) and to induce oscillatory calcium release from intracellular stores (49;216-218). Further downstream, GPR55 activation has been shown to lead to the activation of several transcription factors, such as nuclear factor of activated T-cells (NFAT), nuclear factor kappa of activated B cells (NF-κB), cyclic AMP response element binding protein (CREB) and activating transcription factor 2 (ATF2) (49;216;219). In addition, the activation of MAP-kinases, such as p38 and ERK1/2 MAPKs, were described to be induced after GPR55 stimulation (216;219).

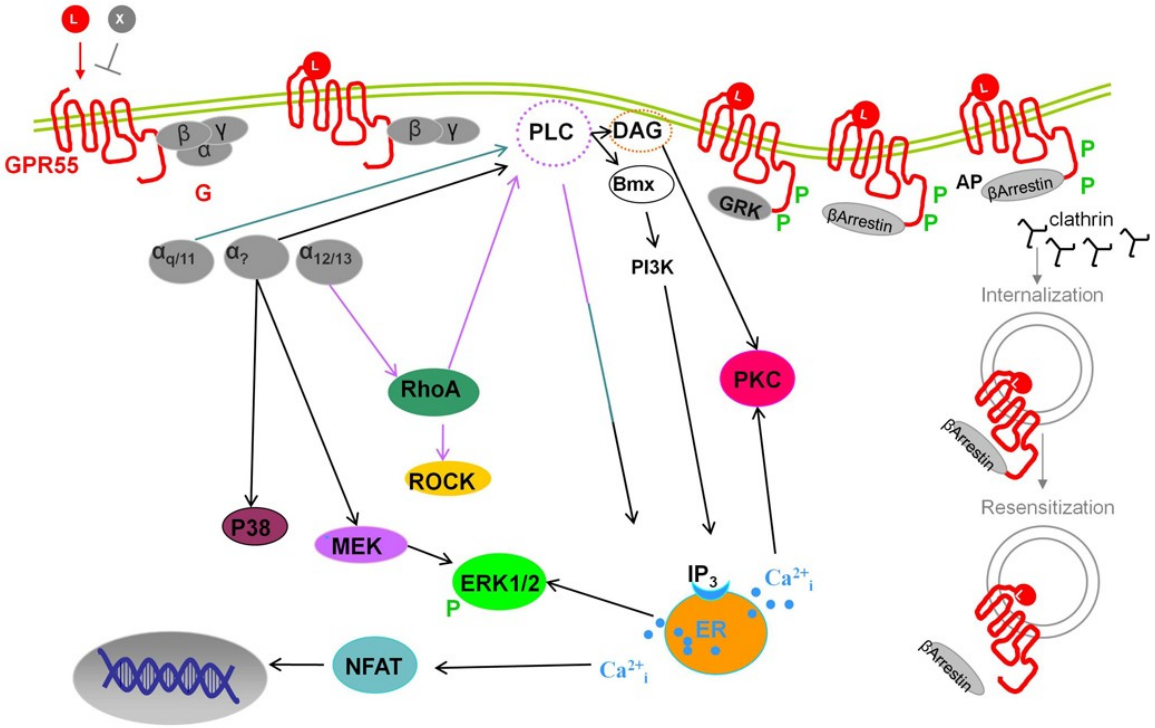

FIGURE 9: Scheme of diverse signaling pathways initiated by GPR55. Multiple signaling pathways can be activated by GPR55 agonists via the activation of Gα12/13 and Gαq. In addition, mitogen-activated protein kinases (MAPK, p38, MEK, ERK1/2), small GTPases, such as RhoA can be activated and intracellular Ca^{2+} is released via the activation of phospholipase C (PLC). GPR55 activation leads to the activation of transcription factors (e.g. NFAT) (taken from (208)).

6.3 GPR55 ligands

The first endogenous ligand for GPR55 has been discovered in 2007 by Oka *et al.* (217), the lipid lysophosphatidylinositol (LPI). In addition, several synthetic CB_1R inverse agonists/antagonists, such as AM251, AM281 and rimonabant (SR141716A, Acomplia ®, Sanofi-Aventis) have been shown to activate GPR55 (49;201;216-218;220;221). In addition, three groups recently described several potent and selective GPR55 agonists and antagonists (209;218;222). These new compounds are reported to be inactive at CB_1 and CB_2 receptors and are promising tools to elucidate the pharmacological, physiological and pathophysiological functions of GPR55. One specific GPR55 agonist is GSK319197A [4-(3,4-Dichloro-phenyl)-piperazin-1-yl]-(4'-fluoro-4-methanesulfonyl-biphenyl-2-yl)-methanone (see patent reference Bradley DM; Example 13), a structural analogue of GSK494581A (218) and CID2440433 (209). It is one of a series of benzoylpiperazines originally identified as inhibitors of the glycine transporter subtype 1 (GlyT1) and shown to

be a selective ligand of GPR55 (209;218). GSK319197A activates human GPR55 (pEC_{50} = 6.4 ± 0.29 (mean ± standard deviation); n=6) in the yeast reporter gene assay described in Brown et al. (218) but does not activate rat GPR55 expressed in the same host. GSK319197A also activates human GPR55 stably expressed in HEK293 cells in combination with apoaequorin and the promiscuous Gα subunit, Gα16z49 (218), causing mobilisation of intracellular calcium (pEC_{50} = 5.2 ± 0.16 (mean ± standard deviation); n=6). In yeast and mammalian HEK293 expression systems, GSK319197A behaves as a full agonist at human GPR55. GSK319197A also inhibits binding of [^{3}H]glycine to HEK293 cells stably expressing GlyT1 in a Scintillation Proximity Assay (pIC_{50} = 6.4 ± 0.02 (mean ± standard deviation); n=2)(218).

Summarized, however, the current pharmacological profile of GPR55 does not merit a classification as a 'novel' or '3rd' member of the cannabinoid receptor family.

6.4 Trafficking of GPR55

Little is known regarding the internalization and post-endocytic trafficking properties of GPR55. Without ligand stimulation, GPR55 is predominantly located on the cell surface and internalizes following agonist stimulation in different recombinant cell models, such as HEK293, U2OS and MCF-7 cells (49;205;216;220). Interestingly, endogenous GPR55 distribution differs from that observed in recombinant cell models. In primary cells, such as in human neutrophils and human promyelocytic leukemia cells (HL60) (197), endothelial cells (199), Mz-ChA-1 cells (206) and decidual cells, as well as in uterine maternal tissues (223), GPR55 is predominantly located intracellulary.

6.5 Physiology and Pathophysiology of GPR55

Accumulating evidence suggests that GPR55 plays a role in diverse (patho-)physiological processes (198), including cancer development (203-206), bone formation (214), pain regulation and inflammation (197;202;224). For instance, it has been demonstrated that GPR55 and CB_2 receptors are co-expressed and crosstalk at the level of small GTPases in human neutrophils. Thereby GPR55 enhances the migratory capacity of neutrophils, while it limits the bactericidal functions of neutrophils, such as reactive oxygen species (ROS)

production and degranulation (197). Further, it has been reported that GPR55 is highly expressed in malignant human tumors (203) and cancer cell lines (204-206) and its expression level is directly correlated to the aggressiveness of the tumors (203). Likewise, GPR55 was shown to be expressed in human osteoclasts and its activation could stimulate osteoclast polarization and resorption *in vitro* (214). In addition, GPR55 KO mice were resistant to inflammatory and neuropathic pain (224) and GPR55 may play a role in inflammatory responses of microglial cells (202).

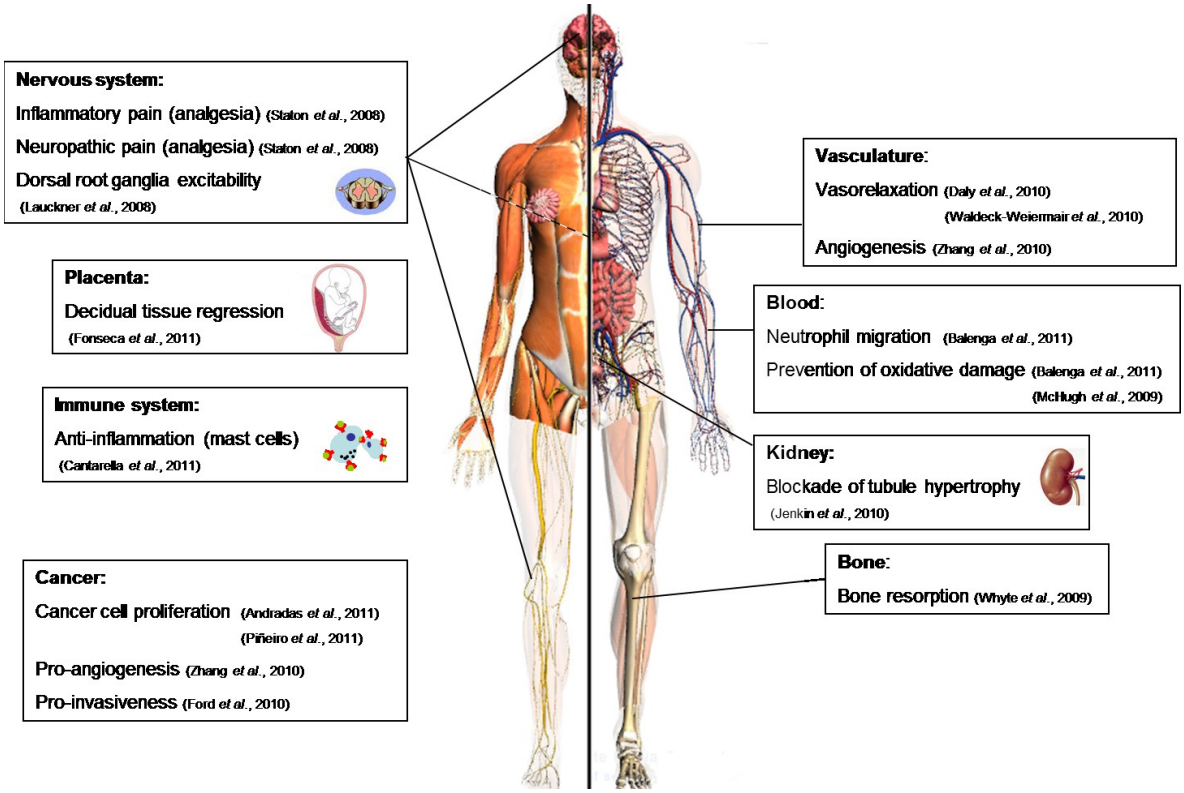

FIGURE 10. Pathophysiological relevance of GPR55 expression. Recent evidence suggests that GPR55 is involved in the control of a variety of physiological functions. In the nervous system, GPR55 regulates dorsal root ganglia excitability (213) and controls inflammatory and neuropathic pain (224). In blood, GPR55 regulates neutrophil migration (197) and may prevent oxidative damage (197;225). GPR55 is also involved in bone metabolism, specifically inducing bone resorption (214). Other studies have suggested additional roles for GPR55 in modulating vascular function [by inducing vasorelaxation (199;226) and controlling angiogenesis (227), renal tubule hypertrophy (228), decidual tissue regression during pregnancy (223), and mast cell-mediated antiinflammatory actions (229)]. However, additional experimental evidence is required to support these hypotheses. Besides controlling these (and most probably many other) physiological functions, GPR55 seems to play an important role in cancer progression by modulating cancer cell proliferation (203;204) and migration (205). (from (198)).

7 AIMs

This thesis consists of two major projects described in two parts.

The aims of this thesis were as following:

- PART II: To elucidate the post-endocytic sorting properties of GPR55 *in vitro* and *in vivo*

- PART III: To determine possible interactions and/or cross-regulation of GPR55 and CB_1 receptors

PART II

THE GPCR – ASSOCIATED SORTING PROTEIN 1 REGULATES LIGAND-INDUCED DOWNREGULATION OF GPR55 *IN VITRO* AND *IN VIVO*

Parts of this chapter were recently published in:

Kargl J, Balenga NA, Platzer W, Martini L, Whistler JL, Waldhoer M. (2011). The GPCR - associated sorting protein 1 regulates ligand-induced downregulation of GPR55. *Br. J. Pharmacol*., doi: 10.1111/j.1476-5381.2011.01562.

1 Abstract

Cannabinoids play an important role in medicine due to their psychoactive, analgesic and anti-inflammatory properties, mediated mainly by the cannabinoid CB_1 and CB_2 receptors. Recently, the lipid receptor GPR55 was 'de-orphanized' and characterized as a novel, receptor for synthetic cannabinoids and small lipid mediators. Of these, rimonabant (SR141716A) – initially developed as a CB_1 receptor inverse agonist/antagonist – has recently attracted significant attention, since it was marketed for weight loss and smoking cessation (Acomplia®; Sanofi-Aventis). Due to adverse side effects, including the development of anxiety and depression, rimonabant was withdrawn from the market. Recently, rimonabant has been shown to be not only an antagonist/inverse agonist at the CB_1 receptor but also an agonist at GPR55, a potential off target effect that could be contributing to the adverse psychotropic events.

One regulatory mechanism to guarantee appropriate 7TM/GPCR expression levels in physiological conditions is that of downregulating 7TM/GPCRs via the GPCR-associated sorting protein 1 (GASP-1), thus leading to an attenuation of cellular signaling events. GASP-1 was originally found to target delta opioid receptors to lysosomes and hence, to the degradative pathway. It was shown that GASP-1 is a key determinant in the development of analgesic tolerance to cannabinoids via its role in facilitating downregulation of the CB_1 receptor *in vitro* and *in vivo*. To date little is known about how trafficking of 7TM/GPCRs alters their responsiveness to neuropsychiatric drugs, such as rimonabant.

Here I investigated the post-endocytic properties of GPR55 after agonist exposure *in vitro* and *in vivo* and tested whether GASP-1 was involved in this process. I show that the prolonged activation of GPR55 with rimonabant or LPI downregulates GPR55 via GASP-1. GASP-1 binds to GPR55 *in vitro* and this interaction is required for targeting GPR55 for degradation. Disrupting the GPR55-GASP-1 interaction prevents post-endocytic receptor degradation, and thereby allows receptor recycling. Further, I demonstrate that long-term rimonabant administration downregulates GPR55 via GASP-1 *in vivo*. GASP-1 knock-out, but not WT mice, show a higher degree of anxiety- and depression-like behavior after

prolonged rimonabant treatment, indicating that proper GPR55 downregulation via GASP-1 is a crucial step in rimonabant induced side-effects.

These data implicate GASP-1 as an important regulator of ligand mediated downregulation of GPR55 *in vitro* and *in vivo*. The absence of GASP-1 enhances rimonabant induced anxiety- and depression-like behavior. By identifying GASP-1 as a key regulator of the trafficking and – by extension - functional expression of GPR55 *in vitro* and *in vivo*, we may be one step closer to gaining a better understanding of this receptor in response to cannabinoid drugs.

2 Materials and Methods

2.1 Materials

Mouse M1 and M2 monoclonal antibodies, β-actin antibody, anti-FLAG M2 affinity matrix, BSA, LGlutathione, Iodoacetamide, Triton X-100, Poly-D-Lysine, Kodak BioMax light films, Lysophosphatidylinositol (LPI), Tween 20, Sigmacote and Bradford substrate were purchased from Sigma Aldrich (Austria). 4-20% Tris-Glycine gels, cell culture reagents, Lipofectamine 2000, Alexa Fluor647nm conjugated IgG2b were from Invitrogen (Austria). HRP-conjugated antibodys were obtained from Jackson Immuno Research (Dianova, Germany). The generation of anti-GASP-1 antibodies has been previously described (115). Complete Protease inhibitor cocktail tablets were from Roche (Austria) and PNGaseF from New England Biolabs (Germany). Immobilon-P Transfer Membrane and a 12-well sampling manifold was purchased from Millipore (Austria). ECL Western Blotting Substrate was from Pierce (THP, Austria). Vectashield mounting medium and Vectastain ABC Kit were purchased from Vector Laboratories (Szabo-Scandic, Austria). EZ-Link Sulfo-NHS-SS-Biotin was from Thermo Scientific (Histocom, Austria). Ethanol, NaOH, KCl and CaCl2*2H2O were obtained from Merck (Austria), NaCl, Tris and Formaldehyde were from Roth (Lactan, Austria). TNT T7 Coupled Reticulocyte Lysate System was purchased from Promega (Austria). EasyTag ^{35}S-methionine and Ultima Gold scinitlation solution were obtained from PerkinElmer and PAGEBlue from Fermentas (Austria). GF/C glass fiber filters were purchased from Whatman. SR141716A (rimonabant, RIM) was from Sanofi-Synthélabo Recherche (Montpellier, France). WIN55,212-2 was purchased from Tocris Cookson (USA), GSK319197A was kindly provided by GlaxoSmithKline, Andrew Brown and [^{3}H]SR141716A was from Amersham Biosciences (USA). The dark–light apparatus was obtained from ENV-510; Med Associates, (USA). LPI was dissolved in H2O and SR141716A in DMSO.

2.2 DNA constructs

The human GPR55 was tagged on the N-terminus with the FLAG epitope (DYKDDDDA) and inserted into a pcDNA3.1(+)vector. The cloning of the FLAG-DOP and FLAG-MOP receptors was previously described in (115). The GPR55-GST-fusion protein was constructed by sub-cloning the last 34 carboxy-terminal amino acids of the human GPR55 into the pGEX-4T-1 plasmid. The generation of the GST-fusion protein constructs of the DOP receptor, the MOP receptor and the HA-tagged GASP-1 construct (HA-GASP-1) were previously described (Whistler *et al.*, 2002). The generation of the lentivirus constructs encoding shRNA against GASP-1 (shGASP-1) or scrambled shRNA (shScr) are described in (134). All DNA constructs were verified by sequencing.

2.3 Cell culture and stable cell lines

HEK293 cells were cultured in Dulbecco´s modified Eagle´s Medium (DMEM) supplemented with 10% FBS at 37°C in 5% CO_2, humidified atmosphere. HEK293 cells stably expressing the human FLAG-GPR55 (GPR55-HEK) were generated by selection with 0.4 mg/ml zeocin containing medium and maintained in 0.2 mg/ml zeocin supplemented media. GPR55-HEK cells were starved over night in Opti-MEM prior to all experiments.

2.4 Co-Immunoprecipitation

FLAG-GPR55 was co-immunoprecipitated with endogenous GASP-1 as previously described (115). Briefly, HEK293 cells were transiently transfected with FLAG-GPR55 using Lipofectamine 2000 and 48 hours post transfection experiments were performed. Cells were washed twice with ice cold PBS and lysed in IPB buffer (0.3 % Triton X-100, 150 mM NaCl, 25 mM KCl, 1mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4 supplemented with complete protease inhibitors). Lysates were centrifuged and supernatant incubated with 20µl of anti-FLAG M2 monoclonal antibody affinity matrix over night at 4°C. 30µl of lysates were kept for FLAG, GASP and β-actin control blots. Precipitates were washed, deglycosylated with PNGase, resolved by SDS-polyacrylamide gel electrophoresis

(SDS/PAGE) and transferred to a PVDF membrane. Membranes were blocked, probed with either anti-FLAG M2 antibody (1:500), anti-GASP antibody (1:1000) or β-actin monoclonal antibody (1:1000) and immunoblotted with HRP-conjugated anti-mouse or anti-rabbit antibody. Blots were visualized with ECL Western Blotting Substrate.

2.5 GST-fusion protein-binding assay

GST-fusion protein-binding assays were carried out as described in (115). GST-fusion proteins were expressed in *E. coli* and bound to glutathione-agarose. Fusion proteins on beads were incubated in blocking buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 5% BSA), while ^{35}S-Methionine labelled HA-GASP-1 was synthesized using a TNT T7 Coupled Reticulocyte Lysate System and subsequently incubated with the fusion proteins in wash buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100) for one hour at 4°C. Probes were washed and resolved on a SDS/PAGE. Gels were stained with PAGEblue, dried and exposed to X-ray films.

2.6 Lentivirus production and shRNA knock-down of GASP-1

GASP-1 knock-down experiments were performed as previously described (134;230). In brief, virus was produced in HEK293T cells and harvested 48 hours post-transfection. Knock-down of GASP-1 levels in GPR55-HEK cells was induced by infection with lenti-shGASP-1 (shGASP-1) or lenti-shScrambled (shScr) virus for 48 hours. EGFP was used to determine successful lenti-virus infection.

2.7 Biotin internalization and protection/degradation assays

GPR55-HEK cells were infected with either shScr or shGASP-1 virus and experiments were conducted as previously described (115;134). In brief, cells were incubated with disulfide cleavable biotin for 10 min at 4°C and washed with TBS (25 mM Tris base,135 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$ * $2H_2O$, pH 7.4). For ***internalization assays,*** cells were placed in warm medium for 0 to 45 minutes in the presence of ligands (RIM or LPI) /vehicle (DMSO or H2O) to allow receptor endocytosis. Remaining cell surface-biotin was

stripped (50 mM glutathione, 75 mM NaCl) (except 100% plates) and quenched (50 mM iodoacetamide, 1% BSA in PBS), followed by cell lysis (0.3 % Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, 1 mM $CaCl_2$, 5 mM iodoacetamide, pH 7.4, with complete protease inhibitors). ***Protection/degradation assays*** was performed as described above. After quench, cells were placed in warm Opti-MEM for the indicated time points to allow receptor degradation (except 100% and Strip) before cell lysates were prepared. Samples of cleared lysates were taken for control blots (anti-GASP, anti β-actin) and the remainder was immunoprecipitated with anti-FLAG M2 affinity matrix. Precipitates were washed, deglycosylated, resolved by SDS/PAGE and visualized with Vectastain ABC. Blots were quantified using IMAGEJ Software.

2.8 Immunocytochemistry

Co-localization experiments with transferrin. GPR55-HEK cells infected with either shScr or shGASP-1 lentivirus were grown on poly-D-lysine coated cover slips to ~50% confluency. Cells were starved in Opti-MEM overnight and subsequently antibody feeding experiments were performed essentially as described (130). In brief, living cells were incubated with anti-FLAG M1 antibody (1:1000) for 45 minutes and treated with agonists (RIM or LPI) for 90 minutes. 30 minutes before fixation, cells were labelled with AlexaFluor 594nm conjugated transferrin (1:500). Cells were fixed in 3.7% formaldehyde in PBS and permeabilized in blotto (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.3% Triton X-100 and 3% milk). Receptors were visualized using AlexaFluor 647-conjugated IgG2b antibody (1:1000, 20 minutes) directed against the M1 antibody.

Co-localization experiments with LAMP1 and LAMP2. Experiments were performed as described above, but incubated with a monoclonal antibody directed against LAMP1/2 (IgG1 1:500) for 1 hour. Receptors were visualized using the AlexaFluor 647-conjugated IgG2b directed against the M1 antibody (1:1000, 20 minutes) and the AlexaFluor 594-conjugated IgG1 directed against LAMP 1/2 (1:500, 20 minutes).

Recycling experiments. ShScr or shGASP-1 virus infected GPR55-HEK cells were grown on poly-D-lysine coated coverslips. Living cells were incubated with the Ca^{2+}-sensitive anti-FLAG M1 antibody and stimulated with agonist (RIM or LPI) /vehicle (DMSO or

H2O) for 45 minutes. Remaining surface receptors – except those at 0 minute and 45 minute time points – were stripped with 0.04% PBS-EDTA and warm Opti-MEM was added for up to 90 minutes. Cells were fixed in 3.7% formaldehyde, blocked (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and 3% milk) and stained with Alexa647-conjugated IgG2b under non-permeabilizing conditions. Receptors were visualized using a laser-scanning confocal imaging system (Zeiss LSM510).

2.9 Animals

All animal experiments were carried out at the Ernest Gallo Clinic and Research Center, UCSF, CA, USA. For all experiments GASP-1 knock-out and control littermates (C57/Bl6 background) were kept in a climate controlled room under a 12:12 light/dark cycle with ad libidum access to food and water. All procedures were conducted according to the guidelines provided by the Institutional Animal Care and Use Committee at Ernest Gallo Clinic and Research Center, CA. Only male mice were used because the female hormonal cycle may affect the responsiveness to cannabinoids (231). For anxiety experiments each animal was only tested once, because the novelty of the experimental setup is only guaranteed the first time the animal is tested.

2.10 Behavioral tests & Drug Treatments

Mice were treated with 10mg/kg rimonabant or vehicle for 21 days. Twenty four hours after the final injection, mice were tested for their behavior and subsequently sacrificed to isolate tissue for radioligand binding analysis. Rimonabant was dissolved in a 1:1:18 solution of ethanol/cremophor/saline in glass vials pretreated with Sigmacote.

2.11 Membrane preparation for saturation binding analysis

Mouse brain membranes were prepared essentially as previously described (141;232). Amygdala and hippocampus from 8 mice undergoing the same treatment paradigms were dissected and pooled into separate vials. Tissues were homogenized (320mM Sucrose, 10mM Tris-HCl and Protease-inhibitors) using a Teflon/glass douncer and centrifuged at

1000 g for 10 minutes at 4°C. The supernatants were then centrifuged at 40000 g for 20 minutes at 4°C and the resulting pellets were washed in homogenization buffer. Membranes were resuspended in buffer (50mM Tris-HCl, 3mM $MgCl_2$, 1mM EDTA; pH 7.4). Protein concentrations were determined by the Bradford method and aliquots were snap frozen in liquid nitrogen and stored at −80°C.

2.12 Radioligand binding

Membrane suspensions were prepared by diluting 50µg amygdala or hippocampus membrane protein in 450µl assay buffer (50mM Tris-HCl, 3mM $MgCl_2$, 1mM EDTA, 0.5% BSA; pH 7.4). Membrane suspensions, which were pre-incubated at 4°C for 10 minutes with either 1µM WIN55,212-2 or 10 µM GSK319197A in Sigmacote-treated glass vials (218) were used for determining non-specific binding (NSB). DMSO-treated membrane suspensions were used for determining total binding (TB). To initiate binding, 50µl of 1.25nM [^{3}H]SR141716A were added to the membranes and the vials were incubated at 30°C for 60 minutes. The reaction was terminated by addition of ice cold wash buffer (50mM Tris-HCl and 0.1% BSA; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass fiber filters using a 12-well sampling manifold. The tubes were rinsed twice over the filters with 5ml of ice cold wash buffer. Finally, the filters were placed into plastic scintillation vials containing 5ml Ultima Gold scintillation cocktail. Bound radioactivity was determined by liquid scintillation counting (Beckman LS6500) the next day. The determination of TB and NSB was performed in duplicates and specific binding was calculated by subtracting NSB from TB.

2.13 Elevated-Plus Maze

The Elevated-Plus Maze consists of two closed arms and two open arms arranged perpendicular to one another. The plus maze was made of wood, painted white, and elevated 41 cm above the floor (arm length 70 cm, width 9 cm, and height 12 cm) (Fig. 11). During the 5 minute trial, the behavior of the mouse was recorded by a camera positioned above the maze in the absence of laboratory personnel. Each mouse was placed in the center of the maze (9 x 9 cm) facing a closed arm. The surface of the maze was

cleaned with disinfectant and dried before the next mouse was tested. Light intensity on the plus maze was 460 lux. The variables measured included the (i) total number of entries into the closed and open arms and (ii) the total time spent in each region. An entry was defined as one-half of a mouse being located within the boundaries of the arm. An increase in the number of entries and time spent in the open arms was indicative of an anxiolytic-like response.

FIGURE 11. Scheme of an Elevated-Plus Maze apparatus. (Taken from: https://www.stoeltingeurope.com/stoelting/2151/2614/1500/EUmaze/Elevated-Plus-Maze)

2.14 Dark–Light Transition Box

The dark–light apparatus consists of an automated activity monitor with a dark box insert to create two equally spaced light and dark compartments (24 cm x 28 cm x 25 cm) (Fig. 12). The entire apparatus was positioned in a sound-attenuating chamber. The light side was illuminated to a degree of 100 lux, compared with 5 lux in the dark side. Each animal was placed facing the entrance of the dark area, and behavior was recorded for 5 min. The dark–light transition box was cleaned with disinfectant and dried before the next mouse was tested. A photobeam-based tracking system was used to track the movement and locomotor activity of the mice within the test box and calculated (i) the time spent in each area, (ii) the number of entries into each area. Anxiolytic-like effects were indicated by increased time spent in the illuminated compartment.

FIGURE 12. Scheme of a Dark-Light Transition Box. (Taken from: http://kc.vanderbilt.edu/mnlcore/OFchamber.html)

2.15 Forced Swim Test

Experiments were performed as previously described (233). A glass cylinder (60cm high, 30 cm in diameter) was filled with 27°C water to a depth of 22 cm (Fig. 13). Each mouse was gently placed in the cylinder for 10 minutes and the last 6 minutes were considered for analysis. In this experimental set up, immobility of the animal is interpreted as depression like behavior. The duration of movement was scored.

FIGURE 13. Scheme of a Forced Swim Test apparatus. (Taken from: http://btc.bol.ucla.edu/neuroscreen.htm)

2.16 Data analysis

Statistical analysis was performed by Prism 4.03 (GraphPad Software, USA) using two-way analysis of variance (ANOVA) for comparisons between multiple groups followed by a Bonferroni´s post-hoc test. A p-value of < 0.05 was considered significant.

3 Results

3.1 GPR55 interacts with GASP-1

In HEK293 cells expressing GPR55, the receptor co-immunoprecipitated with endogenous GASP-1 (Fig. 14A, upper panel, GPR55). Many 7TM/GPCRs interact with GASP-1 through their cytoplasmic tails (123). This was also the case for GPR55. Specifically, a purified recombinant glutathione S-transferase (GST)-fusion protein containing the last 34 carboxy-terminal amino acids of GPR55 bound GASP-1 *in vitro*, similar to the c-terminus of the delta-opioid receptor (Fig. 14B, upper panel, GPR55 and DOP) (115), which served as positive control. Neither the control GST protein nor the carboxy-terminus of the mu-opioid receptor (115) significantly bound GASP-1 (Fig. 14B, upper panel, MOP and GST).

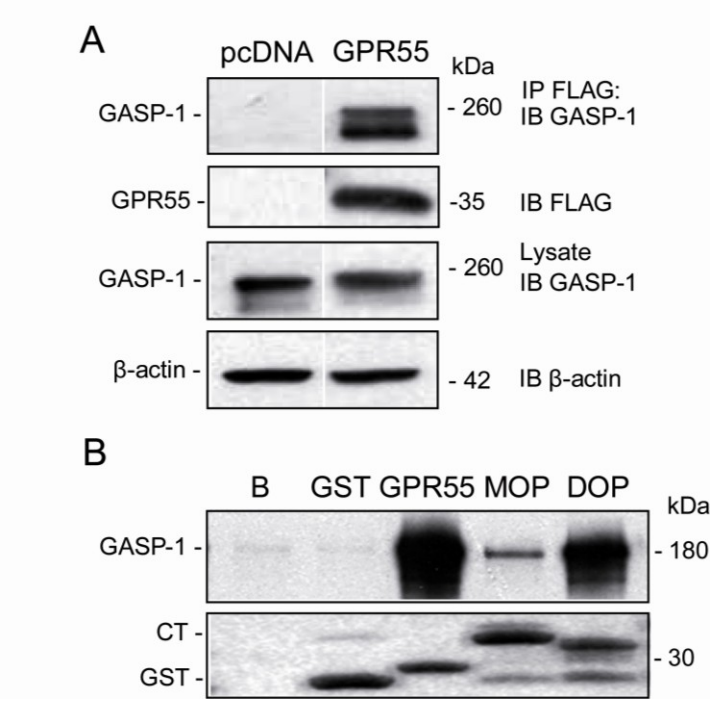

FIGURE 14. GPR55 interacts with GASP-1 *in vitro*. (A) pcDNA3.1 (pcDNA) or FLAG-GPR55 (GPR55) were immunoprecipitated with anti-FLAG affinity matrix (IP) and immunoblotted (IB) for GASP-1 (first panel) and receptor (second panel). Lysates were probed for GASP-1 (third panel) and β actin (fourth panel). (B) GST-fusion proteins containing the c-tail (CT) of GPR55 and the delta-opioid receptor bind ^{35}S-methionine-labelled recombinant GASP-1. In contrast, GASP-1 did not bind to empty beads (B), the GST-protein (GST) alone or GST-MOP. Total protein levels are shown below the autoradiograph. Blots are representative of four independent experiments (from (234)).

3.2 GASP-1 mediates the lysosomal targeting of GPR55 in response to LPI and rimonabant

GASP-1 is known to target many 7TM/GPCRs to lysosomes following prolonged agonist stimulation (98;115). We have recently shown that the GPR55 agonists LPI and rimonabant (SR141716A, RIM) are capable of inducing receptor internalization in a recombinant GPR55-HEK cell model (49;216). However, to date the post-endocytic fate of GPR55 following prolonged agonist stimulation has not been clarified.

I tested whether GPR55 co-localized with the lysosomal markers LAMP1/2 (lysosomal associated membrane proteins 1 and 2) following prolonged stimulation with either LPI or rimonabant and whether this process is regulated by GASP-1. It has recently been described the feasibility of using a lentivirus to knock-down endogenous GASP-1 levels in HEK293 cells using specific shRNAs (134). Lentiviral infection of HEK293 cells stably expressing GPR55 (GPR55-HEK) with shRNA targeting GASP-1 showed an efficient knock-down of GASP-1 protein levels after 48 hours (Fig. 15, shGASP-1, GASP-1 lane at ~260kDa), while scrambled shRNA virus had no effect on GASP-1 levels (Fig. 15, shScr). Lentiviral infection with both shRNAs did not alter GASP-2 levels (Fig. 15, GASP-2 lane at ~ 135kDa).

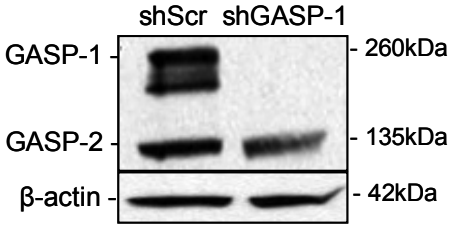

FIGURE 15. GASP-1 knock-down in GPR55-HEK cells. shRNA specifically targeting GASP-1efficiently knocks-down endogenous GASP-1 levels in HEK293 cells stably expressing FLAG-GPR55. GASP-2 levels are not altered by shRNA lenti-virus infection. β-actin served as protein control.

In cells infected with shScr virus, co-localization of GPR55 (Fig. 16A, green) with LAMP 1/2 positive lysosomes (Fig. 16A, red) was observed following treatment with 2.5μM LPI or 2.5μM RIM as early as 30 minutes, but was most prominent after 90 min (16A, magnification of merge, yellow). Hence, both ligands were able to promote a significant and quite similar redistribution of GPR55 to lysosomes in cells expressing GASP-1. In contrast, I could not observe a co-localization of GPR55 with LAMP1/2 in shGASP-1

infected cells (Fig. 16B) following 2.5µM LPI or 2.5µM RIM induced stimulation up to 90 minutes (Fig. 16B, magnification of merge). This suggests that GASP-1 is a key determinant in sorting GPR55 to lysosomes following prolonged agonist stimulation.

In fact, in cells devoid of GASP-1 most of the receptor (Fig 16B, green) remained on - or was detected in vesicles close to - the cell surface, thus suggesting that GASP-1 is involved in either (i) promoting the internalization or (ii) blocking the recycling of GPR55 following agonist stimulation.

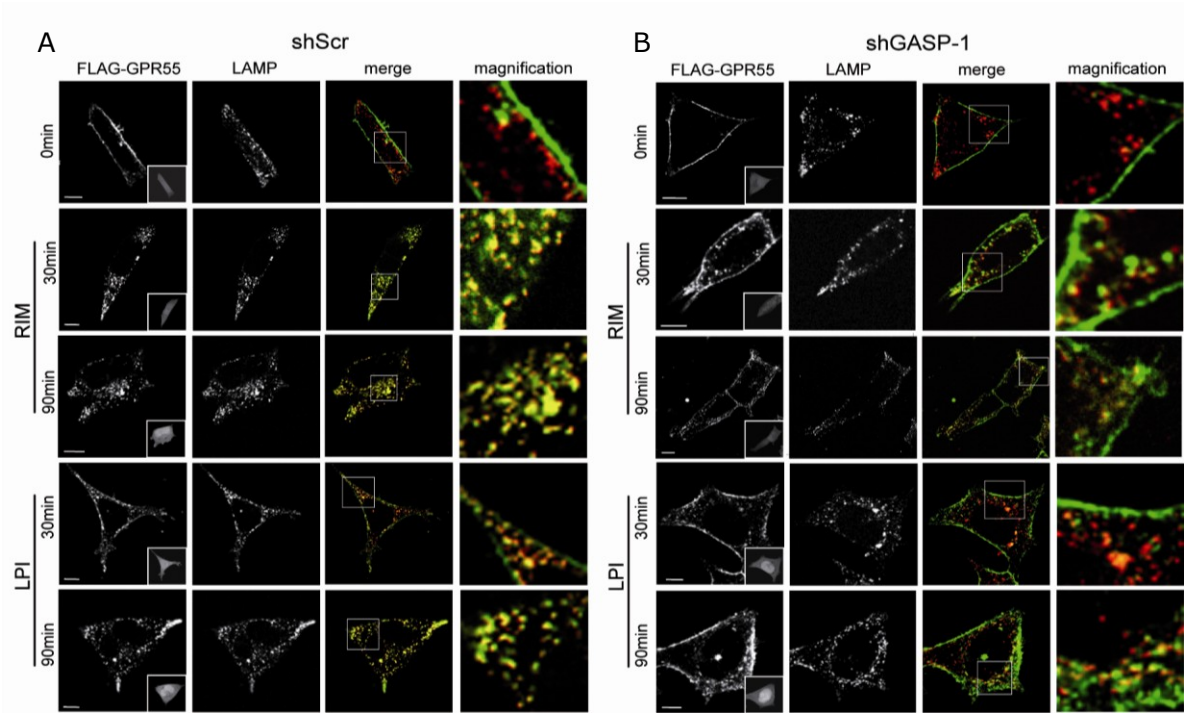

FIGURE 16. GASP-1 promotes the sorting of GPR55 to lysosomes. (A) GPR55-HEK cells infected with shScr lenti-virus were fed anti-FLAG antibody and were either left untreated (0 min) or treated with 2.5 µM of RIM or LPI for 30 or 90 minutes. Receptors (green) were analysed for co-localization with the lysosomal markers LAMP1/2 (red) (B) GPR55-HEK cells were infected with shGASP-1 and treated as in A. No co-localization was observed for GPR55 (green) and LAMP1/2 (red) in shGASP-1 cells, but receptors were predominantly found on - or in vesicles close to - the cell surface. Inserts in FLAG-GPR55 panels indicate EGFP-shRNA-virus expression. Scale bars = 10 µM. (from (234)).

To identify the vesicles to which GPR55 is distributed after ligand stimulation and the fate of the receptor in the absence of GASP-1, I investigated if GPR55 co-localizes with the recycling endosomal marker transferrin. ShSrc lentivirus infected GPR55-HEK cells were stimulated with 2.5µM LPI or 2.5µM RIM for 90 minutes and receptor internalization was observed. As Figure 17 shows, FLAG-GPR55 does not co-localize with transferrin under these conditions (Fig. 17A). In contrast, when GPR55-HEK cells were infected with

shGASP-1 lentivirus, FLAG-GPR55 remained close to the cell surface after ligand stimulation and co-localized with the recycling endosomal marker transferrin (Fig. 17B).

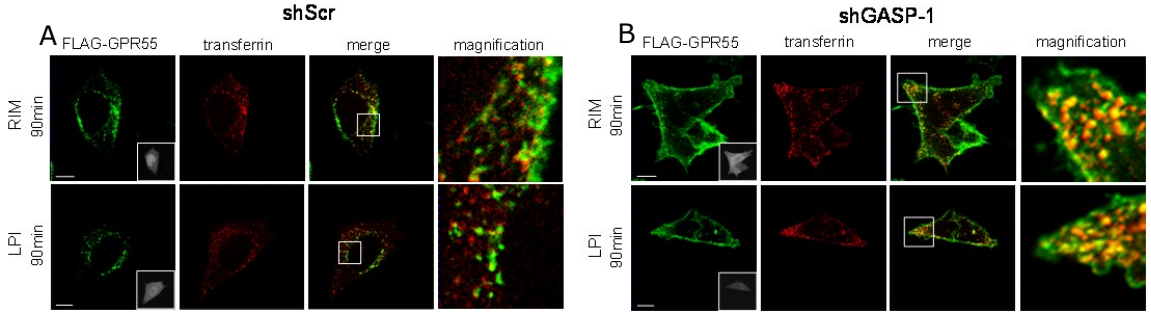

FIGURE 17. Co-localization of GPR55 with the endosomal marker transferrin in the absence of GASP-1. HEK293 cells stably expressing GPR55 were infected with (A) shScr or (B) shGASP-1 lentivirus. Antibody feeding experiments were conducted using anti-FLAG M1 antibodies for 30 minutes and subsequently cells were stimulated with 2.5µM of the GPR55 agonists RIM (upper panels) or LPI (lower panels) for 90 min. AlexaFluor 594-conjugated transferrin was added to each well 30 min before cells were fixed. Cells were analysed for FLAG-GPR55 (green) and transferrin (red) (A) GPR55-HEK cells infected with shScr lentivirus show no co-localization with transferrin, (B) in contrast, in the absence of GASP-1 GPR55 is predominantly located close to the cell surface and co-localizes with the recycling endosomal marker transferrin. Inserts in FLAG-GPR55 panels indicate EGFP-shRNA-virus expression. Scale bars = 10 µM.

Summarized, these data provide first evidence that GPR55 is targeted to the lysosomes after prolonged agonist stimulation in the presence of GASP-1 (Fig. 16A). In the absence of GASP-1, however, the receptor is detected in recycling endosomes following agonist stimulation (Fig. 17B).

3.3 The internalization of GPR55 is not regulated by GASP-1

In various recombinant cell lines - including HEK293, U2OS and MCF-7 cells - GPR55 is predominantly located on the cell surface, but internalizes following agonist stimulation (49;205;216;220). To examine whether GASP-1 affects the internalization of GPR55, I quantified the rate of internalization using a biotinylation protection assay in HEK293 cells where GASP-1 levels were knocked-down with shRNA lentiviruses. This assay allows the selective monitoring of the endocytic fate of a pool of receptors that is expressed on the cell surface and is subsequently stimulated with agonists. In brief, receptors that reached the cell surface of intact cells were labeled with thio-cleavable disulfide-linked biotin (Fig. 18A and B; upper panel, 100%). Cells were then incubated with agonists in warm media to allow biotinylated receptors to internalize for up to 45 minutes. After this incubation step,

cell surface remaining biotin was cleaved with a membrane impermeable reducing agent and the 'protected' internalized biotinylated receptor pool was immunoprecipitated and detected by streptavidin overlay. The shRNA caused efficient knock-down of GASP-1 protein expression 48 hours after infection (Fig. 18B; GASP-1), while scrambled shRNA virus (shScr) had no effect on GASP-1 levels (Fig. 18A; GASP-1). In both, shScr (Fig. 18A) and shGASP-1 (Fig. 18B) infected cells, biotinylated GPR55 internalized following treatment with either 2.5μM rimonabant (Fig. 18A and B; left panels, RIM and 18C) or 2.5μM LPI (Fig. 18A and B; right panels, LPI and 18C). 45 minutes of vehicle (DMSO, final concentration 0.025%) treatment did not lead to significant receptor internalization in the presence and absence of GASP-1, respectively (Fig. 19). Lysates were immunoblotted for GASP-1 and β-actin (Fig. 18A and B; lower panels). GASP-1 does not appear to affect internalization of GPR55.

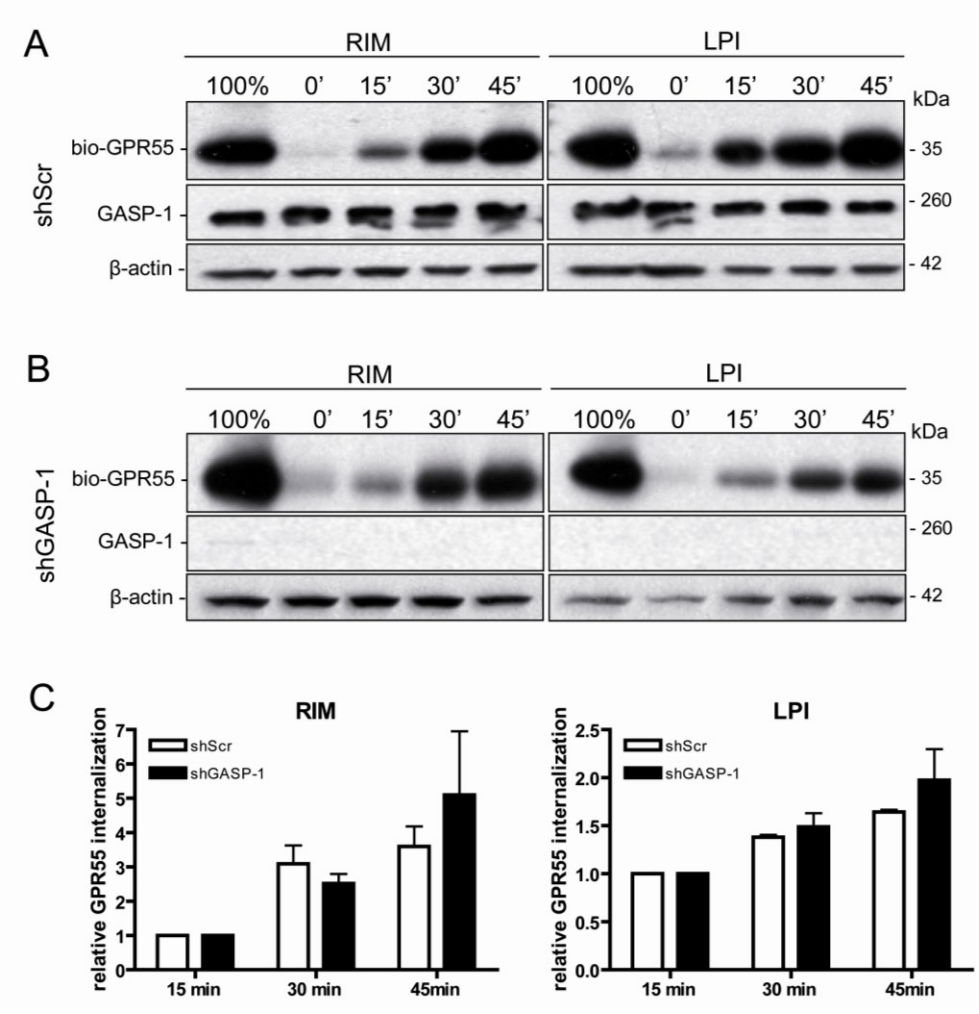

FIGURE 18. Knock-down of GASP-1 does not impair GPR55 internalization. GPR55-HEK cells were infected with either (A) shScr or (B) shGASP-1 lentivirus. Internalization of biotinylated FLAG GPR55 (bio-GPR55) was monitored for the indicated time points (0', 15', 30' and 45'). GPR55 internalized after stimulation with 2.5µM RIM (left panels) or 2.5µM LPI (right panels) in both, shScr and shGASP-1 infected cells. The lower panels show the corresponding lysate samples immunoblotted for GASP-1, indicating successful GASP-1 knock down, and β-actin levels served as protein control. (C) Bio-GPR55 bands were normalized to β-actin and 15 minute values were set at 1. Data are means of three independent experiments ± SEM (from (234)).

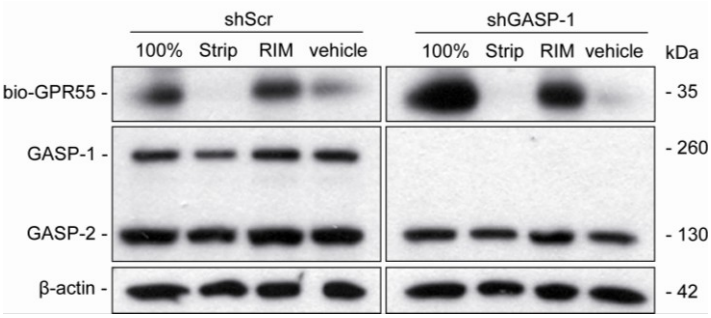

FIGURE 19. GPR55 does not internalize in response to vehicle. GPR55-HEK cells were infected with shScr (left panel) or shGASP-1 (right panel) lentivirus. In the presence and absence of GASP-1 biotinylated GPR55 (bio-GPR55, 100%) internalized in response to 45 minutes of RIM, but not vehicle (DMSO), stimulation (from (234)).

3.4 GASP-1 promotes the degradation of GPR55 in response to LPI and rimonabant

Since GASP-1 has been reported to specifically target 7TM/GPCRs for degradation (98), I next assessed whether GASP-1 altered the post-endocytic sorting of GPR55. Consistent with its ability to bind GASP-1, GPR55 was targeted to lysosomes after internalization (Fig. 16). To quantify the post-endocytic fate of GPR55, I next monitored the stability of a pool of endocytosed GPR55 in the presence or absence of GASP-1 using shRNA knock-down of GASP-1 and a biotin protection/degradation assay. Biotinylated GPR55 receptors were stimulated with either 2.5μM rimonabant (Fig. 20A and C; RIM) or 2.5μM LPI (Fig. 20B and C; LPI) for 45 minutes to allow receptor internalization (45'). Remaining biotin on the cell surface was stripped, and the internalized "protected" pool of receptors was monitored for up to 180 minutes (Fig. 20A, B and C; +30', +90' and + 180'). Lysate samples immunoblotted for β-actin (Fig. 20A and B; lower panels) served as controls for protein level. In GPR55-HEK cells infected with shGASP-1 lentivirus (Fig. 20A and B; right panels, 20C (■)), biotinylated, internalized GPR55 was significantly more stable than in cells infected with the scrambled shScr lenti-virus (Fig. 20A and B; left panels, 20C (□)). Taken together, these results indicate that GASP-1 plays a crucial role in promoting the degradation of GPR55 following endocytosis.

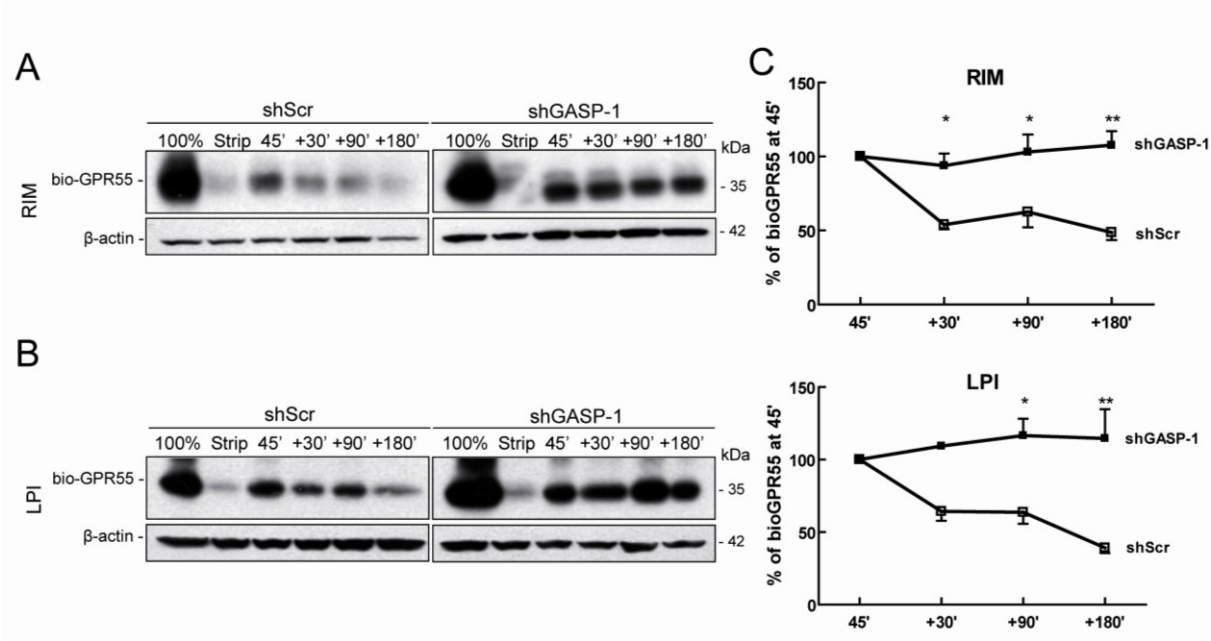

FIGURE 20. Disruption of the GASP-1/GPR55 interaction inhibits the degradation of GPR55. GPR55-HEK cells were infected with shScr virus (left panels) or shGASP-1 virus (right panels). Biotinylated GPR55 (bio-GPR55, 100%) was allowed to internalize in the presence of (A) 2.5μM RIM or (B) 2.5uM LPI for 45 min (45') before cells were stripped of surface-biotin (Strip). The degradation of GPR55 was monitored for additional 180 minutes (+30, +90 and +180). GPR55 degradation was observed in shScr (A and B; left panel, C; □), but not in shGASP-1 (A and B; right panel; C; ■) infected cells. (C) Quantification of biotinylation assays in A and B. Biotinylated GPR55 bands from shScr (□) or shGASP-1 (■) infected cells were normalized to β-actin and 45 minute values were set at 100%. Data are means of three independent experiments ± SEM, *p<0.05, **p < 0.01 (from (234)).

3.5 The recycling of GPR55 is promoted in the absence of GASP-1

In cells devoid of GASP-1 the degradation of some 7TM/GPCRs is disrupted. In some cases receptors are recycled back to the cell surface (115;130;134;235), while in others the receptors are neither degraded nor recycled (132;230), but retained in intracellular compartments. Hence, I next tested whether disrupting the GPR55/GASP-1 interaction facilitated recycling of GPR55. To assess receptor recycling, GPR55-HEK cells were infected with shScr (Fig. 21A and B; upper panels) or shGASP-1 (Fig. 21A and B; lower panels) lentivirus. Surface receptors were labeled with anti-FLAG antibody (0 min) and cells were then stimulated with 2.5μM rimonabant (Fig. 21A) or 2.5μM LPI (Fig. 21B) for 45 minutes to allow receptor internalization (45 min). Since cells stimulated with vehicle (DMSO or H2O, final concentration 0.025%) did not internalize (Fig. 19), recycling could not be monitored. Cells were stripped of the FLAG-antibody (Strip) and receptor

trafficking was monitored for up to 90 minutes (+30 min, +60 min and +90 min). Cells were fixed and any recycled receptor was detected by a fluorescent antibody. Before agonist stimulation, GPR55 was detected primarily on the cell surface (Fig. 21A and B; 0 min), but internalized rapidly following treatment with 2.5µM rimonabant (Fig. 21A; 45min) or 2.5µM LPI (Fig. 21B; 45min). Following the antibody strip (Strip), no recycled receptor was detected in either rimonabant (Fig. 21A; upper panel) or LPI (Fig. 21B; upper panel) treated shScr infected cells even after 90 minutes, consistent with the targeting of GPR55 to the lysosome for degradation (Fig. 16 and 20). In contrast, knockdown of GASP-1 with shGASP-1 facilitated recycling of GPR55 back to the cell surface as early as 30 minutes following the strip (Fig. 21A and B; lower panel, +30min, +60min, +90min).

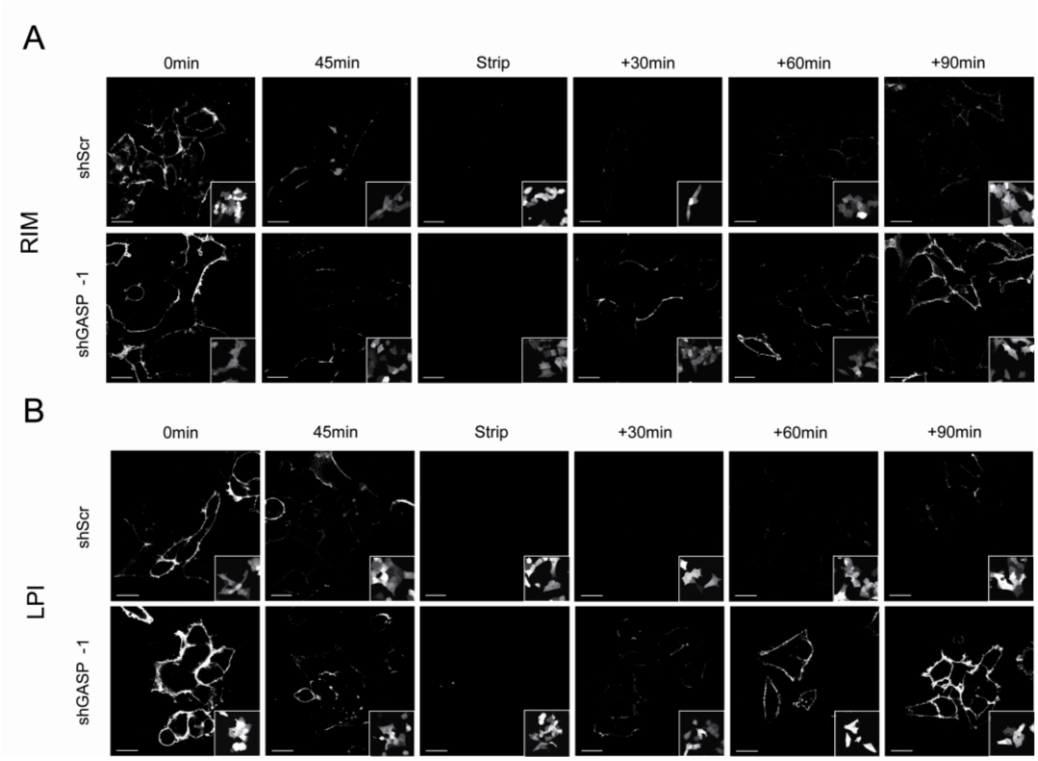

FIGURE 21. Recycling of GPR55 is enhanced in the absence of GASP-1. shScr (upper panels) or shGASP-1 (lower panels) infected GPR55-HEK cells were incubated with anti-FLAG antibody for 45 minutes (0min) to label surface receptors. Cells were incubated with (A) 2.5µM RIM or (B) 2.5µM LPI for 45 minutes (45min) to allow internalization and then stripped of surface antibody (Strip). Cells were incubated in warm medium for up to 90 minutes (+30min, +60min, +90min) to allow degradation. In contrast to shScr infected GPR55-HEK cells (A and B; upper panels), GPR55 recycled in shGASP-1 infected cells (A and B; lower panels). Insets indicate EGFP-shRNA-virus expression. Scale bar = 20 µm (from (234)).

3.6 Repetitive Rimonabant treatment downregluates GPR55 in WT, but not in GASP-1 KO mice

GASP-1 was reported to play a key role in downregulating several 7TM/GPCRs *in vitro* (98;115) and *in vivo* (131;133;141) by targeting the receptors to the lysosomal compartments. As described above, GPR55 can interact with GASP-1 and is downregulated after ligand induced endocytosis in the presence of GASP-1 *in vitro*. In fact, I could show that the absence of GASP-1 allows the receptor to recycle back to the cell surface (Fig. 21).

Here I set out to investigate whether GPR55 is degraded via GASP-1 *in vivo* after repetitive rimonabant treatment. C57BL/6 wild typ (WT) mice and GASP-1 knock-out (GASP-1 KO) mice were treated with vehicle (1:1:18 solution of ethanol/cremophor/saline) or 10mg/kg rimonabant (SR141716A) for 3 weeks. Radioligand binding experiments were performed to quantify GPR55 and CB_1 receptor levels in brain membranes derived from mouse amygdala and hippocampus tissue. Both of these brain areas are involved in anxiety- and depression like behavior (236) and have been reported to express GPR55 as well as CB_1 receptors (198;201). Here, I observe that GPR55 expression is significantly downregulated upon chronic rimonabant treatment (Fig. 22A and B, black bars, left panel) in both amygdala and hippocampus membranes from WT mice, when compared to vehicle treated animals (Fig. 22A and B, white bars, left panel). In contrast, GPR55 receptor levels were not altered in GASP-1 KO mice after treatment with vehicle or rimonabant (Fig. 22A and B, right panel, white vs black bars). CB_1 receptor levels, however, were not significantly different in any of the tissues of both, vehicle versus rimonabant treated animals (Fig. 23A and B, white vs black bars).

Taken together, these data suggest that GASP-1 plays a key role in regulating the expression levels of GPR55 in mouse brain membranes *in vivo*.

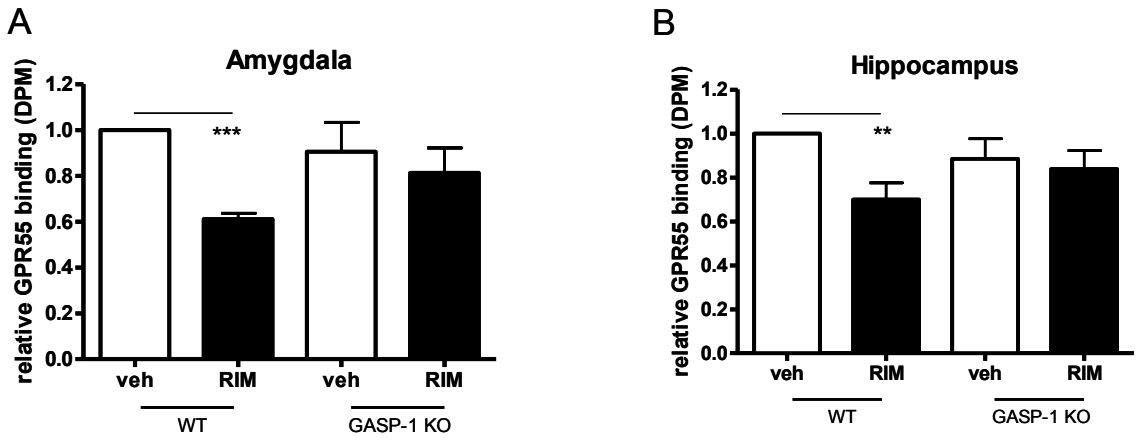

FIGURE 22. GPR55 is downregulated in WT, but not in GASP-1 KO mice, after chronic rimonabant administration. WT and GASP-1 KO mouse tissue from **(A)** amygdala and **(B)** hippocampus was tested for radioligand binding using [^{3}H]SR141716A. WT mice treated with 10mg/kg rimonabant for 21 days showed a significant downreguation of GPR55 receptor levels in the membrane, whereas receptor levels were not altered in GASP-1 KO mice. Data are presented as relative receptor binding ± SEM in drug-treated versus vehicle-treated mice. Relative GPR55 binding of vehicle treated WT control group was set at 1. ***p<0.001; **p<0.01. Data are means of at least three or four independent experiments.

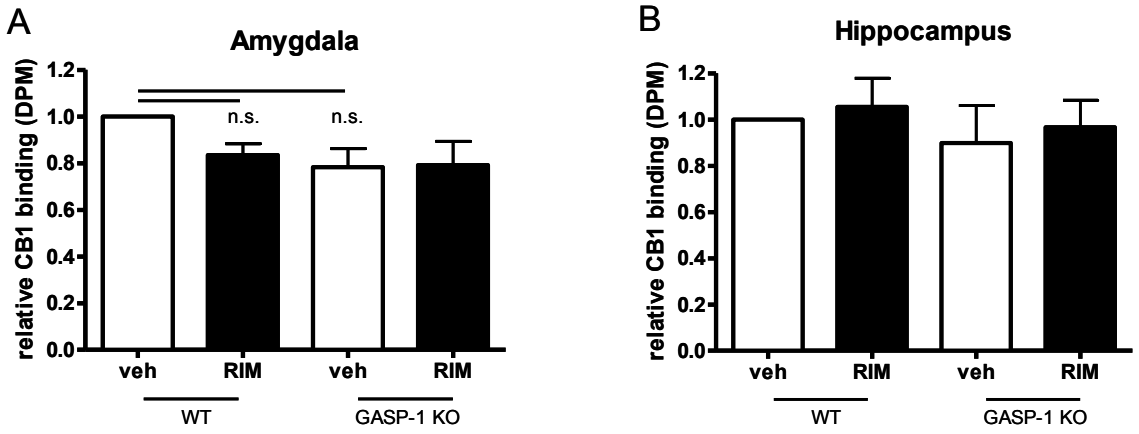

FIGURE 23. CB_1 receptor levels are not altered after repetitive administration of rimonabant. WT and GASP-1 KO mice were chronically treated with vehicle or 10mg/kg rimonabant and total CB_1 receptor numbers of brain membranes were assessed in radioligand binding experiments using [^{3}H]SR141716A. WT and GASP-1 KO mice showed no significant change in CB_1 receptor binding in (A) amygdala and (B) hippocampus membranes. Data are presented as relative receptor binding ± SEM in drug-treated versus vehicle-treated mice. Relative CB_1 receptor binding of vehicle treated WT control group was set at 1. *p<0.05. Data are means of three independent experiments.

3.7 GASP-1 KO mice show anxiety-like behavior upon chronic rimonabant treatment

Rimonabant was developed as a selective CB_1 receptor inverse agonist/antagonist (181;182) and was a promising anti-obesity drug (183). Shortly after market introduction, however, rimonabant was taken off the market because of reports of severe side-effects, such as the development of anxiety and depression in patients. Recently, several groups reported that rimonabant acts as an agonist at GPR55 (201;216;218;220), suggesting this receptor to be a likely candidate for the severe side-effects of the drug.

To further investigate wether GPR55 and/or its proper degradation via GASP-1 are involved in rimonabant induced side-effects, I explored anxiety-like behavior in chronically treated WT and GASP-1 KO mice. It was previously demonstrated that an acute dose of rimonabant induced anxiety-like behavior (237;238). Here, I treated mice for 21 days with 10mg/kg rimonabant (one dose per day). Mice were then subjected to two behavioral tests commonly used to test anxiety-like behavior in mice, i.e. the Elevated-Plus Maze and the Dark-Light Transition Box test. The tests were performed 24 hours following the last drug administration to exclude acute effects of the drug. At the Elevated-Plus Maze, no anxiety-like behavior was detected (Fig. 24A and C), comparing vehicle (white bars) versus rimonabant (black bars) treated WT mice. However, when tested in a Dark-Light paradigm, a significant decrease in the time spent in the light box could be detected in rimonabant treated WT mice (Fig. 24D, white bar vs. black bars). However, entries in the light box (Fig. 24B) and distance travelled in the light box (Fig. 24E) were similar for both vehicle and rimonabant treated mice (Fig. 24B and E, compare black and white bars). Hence, in four out of five tested parameters no effect of chronic rimonabant treatment in WT mice could be detected (Fig. 24).

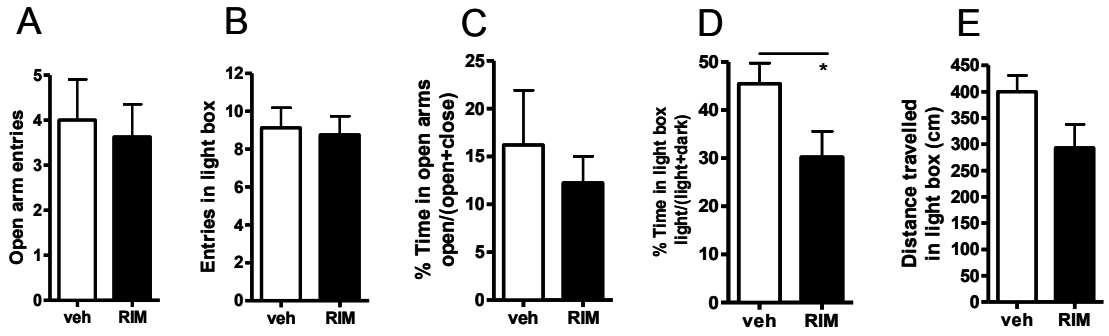

FIGURE 24. WT C57BL/6 mice show a similar degree of anxiety-like behavior following long term vehicle or rimonabant treatment. Anxiety-like behavior was measured by using (A and C) an Elevated-Plus Maze and (B, D and E) Dark-Light Transition Box approach. Mice were treated for 21 days with vehicle or 10mg/kg rimonabant and anxiety like behavior was tested 24 hours after the last drug administration. (A) Open arm entries and (C) % time spent in open arm was analyzed for the Elevated-Plus Maze. For the Dark-Light Transition Box experiments, (B) entries into the light box, (D) % time spent in the light box and (E) distance travelled in the light box were assessed. Vehicle and rimonabant treated mice show no significant changes in the Elevated-Plus Maze and Dark-Light paradigms, except for (C) % time spent in the light box. Data are means ±SEM (n=8 mice per group), $p^* < 0.05$.

Interestingly, however, rimonabant treated GASP-1 KO mice show a significant decrease in the Elevated-Plus Maze approach for open arm entries (Fig. 25A, white vs. black bars). In the Light-Dark Transition Box experiment (i) % of time spent in light box (Fig. 25D, white vs. black bars) and (ii) distance travelled in the light box (Fig. 25E, white vs. black bars) are significantly decreased, when compared to vehicle treated mice. This suggests that GASP-1 KO mice display a significantly higher anxiety-like behavior after repetitive rimonabant treatment compared to vehicle treatment. The other two parameters, i.e. (i) entries into the light box (Fig. 25B) and (ii) % time spent in the open arm (Fig. 25C) suggested a tendency that rimonabant treated GASP-1 KO animals are more anxious, however, these effects were not significant.

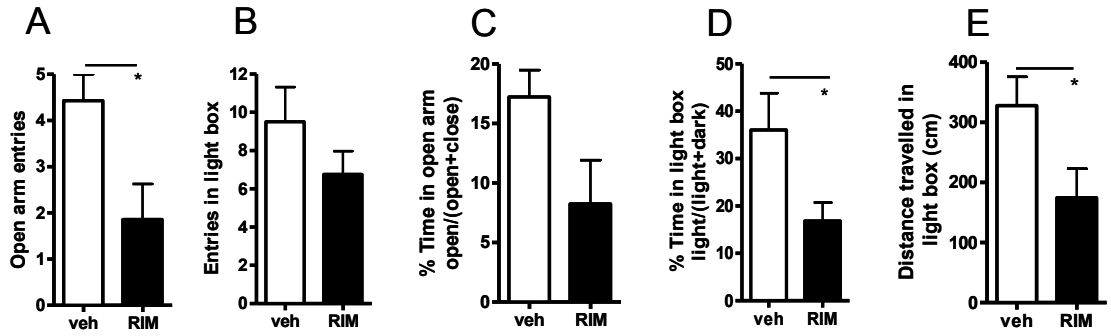

FIGURE 25. GASP-1 KO mice show a higher degree of anxiety-like behavior after repetitive rimonabant administration. GASP-1 KO mice were treated for 21 days with vehicle or 10mg/kg rimonabant and anxiety-like behavior was analyzed 24 hours after the last drug administration. Anxiety-like behavior was measured by performing (A and C) Elevated-Plus Maze and (B, D and E) Dark-Light Transition Box experiments. (A) Open arm entries and (C) % time spent in open arm was measured for the Elevated-Plus Maze. Using the Dark-Light Transition Box, (B) entries into the light box, (D) % time spent in the light box and (E) distance travelled in the light box were assessed. Rimonabant treated GASP-1 KO mice showed a higher degree of anxiety-like behavior in (A, D and E) compared to the control group. Seven or eight mice were used in each group. Data are means ±SEM p*<0.05.

As a control, I tested whether the general locomotor activity of GASP-1 KO versus WT mice was affected following long-term rimonabant treatment. As Figure 26 shows, rimonabant treatment did not affect the locomotor activity of both WT and GASP-1 KO mice (Fig. 26, WT and GASP-1 KO, white vs black bars).

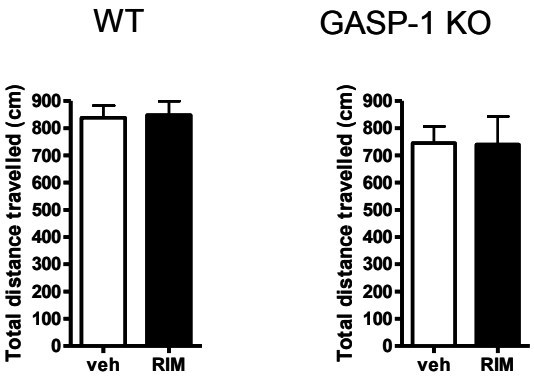

FIGURE 26 Locomotor activity of WT and GASP-1 KO mice. Locomotor activity was assessed by total distance travelled (dark + light box) in the dark-light transition box. GASP-1 KO mice were treated for 21 days with vehicle or 10mg/kg rimonabant and locomotor activity was analyzed 24 hours after the last drug administration. No significant changes in total travelled distance were detected for WT and GASP-1 KO mice comparing vehicle and rimonabant treatment. Eight mice were used in each group. Data are means ± SEM, p*<0.05.

Summarized, these data suggest that GASP-1 KO mice are more sensitive to long-term rimonabant treatment compared to WT mice and therefore are at a higher risk to develop anxiety-like behavior.

3.8 Depression-like behavior was significantly increased in GASP-1 KO mice chronically stimulated with rimonabant

A subset of patients chronically treated with rimonabant developed severe side-effects, including depression (183;239). Here, I further investigated depression-like behavior in WT and GASP-1 KO mice following a long-term rimonabant treatment. Forced Swim Tests were performed with WT and GASP-1 KO mice chronically treated with vehicle or rimonabant. No significant difference in movement was observed for WT mice (Fig. 27A, white vs. black bars), comparing vehicle versus rimonabant administration. In contrast, GASP-1 KO mice move significantly less after rimonabant treatment compared to vehicle (Fig. 27B, white vs. black bars).

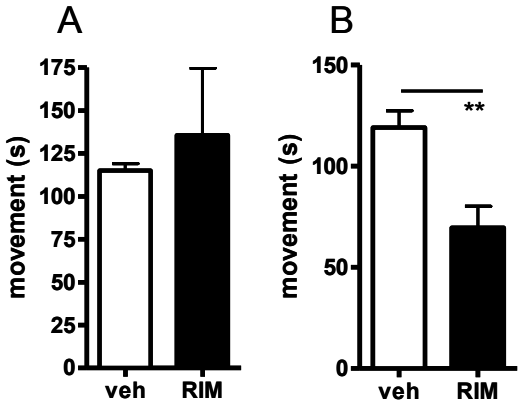

FIGURE 27. Long-term rimonabant administration affects depression-like behavior of GASP-1 KO, but not WT mice. WT and GASP-1 KO C57BL/6 mice were treated with vehicle or 10mg/kg rimonabant for 21 days and depression-like behavior was assessed 24-hours after last drug administration by using a Forced Swim Test approach. Total movement was measured (A) Vehicle and rimonabant treated WT C57BL/6 mice show a similar degree of depression-like behavior. (B) In comparison, long-term rimonabant administration enhances depression-like behavior in GASP-1 KO mice compared to vehicle treated control group. Eight animals were used in each group and data are means ±SEM **$p<0.01$.

Summarized, GASP-1 KO mice show a higher degree of depression-like behavior in the Forced Swim Test paradigm after long-term rimonabant administration in contrast to vehicle treated animals. However, no significant difference was detected in WT mice when they were treated with either vehicle or rimonabant. These data provide strong evidence that GASP-1 is crucially involved in the development of depression-like behavior in rimonabant treated animals.

4 Discussion

The sorting of 7TM/GPCRs after receptor activation is a highly regulated process and includes receptor degradation or recycling back to the cell surface. One regulatory mechanism to guarantee appropriate receptor expression levels in physiological conditions is that of downregulating 7TM/GPCRs via sorting proteins, for example GASP-1, thus leading to an attenuation of cellular signaling events (98). Here, I demonstrate the importance of GASP-1 as a cellular regulator for the post-endocytic sorting of GPR55 both *in vitro* and *in vivo.*

In the first part of this thesis, I show that GPR55 binds directly to GASP-1 (Fig. 14A and B) and this interaction is essential for targeting the receptor to the degradative pathway (Fig. 20A, B and C; shScr). Upon stimulation with the GPR55 agonists rimonabant and LPI, GPR55 co-localizes with the lysosomal markers LAMP1/2 in the presence of GASP-1 (Fig. 16A; shScr). In contrast, the knock-down of GASP-1 results in a predominant co-localization of GPR55 with the recycling endosomal marker transferrin (Fig. 17B, shGASP-1). In agreement with these findings, rapid recycling of GPR55 is observed in cells devoid of GASP-1, (Fig. 21A and B; shGASP-1), however, the agonist induced internalization of GPR55 is not altered in the absence of GASP-1 (Fig. 18B and C). Finally, GPR55 degradation is inhibited in the absence of GASP-1, indicating that GASP-1 is a crucial factor in GPR55 downregulation *in vitro* (Fig. 20A, B and C, shGASP-1). Taken together, these studies implicate GASP-1 as a key player to target GRP55 to the lysosomes/degradative pathway upon agonist exposure *in vitro*.

For centuries cannabinoids have played an important role in medicine due to their psychoactive, analgesic and anti-inflammatory properties. Since then, many natural and synthetic compounds which have the ability to bind CB_1 and CB_2 receptors were discovered. Overweight and obesity have been linked to several serious medical conditions, including heart disease and stroke, hypertension, diabetes, cancer and many more. Obesity is a rising disease among the population in industrial and threshold countries, therefore the development of successful anti-obesity drugs is crucial to minimize overweight and the resulting complications thereof.

Rimonabant – developed as a CB1 receptor inverse agonist/antagonist – has recently attracted significant attention, since it was marketed for weight loss and smoking cessation. Due to adverse side effects, including the development of anxiety and depression in patients (183), rimonabant was withdrawn from the market. Recently, rimonabant has been shown to be not only an antagonist/inverse agonist at the CB_1 receptor but also an agonist at GPR55 (201;216;220) – a potential off target effect that could be contributing to the adverse events. However, as such, rimonabant-like drugs with a better safety profile would be of great benefit. Hence, a better understanding of the off-site targets/effects of rimonabant will allow the development of novel and safer cannabinoid drugs.

Here, I identified GASP-1 as a potential regulator of the trafficking and functional expression of GPR55 in response to rimonabant *in vivo*. I could demonstrate that GPR55 is downregulated upon long-term rimonabant treatment in WT, but not in GASP-1 KO mice (Fig. 22A and B). In contrast, CB_1 receptor levels remain unaltered after treatment with its inverse agonist/antagonist rimonabant (Fig. 23A and B). Importantly, GASP-1 KO mice are more affected by rimonabant induced anxiety- and depression like behavior than their wild type littermates (Fig. 24A-E, Fig. 25A-E and Fig. 27A and B).

However, in light of the ambidextrous role of rimonabant – i.e. being an antagonist on CB_1R and an agonist on GPR55 receptors – the relative impact of rimonabant on GPR55 and CB_1 receptors after prolonged use – and ultimately the adverse effects of this drug – will have to be verified in more detail *in viv*o. However, although much higher concentrations of rimonabant than typically used for CB1 receptor antagonsim (5-15 nM, see (180)) are required to elicit an effect on GPR55 (201;216), a role for GPR55 activation always needs to be considered where micromolar concentrations of rimonabant are present, i.e. during long-term treatment and/or accumulation in fatty tissues, such as the brain. Intriguingly, the side effects of rimonabant in humans were pervasive only after prolonged drug use (183) suggesting that they were not a consequence of acute activation/inhibition of GPR55/CB_1 receptors - but rather could have been a consequence of prolonged agonist exposure.

Summarized, this study enforces the importance of GPR55 trafficking to drug responses in general and suggests the involvement of the sorting protein GASP-1 as an underlying cause to the severe side effects of the weight-reducing agent rimonabant.

PART III

THE CANNABINOID RECEPTOR 1 MODULATES THE SIGNALING PROPERTIES OF THE G PROTEIN-COUPLED RECEPTOR 55

1 Abstract

GPR55 and the CB_1Rs are co-expressed in many tissues, predominantly in the central nervous system. 7TM/GPCRs can form homo- and heteromers and initiate distinct signaling pathways.

Here, I tested whether GPR55 and CB_1 receptors are capable of (i) forming heteromers and (ii) whether such heteromers could exhibit novel signaling patterns. I show that GPR55 and CB_1 receptors alter each others signaling properties in human embryonic kidney (HEK293) cells. I demonstrate that the co-expression of FLAG-CB_1 receptors in cells stably expressing HA-GPR55 specifically inhibits GPR55-mediated NFAT and SRE induction, as well as ERK1/2 MAP-Kinase activation. However, once activated, the CB_1 receptor restores GPR55-mediated signaling. GPR55 and CB_1 receptors can form heteromers, but the internalization of both receptors is not affected. In addition, I observe that the presence of GPR55 enhances CB_1R-mediated ERK1/2 and NFAT activation.

My data provide first evidence that GPR55 can form heteromers with another 7TM/GPCR and that this interaction with the CB_1 receptor has functional consequences *in vitro*. The GPR55–CB_1R heteromer may play an important physiological and/or pathophysiological role in tissues endogenously co-expressing both receptors.

2 Materials and methods

2.1 Material

Dulbecco´s modified Eagle´s Medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000, zeocin, Opti-MEM, PBS, SDS-polyacrylamide gels, AlexaFluor 488nm-conjugated IgG2b and AlexaFluor 594-conjugated IgG1 were purchased from Invitrogen (Austria). G418 was from PAA (Austria). NFAT and CREB reporter plasmids were obtained from PathDetect; Stratagene (Austria) and SRE reporter plasmid was kindly provided by J Silvio Gutkind, National Institutes of Health, Bethesda, MD. Steadylite Plus Kit was purchased from Packard Instrument Company (USA). Anti-FLAG M1, anti-FLAG M2 and β-actin antibody, anti-FLAG M2 monoclonal antibody affinity matrix, TMB (3, 3′, 5, 5′-Tetramethylbenzidine), BSA, Triton X-100, Poly-D-Lysine, Kodak BioMax light films, Lysophosphatidylinositol (LPI), Tween 20 and Bradford substrate were obtained from Sigma Aldrich (Austria). HRP-conjugated anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were from Jackson ImmunoResearch (Dianova, Germany). Anti-HA-11 antibody was obtained from Covance Complete Protease inhibitor cocktail tablets were purchased from Roche (Austria) and PNGaseF, rabbit anti-pERK1/2 and rabbit anti-tERK1/2 antibodies from New England Biolabs (Germany). ECL Western Blotting Substrate was obtained from Pierce (THP, Austria) and Vectashield mounting medium was purchased from Vector Laboratories (Szabo-Scandic, Austria). Immobilon-P Transfer Membrane (PVDF) was purchased from Millipore (Austria). Ethanol, DMSO NaOH, KCl and CaCl2*2H2O were obtained from Merck (Austria), NaCl, Tris, sulfuric acid, glycerol, SDS, bromphenol blue, dithiothreitol and Formaldehyde were from Roth (Lactan, Austria). SR141716A (rimonabant, RIM) was from Sanofi-Synthélabo Recherche (Montpellier, France), WIN55,212-2 was from Tocris Cookson (USA) and GSK319197A was kindly provided by GlaxoSmithKline, Andrew Brown (description see introduction). LPI was dissolved in H2O, SR141716A, GSK319197A and WIN55,212-2 in DMSO and anandamide in EtOH.

2.2 Cell culture, transfections and stable cell lines

HEK293 cells were cultured in Dulbecco´s modified Eagle´s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2, humidified atmosphere. HEK293 cells stably expressing the human 3xHA-GPR55 (HEK-GPR55) were previously described (49) and maintained in G418 containing medium (0,4 mg/ml). To generate HEK293 cells stably expressing FLAG-CB_1R alone (HEK-CB1) or 3xHA-GPR55 and FLAG-CB_1 receptor (HEK-GPR55+CB1), HEK293 or HEK-GPR55 cells were transfected with pcDNA3.1 encoding the FLAG-CB_1 receptor using Lipofectamine 2000. Cells were generated in selection media (0,5mg/ml zeocin for HEK-CB1) or (0,8mg/ml G418 and 0,5mg/ml zeocin for HEK-GPR55 + CB1) and single colonies were propagated. HEK-CB1 cells were cultered in DMEM media containg 0,2mg/ml zeocin and HEK-GPR55+CB1 cells were maintained in DMEM media containing 0,4 mg/ml G418 and 0,2 mg/ml zeocin. All cells were serum starved over night in Opti-MEM prior to all experiments. Transient transfections were performed using Lipofectamine 2000 following the manufacturer's instructions.

2.3 Reporter gene assay

Transcription factor luciferase assays were carried out as previously described (49). Briefly, HEK293, HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1cells were seeded in 96-well plates (40,000 cells/well) and transiently transfected with the cis-reporter plasmids for NFAT-luc (100-200ng), SRE-luc (50 ng/well) (kindly provided by J Silvio Gutkind, National Institutes of Health, Bethesda, MD) or CREB-luc (200ng/well) alone or in combination with pcDNA FLAG-CB_1R, pcDNA FLAG-CCR5 or pcDNA 3.1 (25-100ng) using Lipofectamine 2000 for gene dose experiments. 24 to 48 hours post-transfection, cells were incubated with indicated ligand concentrations for 4 hours in serum free media at 37°C. Using a FlexStationII, the cell number was determined and subsequently luciferase activity was visualized using the Steadylite Plus Kit and was measured in a TopCounter for 5 s. Luminescence values are given as relative light units (RLU).

2.4 ELISA

Enzyme-linked immunosorbent assay was carried out as previously described (134). Experiments were performed in parallel to the reporter gene assays (see above). Cells were fixed with 3.7% formaldehyde, blocked and permeabilized in blotto (50 mm Tris–HCl, pH 7.5, 1 nm CaCl2, 0.1% Triton X-100 and 3% milk) for 1h. The expression of FLAG-CB_1R and FLAG-CCR5, respectively, was determined by incubating cells with anti-FLAG M1 antibody (1:500) overnight at 4°C, followed by incubation with an HRP conjugated anti-mouse antibody (1:2500) for 2h at room temperature. Cells were washed with TBS (25 mm Tris base, 135 mm NaCl, 2.5 mm KCl, 1mm CaCl2* 2H2O, pH 7.4) and cell numbers were determined by means of optical density using a FlexStation II device. TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate was added and the colouring reaction was stopped by the addition of 0.5 M sulfuric acid after 2 min at room temperature. Colour intensity was measured at 450 nm in a BioRad xMark Microplate Spectrophotometer.

2.5 MAP-Kinase Western Blot

ERK1/2 phosphorylation was detected as previously described (216). In brief, HEK293, HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells were seeded in 6-well plates and confluent wells were serum starved over night. Then cells were incubated with pre-warmed Opti-MEM containing vehicle (DMSO or EtOH, final concentration: 0,025%), SR141716A (Rimonabant), GSK319197A (kindly provided by Andrew Brown, GlaxoSmithKline), WIN55-212,2, anandamide or combinations thereof for 25 min at 37°C. Cells were washed once with ice cold PBS, snap-frozen in liquid nitrogen and lysed in IPB (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 25 mM KCl, 1 mM $CaCl_2$, 0.3% Triton X-100, 92mg/ml sucrose and protease inhibitors). Lysates were centrifuged and supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to a PVDF membrane. Membranes were blocked in TBST buffer (1 mM $CaCl_2$, 136 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, 0.1% (v/v) Tween 20) containing 5% milk, washed in TBST without milk and incubated with rabbit anti-pERK1/2 (1:1000) or rabbit anti-tERK1/2 (1:1000) antibodies overnight at 4°C. Membranes were immunoblotted with HRP-conjugated goat anti-rabbit antibody (1:4000) for 2 hrs at RT and protein bands were visualized with ECL Western Blotting Substrate.

2.6 Co-Immunoprecipitation

HA-GPR55 was immunoprecipitated with the FLAG-CB_1 receptor using the stable HEK-GPR55+CB1 cells. In brief, cells were washed twice with ice cold PBS and lysed in IPB (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 25 mM KCl, 1 mM $CaCl_2$, 0.3% Triton X-100 and protease inhibitors). Cell lysates were centrifuged and the supernatant was incubated with 20µl of anti-FLAG M2 monoclonal antibody affinity matrix for 2 hours at 4°C. 30µl of lysate was kept for FLAG, HA, and β-actin control blots. Samples were washed, deglycosylated with PNGase for 1 hour at 37°C and incubated with reducing sample buffer (100 mmol/L Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 0.2% bromphenol blue, and 200 mmol/L dithiothreitol) for 5min at 95°C. Proteins were resolved by SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked, probed with either anti-FLAG M2 antibody (1:500), anti-HA-11 antibody (1:1000) or β-actin monoclonal antibody (1:1000) for 2 hours and immunoblotted with HRP-conjugated anti-mouse antibody (1:4000) for 2 hours. Blots were visualized with ECL Western Blotting Substrate. HEK293, HEK-GPR55 and HEK-CB1 cells were used as control.

2.7 Immunocytochemistry and confocal microscopy

Cells were grown on poly-D-lysine coated coverslips to 50% confluency, starved in Opti-MEM over night and antibody feeding experiments were performed essentially as described in (234). In brief, living cells were fed with anti-FLAG M1 antibody (1:1000) and/or anti-HA-11 (1:1000) for 30 minutes at 37˚C. Subsequently, cells were stimulated with selective agonists or DMSO (control, final concentration: 0,025%) for 45 minutes. Then cells were fixed in 3.7% formaldehyde, permeabilized in blotto (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.3 % Triton X-100 and 3% milk) and labelled with secondary antibodies; AlexaFluor 488nm-conjugated IgG2b against the FLAG-tag (1:1000) and/or AlexaFluor 594-conjugated IgG1 against the HA-tag (1:1000) for 20 minutes. Immunolabelled receptors were visualized by using a laser-scanning confocal imaging system (Zeiss LSM510).

2.8 Statistical analysis

At least three independent blots were analyzed for quantification of phosphorylated ERK1/2 and total ERK1/2 levels by using Image J software (NIH) and pERK1/2 was normalized to tERK1/2. For reporter gene experiments, measured relative light units (RLU) were normalized to the cell number. Statistical analysis for comparisons between multiple groups was performed by using one way ANOVA followed by a Bonferroni´s post-hoc test using GraphPad Prism software (Inc, San Diego, California). A p-value of < 0.05 was considered statistically significant.

3 Results

3.1 The CB1 receptor modulates GPR55 transcription factor activation

It has previously been reported that CB_1 receptors can form heteromers with other 7TM/GPCRs and thereby modulate the signaling properties of the receptors involved (180). In contrast, to date no reports exist that GPR55 is able to form functional heteromers with other 7TM/GPCRs. However, we have recently described that GPR55 modulates the signaling capacities of the CB_2 receptor in human neutrophils, where both receptors are naturally co-expressed (197). Likewise, GPR55 and CB_1 receptors have been shown to be co-expressed in several cell types (see review (198)) and, importantly, some cannabinoid ligands have been reported to modulate both receptors in opposite ways (e.g. SR141716A is an inverse agonist/antagonist on the CB_1 receptor and an agonist on GPR55) (181;182;216;218;220). Hence, I was interested if GPR55 and CB_1 receptors heteromerize and/or modulate each others signaling properties. In addition, I set out to elucidate the properties of the 'dual acting' ligand SR141716A in a cell system where both GPR55 and CB_1 receptors are co-expressed.

I engineered HEK293 cells stably co-expressing HA-tagged GPR55 and FLAG-tagged CB_1 receptor (referred to as HEK-GPR55+CB1) and control cells expressing FLAG-CB_1 receptor (HEK-CB1) alone. Control cells stably expressing HA-GPR55 receptor (HEK-GPR55) were previously published (49) (Fig. 28A and B).

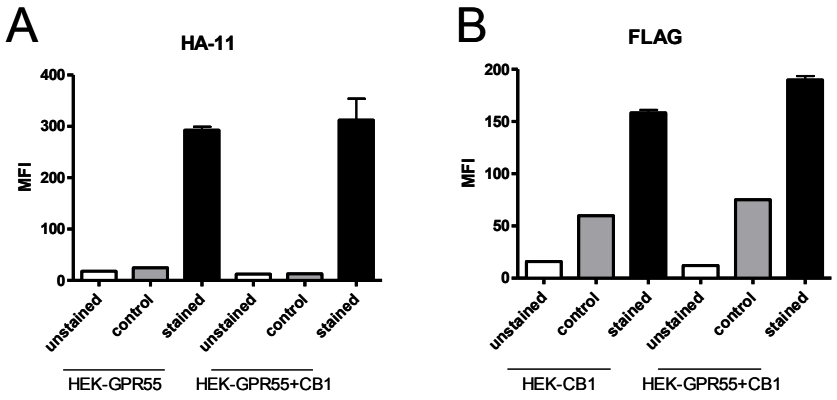

FIGURE 28. Expression of GPR55 and CB_1 receptors in HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells was determined by flow cytometry with (A) anti-HA and Alexa488-conjugated IgG1 antibodies as well as (B) anti-FLAG and Alexa488-conjugated IgG2b antibodies under non-permeabilizing conditions.

In earlier studies we have shown that various transcription factors, i.e. NF-κB, CREB and NFAT can be activated by GPR55 (49;216). Here I show for the first time that the serum response element (SRE) can be activated by GPR55 (Fig. 29B, D and F). I first tested whether NFAT and SRE induction via the GPR55 receptor was modulated by co-expressing the CB_1 receptor. I observed NFAT activation and SRE induction in HEK-GPR55 (white bars), but not in HEK-CB1 (grey bars) cells after stimulation with the GPR55 agonists LPI or SR141716A (Fig. 29 A-D). In the presence of the CB_1R, GPR55-mediated transcription factor activation was reduced up to ~ 50% after LPI treatment when compared to HEK cells expressing the GPR55 receptor alone (Fig. 29A and B, compare white bars vs. black bars). Interestingly, no activation of NFAT and SRE activity was observed in HEK-GPR55+CB1 cells, following treatment with 1μM SR141716A (Fig. 29C and D, compare white bars vs. black bars). Likewise, the GPR55 specific agonist GSK319197A completely abrogated NFAT- and SRE-luciferase activity in HEK-GPR55+CB1 cells (Fig. 29E and F, ▼).

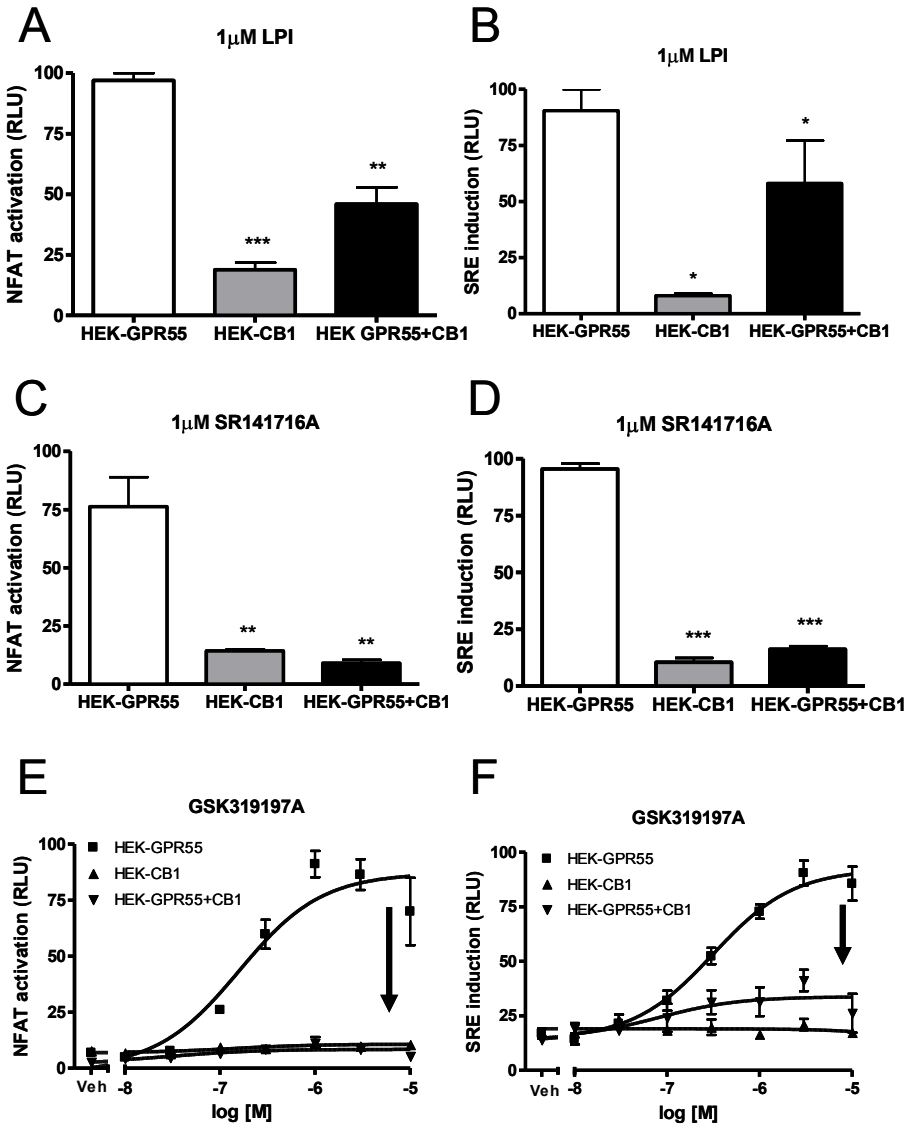

FIGURE 29. The CB_1 receptor modulates GPR55 transcription factor activation in HEK-GPR55+CB1 cells. HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells were transfected with (A, C and E) NFAT or (B, D and F) SRE transcription factor-luciferase-reporter plasmids and 24 h post transfection, cells were stimulated with either (A and B) 1μM of LPI, (C and D) 1μM SR141716A or (E and F) with increasing concentrations of the selective GPR55 agonist GSK319197A for 4h in serum-free medium. (A, C and E) NFAT activation and (B, D and F) SRE induction was observed in HEK-GPR55 (white bars or ■), but not in HEK-CB1 (grey bars or ▲) cells. NFAT activation and SRE induction was (A – B) reduced or (C – F) abolished in HEK-GPR55+CB1 cells (black bars or ▼). Data are means ± SEM from one of four independent experiments performed in triplicates. Data were normalized and expressed as percent of maximum activation which was set as 100%, RLU (relative light units) *p<0.05, **p < 0.01, ***p < 0.001.

To further investigate whether activation of transcription factors by GPR55 is specifically altered in the presence of the CB_1 receptor, I performed gene dose experiments. HEK-GPR55 cells were transfected with increasing concentrations of either CB_1 or CCR5 receptor DNA. Cells were stimulated with either 1μM GSK319197A (Fig. 30A and C) or 1μM SR141716A (Fig. 30B and D). Increasing CB_1R expression results in a loss of GPR55-mediated NFAT (Fig. 30A and B) or SRE (Fig. 30C and 2D) induction in a dose dependent manner (Fig. 30A-D, left panels). To control whether the presence of an unrelated Gαi-coupled 7TM/GPCR would likewise modulate GPR55 mediated signaling, HEK-GPR55 cells were transfected with increasing amounts of the chemokine CCR5 receptor. In the presence of CCR5, no changes in NFAT (Fig. 30A and B, right panels) or SRE (Fig. 30C and D, right panels) induction were observed. Gene-dose dependent CB_1R (Fig. 30E) and CCR5 (Fig. 30F) expression was confirmed by ELISA.

In summary, these data show that the presence of the CB_1 receptor inhibits GPR55-induced NFAT and SRE transcription factor activity, while an unrelated Gαi-coupled 7TM/GPCR – i.e. CCR5 – had no effect on GPR55 mediated signaling.

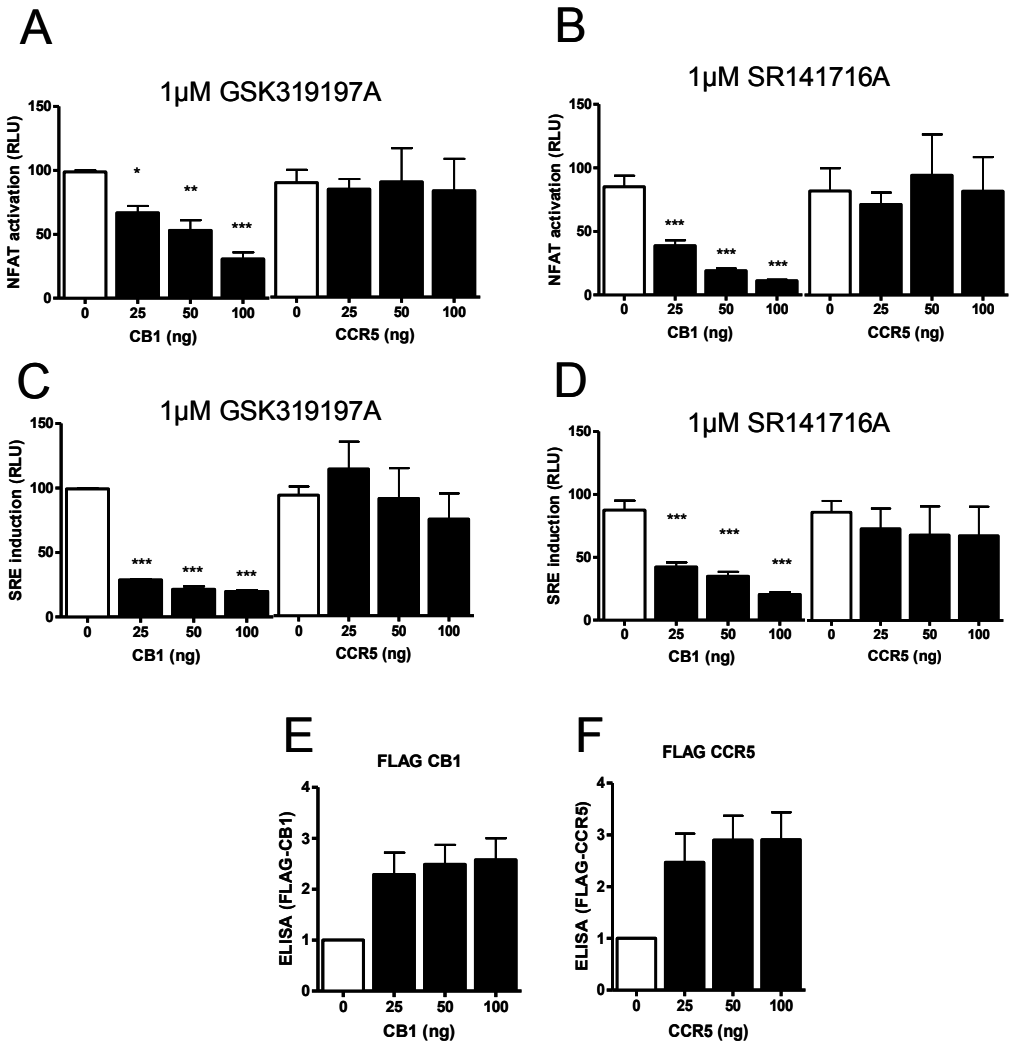

FIGURE 30. Increasing CB_1 receptor levels block GPR55 transcription factor activation in a dose dependent manner. HEK-GPR55 cells were transfected with (A and B) NFAT or (C and D) SRE transcription factor luciferase-reporter plasmids and increasing amounts of either (A – D, left panels) FLAG-CB_1 pcDNA or (A – D, right panels) FLAG-CCR5 pcDNA. DNA content in each well was kept constant by co-transfecting with empty pcDNA vector. 48 h post transfection, cells were stimulated with (A and C) 1µM GSK319197A or (B and D) 1µM SR141716A for 4 h in serum-free medium. (A and B) NFAT activation and (C and D) SRE induction was reduced in the presence of increasing amounts of CB_1 receptor (A – D, left panels), but remained unaffected by increasing amounts of CCR5 receptor (A – D, right panels). (E) FLAG-CB_1R and (F) FLAG-CCR5 protein expression levels were measured by ELISA. Data are means ± SEM from one of three independent experiments performed in triplicates. Data were normalized and expressed as percent of maximum activation of control (white bars), which was set as 100%, RLU (relative light units)*$p<0.05$, **$p < 0.01$, ***$p < 0.001$.

3.2 ERK 1/2 phosphorylation is altered in HEK-GPR55+CB1 cells compared to HEK-GPR55 and HEK-CB1 cells

We and others have previously shown that ERK1/2 MAPKs are activated upon stimulation of GPR55 in a variety of cellular backgrounds (203;216;217;219). In order to test whether the presence of the CB_1R interfered with GPR55-mediated signaling at a more upstream level than transcription factors, I tested MAP kinase activation in HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells. As expected, ERK1/2 phosphorylation was significantly increased in HEK-GPR55 cells after stimulation with 2.5µM of the GPR55 agonists SR141716A and GSK319197A (Fig. 31A, upper panel, C and D, white bars), but not after treatment with the CB_1R agonist WIN55,212-2 (Fig. 31A, upper panel and B, white panel). ERK1/2 activation was observed in HEK-CB1 cells only after stimulation with 2.5µM WIN55,212-2 (Fig. 31A, middle panel and B, grey bar). In HEK-GPR55+CB1 cells, stimulation with 2.5µM of the respective GPR55 agonists GSK319197A and SR141716A resulted in only marginal ERK1/2 phosphorlylation (Fig. 31A, lower panel, C and D, black bars). Interestingly, the treatment of HEK-GPR55+CB1 with 2.5µM WIN55,212-2 induced the strongest ERK1/2 phosphorylation measured in these experiments (Fig. 31A, compare middle and lower panels, and B, black bar).

In summary, these data suggest that the CB_1 receptor impairs GPR55-mediated signaling at the level of ERK1/2 MAP kinases. On the contrary, the presence of GPR55 seems to enhance ERK-phosphorylation mediated by WIN55,212-2-activated CB_1 receptors.

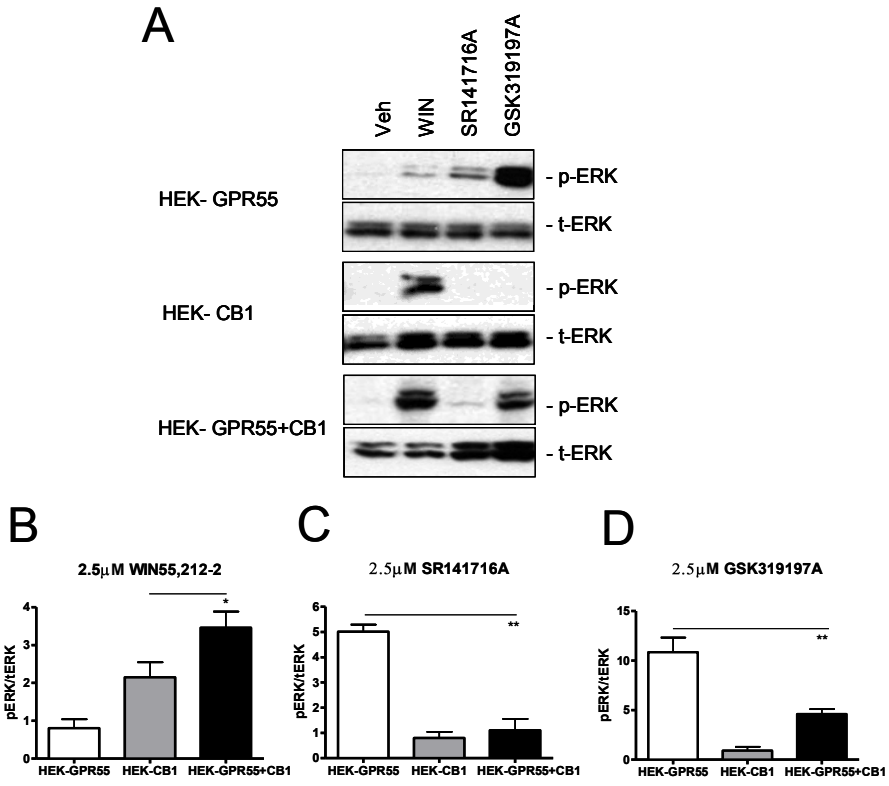

FIGURE 31. ERK1/2 phoshorylation state is altered in HEK-GPR55+CB1 cells. (A-D) HEK-GPR55 (upper panel or white bars), HEK-CB1 (middle panel or grey bars) and HEK-GPR55+ CB1 cells (lower panel or black bars) were serum starved over night and stimulated with either vehicle, (B) 2.5µM WIN55,212-2, (C) 2.5µM SR141716A or (D) 2.5µM GSK319197A for 25 min. Cell lysates were resolved on a 12% SDS-gel followed by antibody staining. (A) A representative blot out of 3 independent experiments is shown. Control stimulation with vehicle shows baseline pERK1/2 levels in single and double expression cell lines and the corresponding total ERK levels (tERK) are presented below the phospho-ERK (pERK) bands. WIN55,212-2 induces ERK1/2 phosphorylation in HEK-CB1 and HEK-GPR55+CB1 (A and B, grey and black bars), but not in HEK-GPR55 cells (A, white bar), pERK1/2 levels were significantly increased in HEK-GPR55+CB1 cells compared to HEK-CB1 cells (A and B, compare grey and black bars). The stimulation with (B) 2.5µM SR141716A or (C) 2.5µM GSK319197A mediates ERK1/2 phosphorylation in HEK-GPR55 (B and C, white bars), but ERK1/2 phosphorylation is significantly lower in HEK-GPR55+CB1 cells (B and C; black bars). Graphs show the means ± SEM from 3 independent experiments, whereby pERK1/2 bands were normalized to total ERK1/2 levels using densitometric analysis. *$p<0.05$, **$p < 0.01$.

To further test the relative impact and/or cross-regulation of GPR55 or CB_1 receptors on ERK1/2 MAP-kinase phosphorylation and reporter gene activity, I stimulated the respective receptors in single or co-expressing cells with the following ligand combinations: HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells were stimulated with WIN55,212-2, WIN55,212-2 + GSK319197A or WIN55,212-2 + SR141716A (2.5µM for ERK1/2 activation and 1µM for reporter gene experiments). Stimulation of HEK-CB1 and HEK-GPR55+CB1 cells with WIN55,212-2 lead to the phosphorylation of ERK1/2 and

activation of NFAT in both cell lines, but signals were significantly increased in the double expressing cell line (Fig. 31A and B, and 32A, D and G). Concomitant activation of both receptors in HEK-GPR55 + CB1 cells with WIN55,212-2 and GSK319197A resulted in a significant increase in both ERK1/2 phosphorylation (Fig. 32B and 4E, black bar) and NFAT activity (Fig. 32H, black bar) when compared to HEK-GPR55 (white bars) or HEK-CB1 (grey bars) cells. This observation suggests that the inhibitory effect of the CB_1 receptor on GPR55 signaling may be abolished when the CB_1 receptor is activated.

As expected, a different picture arises when HEK-GPR55+CB1 cells were co-treated with WIN55,212-2 and the GPR55 agonist SR141716A instead of GSK319197A. Since SR141716A is both an inverse agonist/antagonist on CB_1R and an agonist on GPR55, it was not surprising to see a significant inhibition in both ERK1/2 (Fig. 32C and F, black bar) and NFAT- activity (Fig. 32I, black bar).

Taken together, these results indicate that only “inactive” CB_1 receptors block GPR55-mediated signaling in HEK-GPR55+CB1 cells.

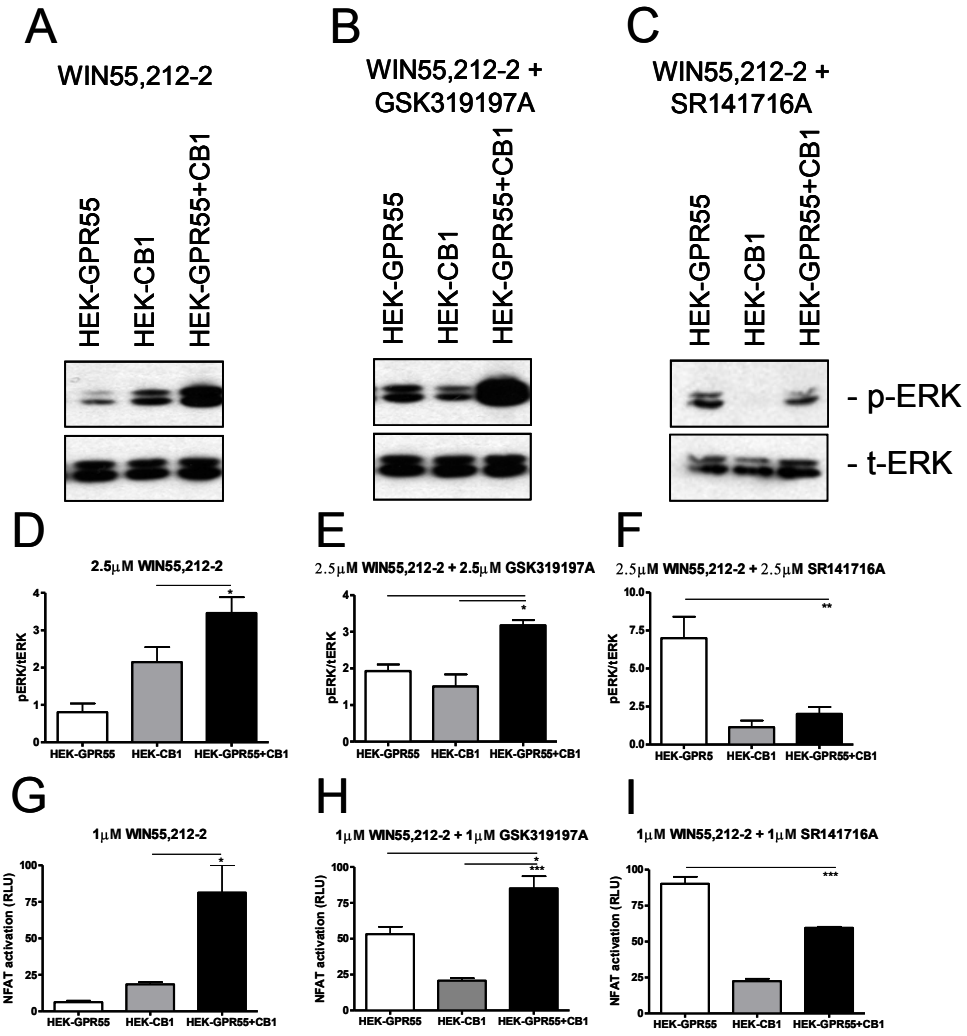

FIGURE 32. Combinatorial effects of the CB_1R agonist WIN55,212-2 and GPR55 ligands on ERK1/2 phosphorylation and NFAT activation in HEK-GPR55+CB1 cells. Immunoblots showing ERK1/2 phosphorylation in response to (A) 2.5μM WIN55,212-2, (B) 2.5μM WIN55,212-2 + 2.5μM GSK319197A or (C) 2.5μM WIN55,212-2 + 2.5μM SR141716A in HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells. Individual stimulation with 2.5μM WIN55,212-2 leads to (A and D) ERK1/2 and (G) NFAT activation in HEK-CB1 cells when compared to HEK-GPR55 cells (D and G, compare grey vs white bars), whereby the strongest activation was detected in HEK-GPR55+CB1 cells (D and G, black bars). Co-stimulation with 2.5μM of each, WIN55,212-2 and GSK319197A (B and E) elevates ERK1/2 phosphorylation and (H) NFAT activation in all tested cell lines, whereby the strongest activation is seen in HEK-GPR55+CB1 cells (E and H, black bars). In contrast, (C and F) ERK 1/2 and (I) NFAT activation was significantly reduced by co-stimulating HEK-CB1 and HEK-GPR55+CB1 cells with 2.5μM WIN55,212-2 and 2.5μM SR141716A (F and I, grey and black bars). Representative blots from 3 independent experiments are shown. pERK1/2 was normalized to total ERK1/2 and data are means of three independent experiments ± SEM. Reporter gene assay data are means ± SEM from one of three independent experiments performed in triplicates. Data were normalized and expressed as percent of maximum activation which was set as 100%. *$p<0.05$, **$p < 0.01$, ***$p < 0.001$.

In addition, I tested whether endogenous cannabionid ligands, such as anandamide (AEA), could equally well regulate GPR55 signaling in HEK-GPR55+CB1 cells. In fact, I found a very similar profile of ERK1/2-phosphorylation and NFAT-activity when HEK-GPR55+CB1 cells were stimulated with AEA instead of WIN55,212-2 in all combinations described above. While stimulation with AEA had no effect on ERK1/2- and NFAT-activity in HEK-GPR55 cells (Fig. 33A, B and E, white bar), a significant increase was observed in HEK-GPR55+CB1 cells (Fig. 33A, B and E, grey vs. black bars), when compared to HEK-CB1 cells. Concomitant stimulation of GSK319197A with AEA in the double expressing cell line led to an increase in pERK1/2 and NFAT activation (Fig. 33A, C and F, black bars), while the combination of AEA and SR141716A significantly reduced both pERK1/2 and NFAT- levels (Fig. 33A, D and G, black bars).

Taken together, these data suggest that both synthetic (WIN55,212-2) and endogenous (AEA) CB1R agonists are capable of restoring GPR55 mediated signaling properties in cells co-expressing these receptors.

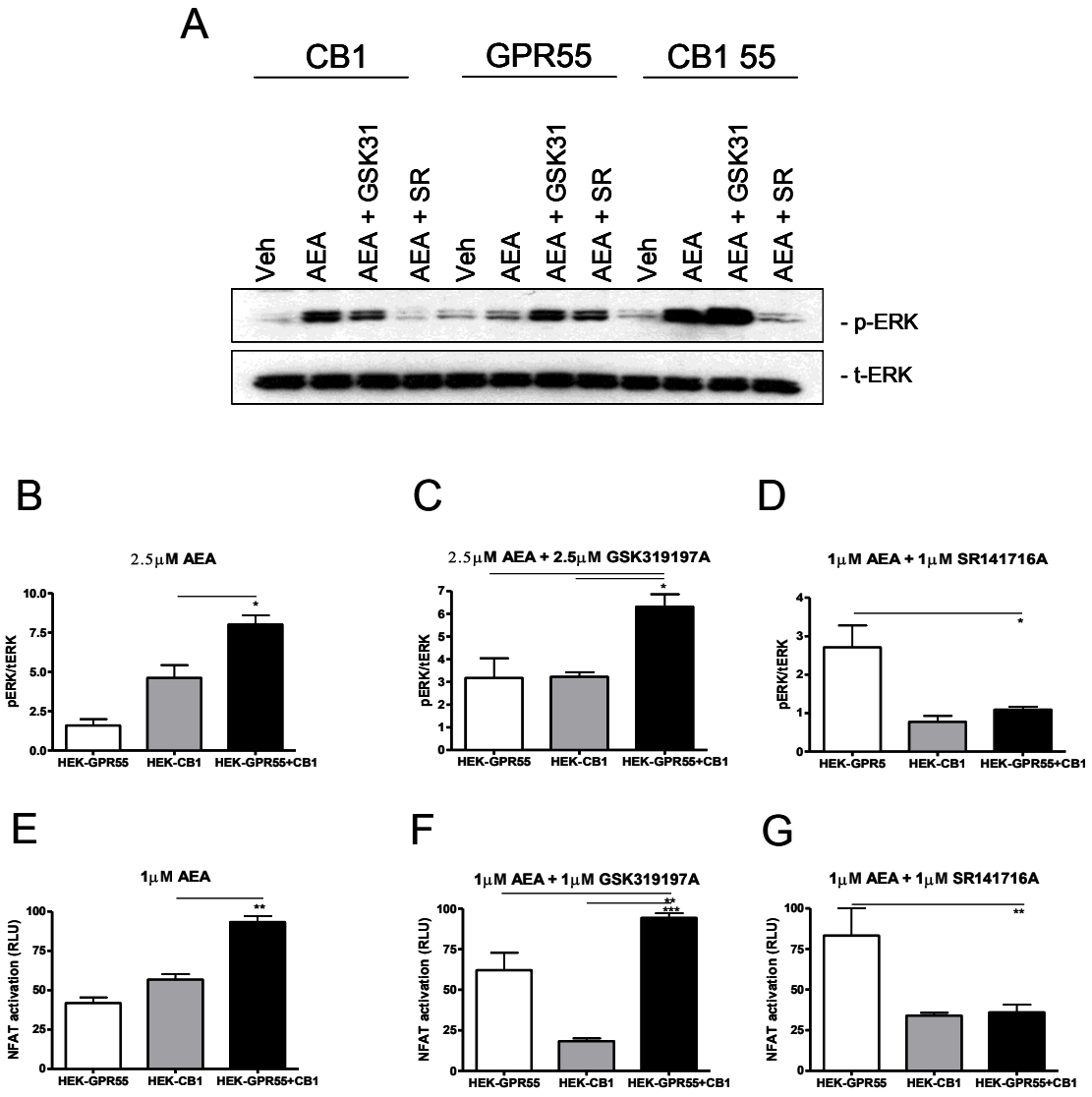

FIGURE 33. Combinatorial effects of the CB_1R agonist anandamide and GPR55 ligands on ERK1/2 phosphorylation and NFAT activation in HEK-GPR55+CB1 cells. (A) Representative immunoblot showing ERK1/2 phosphorylation in response to vehicle, 2.5µM AEA, 2.5µM AEA + 2.5µM GSK319197A and 2.5µM AEA + 2.5µM SR141716A in HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells. (A – D) Activation of ERK1/2 MAP-Kinases and (E – G) NFAT were altered in HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cell after individual stimulation with (A, B and E) 2.5µM anandamide (AEA) or co-stimulation with (A, C and F) 2.5µM AEA + 2.5µM GSK319197A or (A, D and G) 2.5µM AEA + 2.5µM SR141716A. Stimulation with 2.5µM AEA leads to a significant higher level of (A and B) ERK1/2 and (E) NFAT activation in HEK-GPR55 + CB1 compared to HEK-CB1 cells. No activation over baseline was observed in HEK-GPR55 cells. Increased (A and C) ERK1/2 phosphorylation and (F) NFAT activation occurred in all three cell lines after co-stimulation with 2.5µM AEA and 2.5µM GSK319197A. HEK-GPR55+CB1 cells show significant higher activation levels compare to single cell lines. (A and D) pERK1/2 and (G) NFAT activation is inhibited by co-stimulation of HEK-CB1 and HEK-GPR55+CB1 cells with 2.5µM AEA and 2.5µM SR141716A, but induced in HEK-GPR55 cells. pERK1/2 was normalized to total ERK1/2 and data are means of three independent experiments ± SEM. Reporter gene assay data are means ± SEM from one of four independent experiments performed in triplicates. Data were normalized and expressed as percent of maximum activation which was set as 100%. *p<0.05, **p < 0.01, ***p < 0.001.

3.3 Gαi-signaling is not involved in the cross-talk between CB_1R and GPR55

CB_1 receptors have been reported to couple to Gαi proteins (147;154). Here, I wanted to test whether Gαi signaling is involved in the CB_1R-mediated abrogation of GPR55 signaling. I tested whether treatment of cells with pertussis toxin (PTX) was able to restore GPR55 mediated NFAT and ERK1/2 activation in HEK-GPR55+CB1 cells. PTX treatment did not alter GPR55 mediated NFAT or ERK1/2 activation in HEK-GPR55 cells (Fig. 34A, compare □ vs. ■, and D) or HEK-CB1 cells (Fig. 34B, compare □ vs. ■, and D) when stimulated with the GPR55 agonist GSK319197A. Control experiments on HEK-CB1 cells showed that PTX was active and could block transcription factor activation and ERK1/2 phosphorylation in WIN55,212-2 stimulated cells (Fig. 35). However, I detected a decrease in both NFAT and ERK1/2 activity in GSK319197A stimulated HEK-GPR55+CB1 cells after PTX-treatment (Fig. 34C, compare □ vs. ■, and D), pointing towards an involvement of Gαi proteins in the signaling capacity of the GPR55-CB_1R heteromer when it is activated by a GPR55 selective ligand.

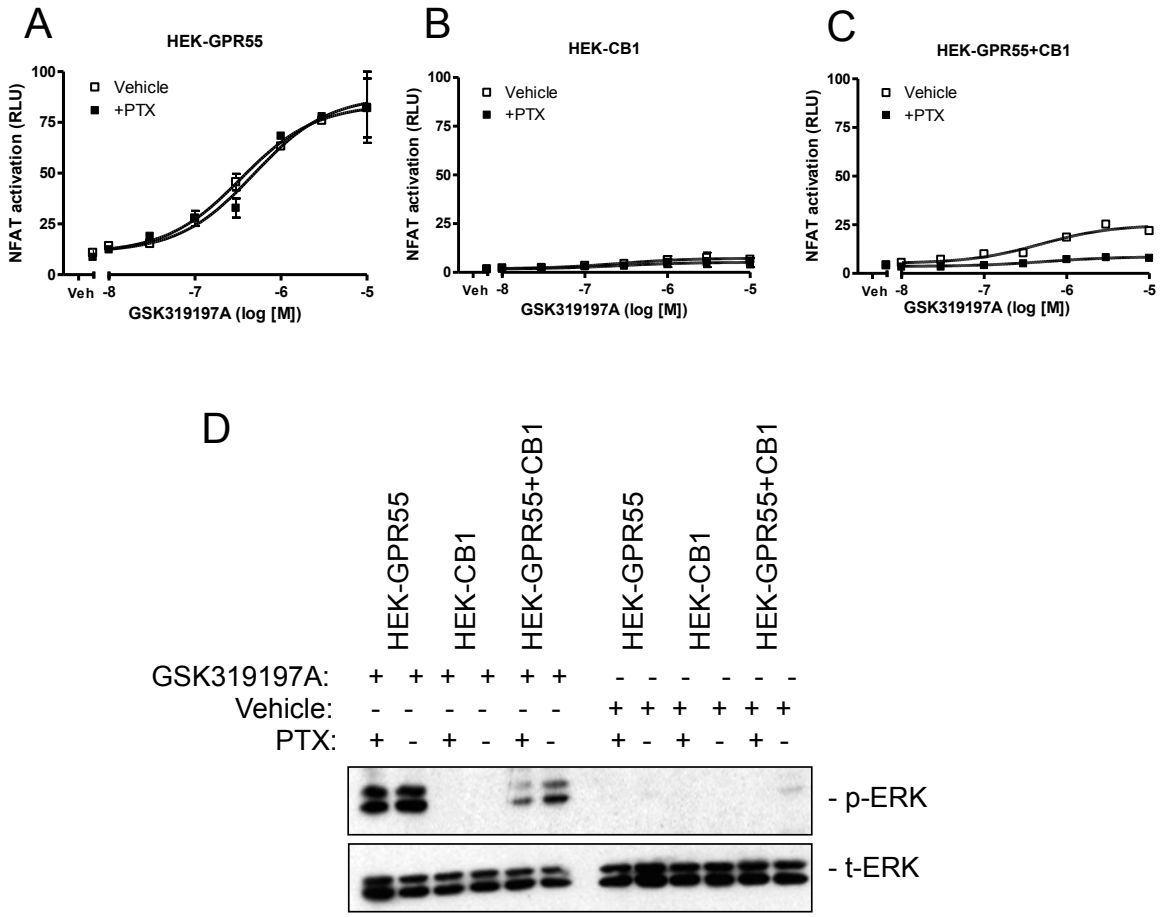

FIGURE 34. CB_1R mediated Gαi-activation is not responsible for the signal abrogation via GPR55 in HEK-GPR55+CB1 cells. (A) HEK-GPR55, (B) HEK-CB1 or (C) HEK-GPR55+CB1 cells were transfected with NFAT transcription factor plasmid. 24h post-transfection cells were pre-incubated for 4h with either vehicle (□) or 100ng/ml PTX (■) and stimulated with increasing concentrations of GSK319197A. NFAT activation was not altered by PTX in HEK-GPR55 cells (A, compare veh (□) vs PTX (■)). (B) No NFAT activation was measured in HEK-CB1 cells after stimulation with the GPR55 agonist GSK319197A. (C) In HEK-GPR55+CB1 cells, NFAT signaling was impaired in PTX treated cells (■) when compared to cells treated with vehicle (□). (D) ERK 1/2 phosphorylation in the presence or absence of PTX. HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells were serum starved over night, pre-incubated with vehicle or 100ng/ml PTX for 4h and stimulated with vehicle or 2.5μM GSK319197A for 25 min. ERK1/2 phosporylation was not altered by PTX in HEK-GPR55 cells. No ERK1/2 activity was observed after vehicle treatment in all cell lines and stimulation with the GPR55 agonist GSK319197A in HEK-CB1 cells. HEK-GPR55 + CB1 cells pre-incubated with PTX showed decreased pERK1/2 when compared to vehicle pre-incubated double expressing cell line. Reporter gene assay data are means ± SEM from one of three independent experiments performed in duplicates. Data were normalized and expressed as percent of maximum activation which was set as 100%. Representative ERK1/2 blot from 3 independent experiments are shown.

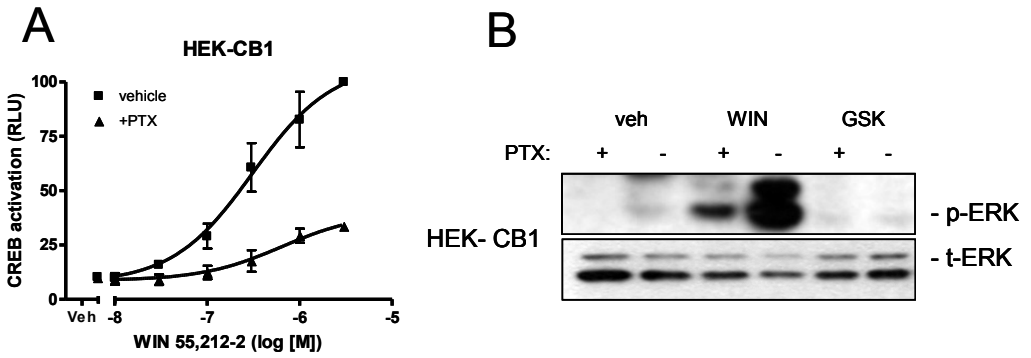

FIGURE 35. Control PTX transcription factor and ERK1/2 MAP-Kinase activation (A) CREB activation and (B) ERK1/2 phosphorylation in HEK-CB1 cells in response to 2.5μM WIN55,212-2 in the presence and absence of PTX. PTX blocks Gαi-signaling from CB_1 receptor in both experimental approaches.

3.4 Ligand-independent heteromerization of GPR55 and CB_1R

It is a well established concept that 7TM/GPCRs form heteromers and these can function as novel signaling entities (74). I tested a possible heteromerization of GPR55 and CB_1Rs by co-immunoprecipitation of the respective receptors from cell lysates derived from the HEK-GPR55+CB1 cell line (Fig. 36A). Indeed, as can be seen in Fig. 36A, lane 4, GPR55 co-immunoprecipitated with the CB_1R. Control lysates were immunoblotted for HA-GPR55 (Fig. 36A, 2nd panel, lane 2 and 4), FLAG-CB_1R (Fig. 36A, 3rd panel, lane 3 and 4) and β-actin (Fig. 36A, 4th panel). HEK293 served as negative controls (Fig. 36A, lanes 1). These data suggest that GPR55 and CB_1 receptors form heteromers under non-stimulated conditions.

3.5 Internalization properties of both, GPR55 and CB_1Rs, are not altered in HEK-GPR55 + CB1 cells

Both GPR55 and CB_1 receptors have been described to rapidly internalize following agonist stimulation (49;130;216;234). Interestingly, it has been reported that the mu-opioid receptor can 'co-internalize' with an activated delta-opiod receptor, i.e. internalizes as a heteromer (86). Hence I was interested if GPR55 and CB_1R could likewise internalize as a heteromer upon stimulation with one of the respective agonists. Antibody feeding experiments in live cells revealed that upon treatment of cells with selective CB_1R or

GPR55 agonists for 45 minutes, GPR55 and CB_1 receptors do not co-internalize (Fig. 36B, WIN, SR and GSK). Non-stimulated GPR55 and CB_1 receptors were detected on the cell surface (Fig. 36B, 1st lane). GPR55 was internalized following treatment with 2.5µM SR141716A, 2.5µM GSK319197A, 2.5µM WIN55,212-2 + 2.5µM GSK319197A and 2.5µM WIN55,212-2 + 2.5µM SR141716A in HEK-GPR55 and HEK-GPR55 + CB1 cells (Fig. 36B, 2st and 3rd panel, red). Stimulation with 2.5µM WIN55,212-2 and 2.5µM WIN55,212-2 + 2.5µM GSK319197A induced CB_1 receptor endocytosis in single and double expressing cells (Fig. 36B, 1nd and 3rd panel, green). CB_1 receptor internalization was inhibited by co-stimulation of 2.5µM WIN55,212-2 + 2.5µM SR141716A in HEK-CB1 and HEK-GPR55 + CB1 cells (Fig. 36B, last lane, GPR55 red and CB_1R green).

In summary, I could not observe any changes in the internalization properties of either GPR55 or CB_1R in single receptor-expressing versus the double receptor-expressing cell lines, suggesting that GPR55 and CB_1R do not internalize as a heteromer.

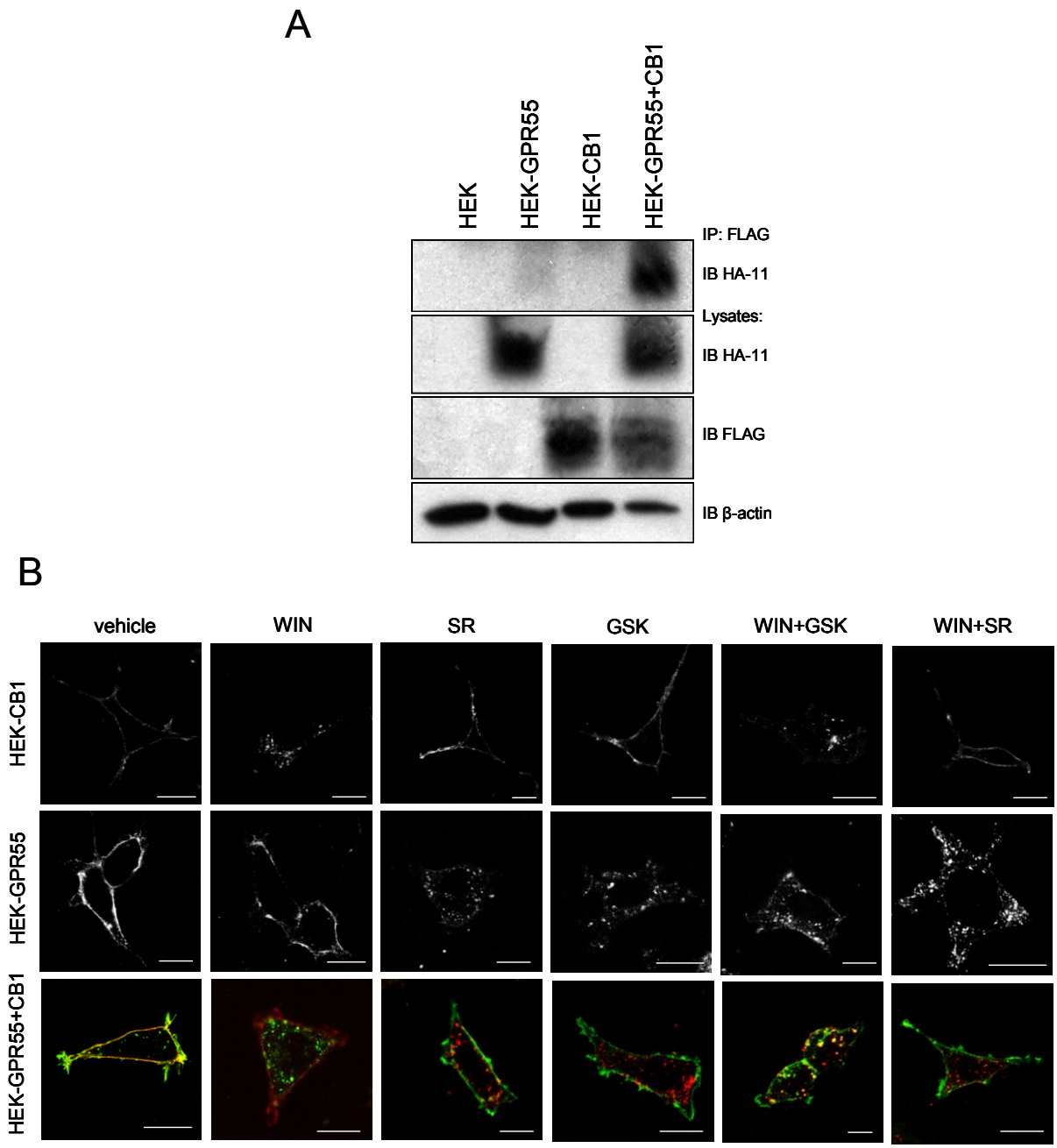

FIGURE 36. Co-immunoprecipitation and internalization of GPR55 and CB_1R in HEK293 cells. (A) HEK293, HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cell lysates were co-immunoprecipitated with anti-FLAG affinity matrix (IP) and immunoblotted (IB) for HA-GPR55 (first panel). Lysates were probed for HA-GPR55 (second panel), FLAG-CB_1 receptor (third panel) and β-actin (fourth panel). GPR55 strongly interacts with CB_1 receptor in the double expressing cell line. Blots are representative of three independent experiments. (B) HEK-GPR55, HEK-CB1 and HEK-GPR55+CB1 cells were stimulated with 2.5μM agonist for 45 min and receptor internalization was monitored. GPR55 (red) and CB_1 receptor (green) are located on the cell surface without agonist treatment and internalize following agonist stimulation in single and double expressing cell lines. Scale bar = 20 μm.

4 Discussion

GPR55 pharmacology and signaling properties were extensively studied in the past years, using many different cell systems and signaling readouts (198;212). Nevertheless, the pharmacology of GPR55 is still rather controversial and seems to be highly cell system dependent (180;212). It is well known that the co-expression of 7TM/GPCRs in specific tissues can lead to altered binding and/or signaling properties via the respective receptors (89;240-244). Both GPR55 and CB_1 receptors are highly expressed at approximately the same range in several brain regions, such as the striatum, hypothalamus and the brain stem (201). In light of these studies, I set out to investigate whether the co-expression of CB_1R and GPR55 in a recombinant HEK293 cell line could explain some of the controversial pharmacology reported for GPR55.

Here I demonstrate that GPR55 and CB_1 receptors form heteromers and influence each others signaling properties in HEK293 cells co-expressing both receptors. Specifically, I show that GPR55 mediated signaling is inhibited in the presence of the CB_1 receptor. Interestingly, this effect is only apparent when the CB_1 receptor is inactive. In contrast, I show that the signaling capacity of the CB_1R is amplified in the presence of GPR55.

GPR55-mediated signaling is lowered or completely abrogated in the presence of unstimulated CB_1 receptor at the level of MAP-Kinases (Fig. 31) and transcription factors, such as NFAT and SRF (Figs. 29 and 30). The signaling capacity of the CB_1 receptor is enhanced in the presence of GPR55. Upon stimulation of the CB_1 receptor with the synthetic ligand WIN55, 212-2 (Fig. 31 and 32) or the endogenous ligand anandamide (Fig. 33), I observed elevated ERK1/2 signals and NFAT activation in HEK-GPR55+CB1 cells compared to HEK-CB1 cells (compare Fig. 32A, D and G and Fig. 33A,B and E, black bars). Interestingly, signaling via GPR55 is restored in the presence of activated CB_1 receptors, since co-stimulation of HEK-GPR55+CB1 cells with both GSK319197A and WIN55,212-2 (or AEA) resulted in a dramatic increase of pERK and NFAT activity (Fig. 32B, E and H, and Fig. 33A, C and F). In line with these data, the GPR55 signal was effectively blocked when HEK-GPR55+CB1 cells were co-stimulated with WIN55,212-2 (or AEA) and SR141716A (Fig. 32C, F and I, and Fig. 33A, D and G). SR141716A is both an inverse agonist/antagonist on CB_1R and an agonist on GPR55 (see also Fig. 29 and D,

white bar and Fig. 32C, F and I). Hence, when the CB_1 receptor is inactivated in the presence of SR141716A, GPR55 is incapable of inducing ERK and/or NFAT activation (Fig. 32C, F and I, and Fig. 33A, D and G) – despite the fact that SR141716A is a potent agonist on GPR55. These data further substantiate the hypothesis that only inactive CB_1 receptors are able to inhibit GPR55 mediated signaling.

I next observed that the disruption of CB_1 receptor coupling to Gαi proteins with PTX does not restore GPR55 mediated transcription factor activation or pERK1/2 activation in HEK-GPR55+CB1 cells. Interestingly, I noticed a further decrease in GPR55 mediated NFAT and ERK1/2 activation in HEK-GPR55+CB1 cells (Fig. 34C and D). It has been reported that CB_1 receptors are able to constitutively activate Gαi proteins (147;154;155). PTX irreversibly interacts with the Gαi-subunits and thereby inhibits the interaction with 7TM/GPCRs. I hypothesize that the inhibition of the CB_1 receptor – Gαi-subunit interaction keeps the CB_1 receptor in its inactive state and thereby further renders GPR55 inactive when both receptors are co-expressed. In line with these observations, I show that GPR55 and CB_1 receptors physically interact in HEK-GPR55+CB1 cells in the absence of any ligand (Fig. 36A). However, I did not observe any altered internalization patterns following agonist activation of both receptors in double expressing cells when compared to single expressing cells (Fig. 36B).

In summary, when co-expressed, GPR55 and CB_1 receptors alter each others signaling properties at the level of MAP-Kinases and that of transcription factor activation. Unstimulated - and therefore inactive - CB_1 receptors form heteromers (Fig. 36A) with GPR55 and both receptors are located on the cell surface (Fig. 36B, vehicle). When CB_1R remains unstimulated, it prevents the activation/signaling of GPR55. A similar mechanism has been reported for the opioid receptor system. Here, the presence of the DOP decreases the activity of the MOP on stimulation with selective MOP agonists (89). However, once activated, CB_1R internalizes (Fig 36B, WIN) and thereby restores GPR55 mediated signaling when co-stimulated with the specific GPR55 agonist GSK319197A (Fig. 32B, E and H, and Fig. 33A, C and F). Another interesting finding of this study is that CB_1R mediated signaling is greatly amplified in the presence of activated GPR55. A recent report on the CRTH2 and DP receptor heteromers (90) describes a similar cross-talk mechanism, i.e. the DP receptor is able to amplify a CRTH2-induced Ca^{2+} release from intracellular stores and, coincidentally, loses its own signaling capacity. In addition, a new study by

Rozenfeld *et al.* shows that the heteromerization of CB_1R and DOP affects receptor signaling. The signaling ability of the CB_1R is impaired in the precence of DOP and has been suggested to be caused by an increased PLC-dependent β-arrestin recruitment to the heteromer (245).

GPR55 plays an important role in several physiological and pathophysiological processes (198), such as the development of cancer (203-206;227), bone formation (214), pain and inflammation (197;202;224). Since both CB_1 and GPR55 receptors are endogenously co-expressed in several tissues, I propose that potential cross-regulatory effects between the two receptors should be taken into account when studying these receptors in their native environment.

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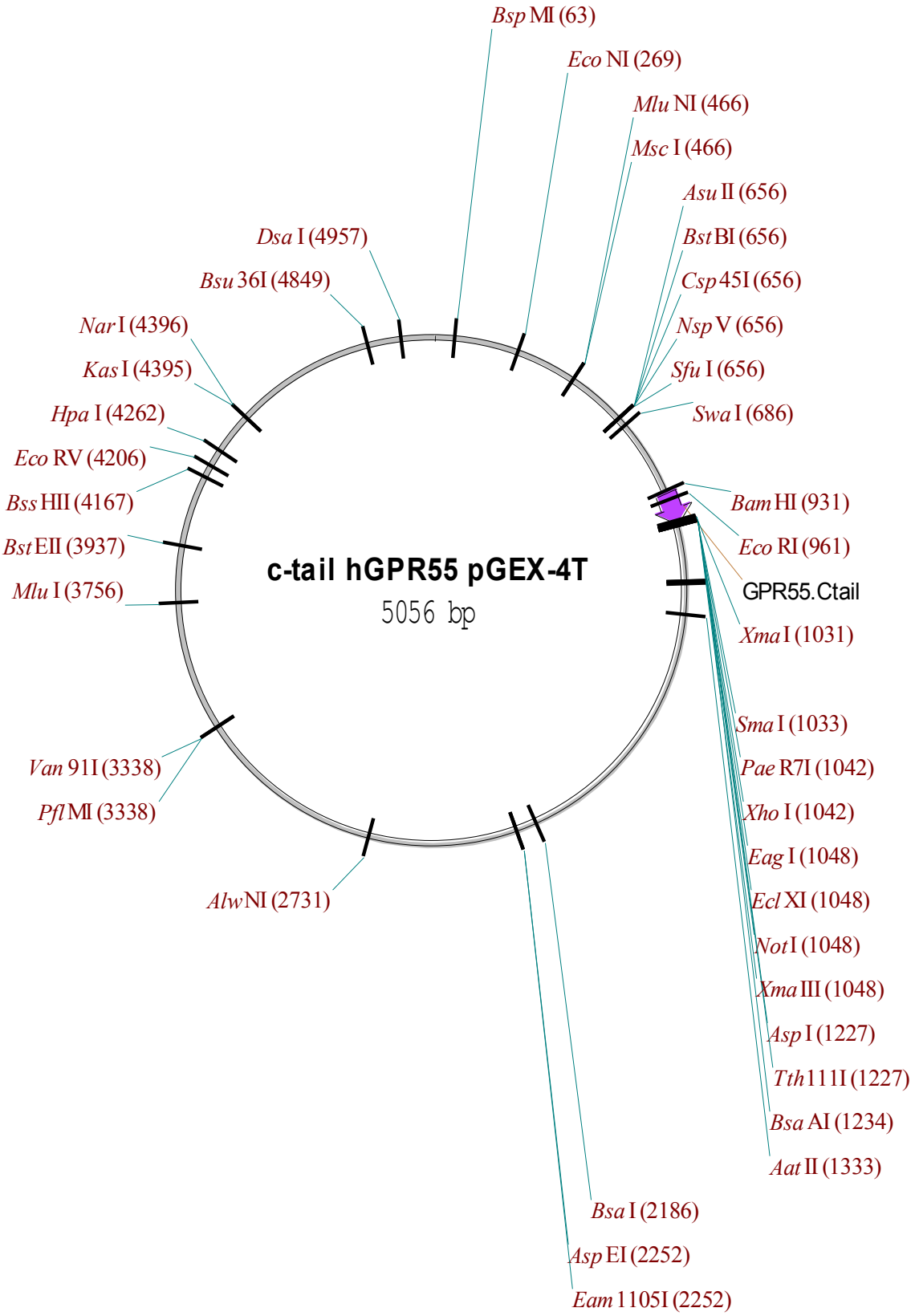
GlaxoSmithKline

Bradley DM et al. 2006

Patent number: WO/2006/094843 A1.

Preparation of N-benzoyl piperazines as glycine transporter inhibitors.

PART V APPENDIX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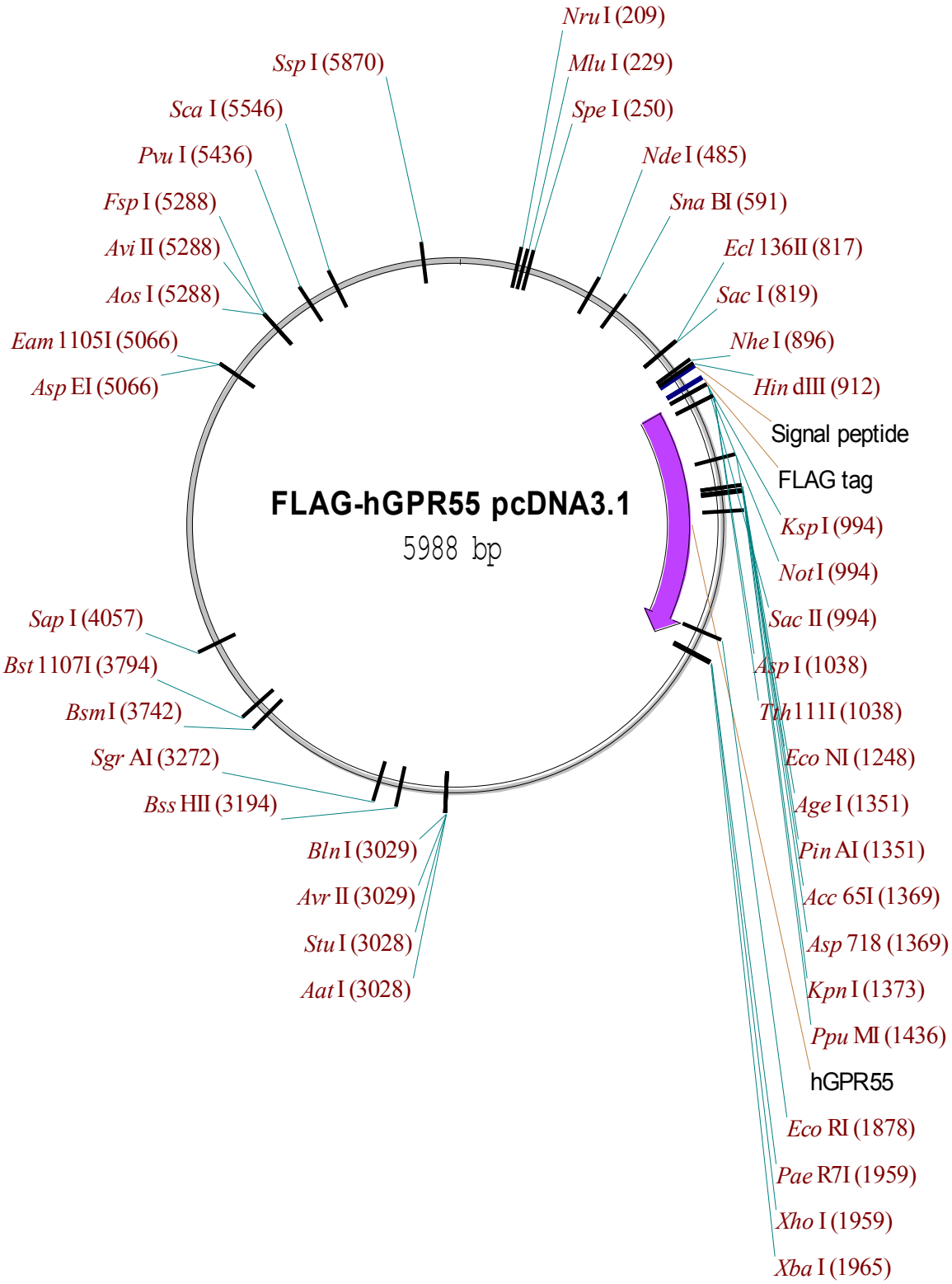
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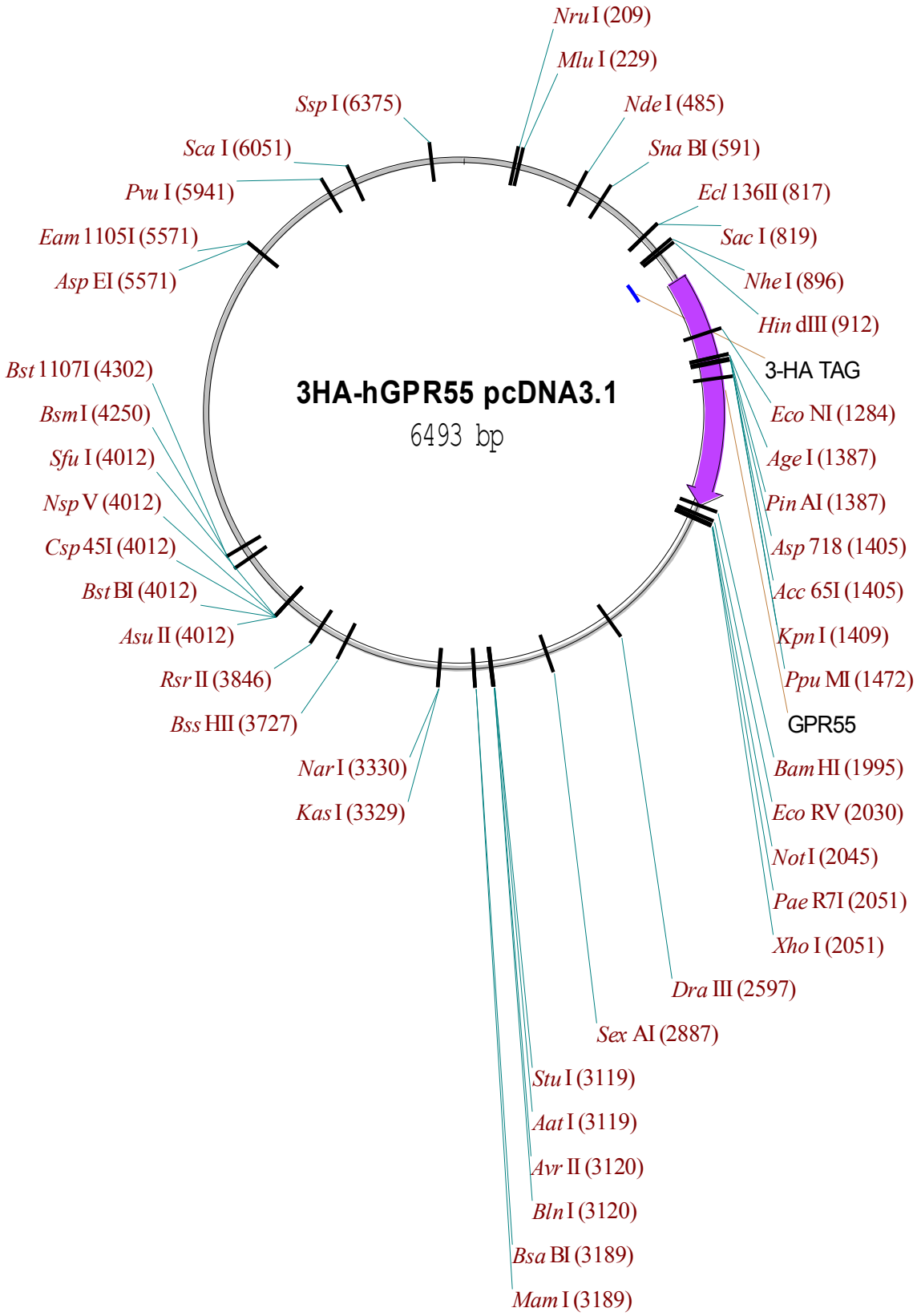
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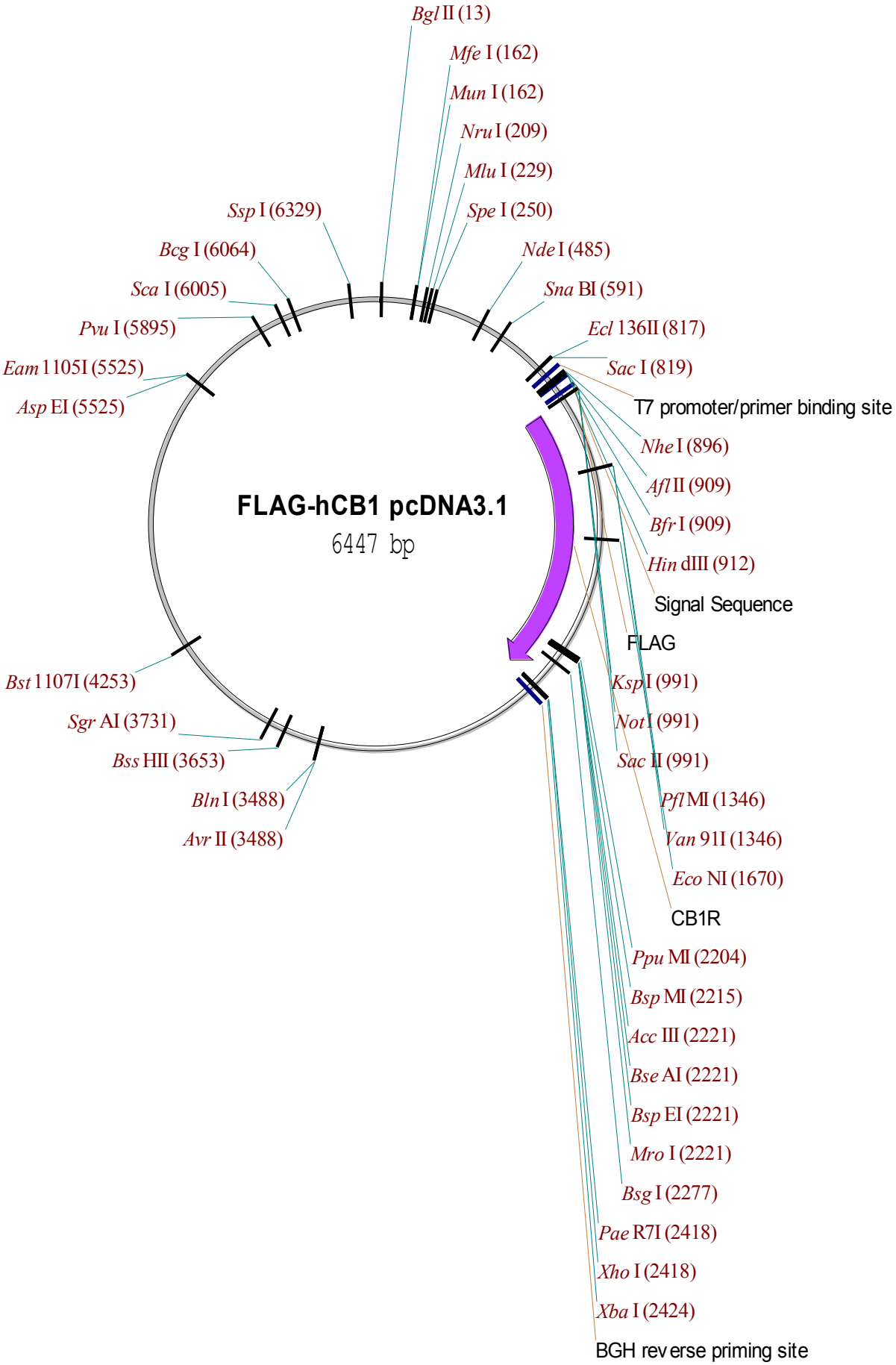
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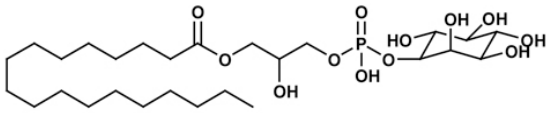
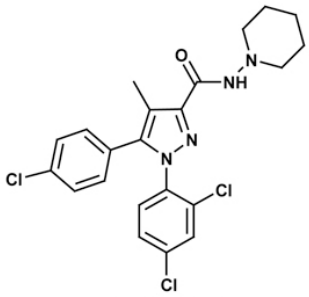
Asp EI (5525)
Bst 1107I (4253)
Sgr AI (3731)
Bss HII (3653)
Bln I (3488)
Avr II (3488)

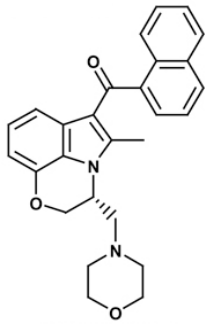
L-α-lysophosphatidylinositol (LPI):



Rimonabant (SR141716A):



WIN55-212,2:



(All three structures were adapted from (208)).

MINIREVIEW

Minireview: Recent Developments in the Physiology and Pathology of the Lysophosphatidylinositol-Sensitive Receptor GPR55

Christopher M. Henstridge, Nariman A. B. Balenga, Julia Kargl, Clara Andradas, Andrew J. Brown, Andrew Irving, Cristina Sanchez, and Maria Waldhoer

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Emerging data suggest that off-target cannabinoid effects may be mediated via novel seven-transmembrane spanning/G protein-coupled receptors. Due to its cannabinoid sensitivity, the G protein-coupled receptor 55 (GPR55) was recently proposed as a candidate; however, GPR55 is phylogenetically distinct from the traditional cannabinoid receptors, and the conflicting pharmacology, signaling, and functional data have prevented its classification as a novel cannabinoid receptor. Indeed, the most consistent and potent agonist to date is the noncannabinoid lysophospholipid, lysophosphatidylinositol. Here we present new human GPR55 mRNA expression data, providing supportive evidence of GPR55 expression in a vast array of tissues and cell types. Moreover, we summarize major recent developments in GPR55 research and aim to update the reader in the rapidly expanding fields of GPR55 pharmacology, physiology, and pathology. (***Molecular Endocrinology*** **25: 1835–1848, 2011)**

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Pharmacology, Signaling and Physiological Relevance of the G Protein-coupled Receptor 55

Abstract

According to The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), ~70 million European adults have consumed cannabis on at least one occasion. Cannabis consumption leads to a variety of psychoactive effects due to the presence of the constituent Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC interacts with the endocannabinoid system (ECS), which consists of the seven transmembrane spanning (7TM)/G protein-coupled receptors (GPCRs) CB_1 and CB_2, their respective ligands (endocannabinoids), and enzymes involved in their biosynthesis and degradation. This system plays a critical role in many physiological processes such as learning and memory, appetite control, pain sensation, motor coordination, lipogenesis, modulation of immune response, and the regulation of bone mass. Therefore, a huge effort has been spent trying to fully elucidate the composition and function of the ECS. The G protein-coupled receptor 55 (GPR55) was recently proposed as a novel component of this system; however, its classification as a cannabinoid receptor has been significantly hampered by its complex pharmacology, signaling, and cellular function. GPR55 is phylogenetically distinct from the traditional cannabinoid receptors, but in some experimental paradigms, it is activated by endocannabinoids, phytocannabinoids, and synthetic cannabinoid ligands. However, the most potent compound appears to be a lysophospholipid known as lysophosphatidylinositol (LPI). Here, we provide a comprehensive evaluation of the current

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ORIGINAL ARTICLE

GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils

Nariman A B Balenga[1], Elma Aflaki[2], Julia Kargl[1], Wolfgang Platzer[1], Ralf Schröder[3], Stefanie Blättermann[3], Evi Kostenis[3], Andrew J Brown[4], Akos Heinemann[1], Maria Waldhoer[1]

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The directional migration of neutrophils towards inflammatory mediators, such as chemokines and cannabinoids, occurs via the activation of seven transmembrane G protein coupled receptors (7TM/GPCRs) and is a highly organized process. A crucial role for controlling neutrophil migration has been ascribed to the cannabinoid CB_2 receptor (CB_2R), but additional modulatory sites distinct from CB_2R have recently been suggested to impact CB_2R-mediated effector functions in neutrophils. Here, we provide evidence that the recently de-orphanized 7TM/GPCR GPR55 potently modulates CB_2R-mediated responses. We show that GPR55 is expressed in human blood neutrophils and its activation augments the migratory response towards the CB_2R agonist 2-arachidonoylglycerol (2-AG), while inhibiting neutrophil degranulation and reactive oxygen species (ROS) production. Using HEK293 and HL60 cell lines, along with primary neutrophils, we show that GPR55 and CB_2R interfere with each other's signaling pathways at the level of small GTPases, such as Rac2 and Cdc42. This ultimately leads to cellular polarization and efficient migration as well as abrogation of degranulation and ROS formation in neutrophils. Therefore, GPR55 limits the tissue-injuring inflammatory responses mediated by CB_2R, while it synergizes with CB_2R in recruiting neutrophils to sites of inflammation.

Keywords: GPR55; CB_2R; chemotaxis; ROS production; Rac2; Cdc42

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The GPCR - associated sorting protein 1 regulates ligand-induced downregulation of GPR55

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Running title: GASP-1 regulates ligand induced GPR55 sorting

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Summary

Background and Purpose: Many G protein coupled receptors (GPCRs), including the CB1 cannabinoid receptor, are downregulated following prolonged agonist exposure by interacting with the GPCR associated sorting protein-1 (GASP-1). The CB1 cannabinoid receptor antagonist rimonabant has also recently been described to be an agonist at GPR55, a cannabinoid-related receptor. Here we investigated the post-endocytic properties of GPR55 after agonist exposure and tested whether GASP-1 was involved in this process.

Experimental Approach: We evaluated the direct protein-protein interaction of GPR55 with GASP-1 using i) GST-binding assays and ii) co-immunopreciptiation assays in GPR55-HEK293 cells with endogenous GASP-1 expression. We further tested the internalization, recycling and degradation of GPR55 using confocal fluorescence microscopy and biotinylation assays in the presence and absence of GASP-1 (lentiviral shRNA knockdown of GASP-1) under prolonged agonist (rimonabant (RIM), lysophosphatidylinositol (LPI)) stimulation.

Key Results: We show that the prolonged activation of GPR55 with rimonabant or LPI down-regulates GPR55 via GASP-1. GASP-1 binds to GPR55 *in vitro* and this interaction is required for targeting GPR55 for degradation. Disrupting the GPR55-GASP-1 interaction prevents post-endocytic receptor degradation, and thereby allows receptor recycling.

Conclusion and Implications: These data implicate GASP-1 as an important regulator of ligand-mediated downregulation of GPR55. By identifying GASP-1 as a key regulator of the trafficking and - by extension - functional expression of GPR55, we may be one step closer to gaining a better understanding of this receptor in response to cannabinoid drugs.

Keywords: GPCR, GASP-1, degradation, GPR55, LPI, rimonabant

Review

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G Protein-Coupled Receptor-Associated Sorting Protein 1 Regulates the Postendocytic Sorting of Seven-Transmembrane-Spanning G Protein-Coupled Receptors

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Key Words
G protein-coupled receptor-associated sorting protein 1 · G protein-coupled receptor-associated sorting protein 2 · Postendocytic receptor sorting · Seven-transmembrane domain G protein-coupled receptor · Trafficking · Degradation · Receptor, downregulation · Adaptor protein

Abstract
The largest superfamily of membrane proteins that translate extracellular signals into intracellular messages are the 7-transmembrane-spanning (7TM) G protein-coupled receptors (GPCR). One of the ways in which their activity is controlled is by the process of desensitization and endocytosis, whereby agonist-activated receptors are rapidly and often reversibly silenced through removal from the cell surface. Indeed, following endocytosis, individual receptors can be sorted differentially between recycling endosomes and lysosomes, which controls the reversibility of the silencing. Thus, endocytosis can either serve as a mechanism for receptor resensitization by delivering receptors back to the plasma membrane or facilitate receptor downregulation by serving as the first step towards targeting the receptors to lysosomes for degradation. The sorting of receptors to the lysosomal pathway can be facilitated by interaction with an array of accessory proteins. One of these proteins is the GPCR-associated sorting protein 1 (GASP-1), which specifically targets several 7TM-GPCR to the lysosomal pathway after endocytosis. Furthermore, GASP-1 was recently found to directly affect the signaling capacity of a 7TM-GPCR. Importantly, the in vivo relevance of GASP-1-dependent receptor sorting has also begun to be verified in animal models. Here, we summarize the recent advances in elucidating GASP-1-dependent receptor sorting functions and their potential implications in vivo.

Introduction

Endocytosis of 7-Transmembrane-Spanning G Protein-Coupled Receptors

Seven-transmembrane-spanning G protein-coupled receptors (7TM-GPCRs) comprise the largest superfamily of membrane proteins that translate extracellular signals into intracellular messages and can be activated by a wide range of compounds including odorants, photons, hormones and neurotransmitters [1, 2]. Due, likely, to their involvement in a variety of physiological and pathophysiological processes, the activity of 7TM-GPCRs is extensively regulated. One of the ways in which receptor-mediated signaling is modulated is by the process of

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BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 604–614

0007-1188/10
www.brjpharmacol.org

THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

GPR55 ligands promote receptor coupling to multiple signalling pathways

Christopher M Henstridge[1], Nariman AB Balenga[2], Ralf Schröder[3], Julia K Kargl[2], Wolfgang Platzer[2], Lene Martini[4], Simon Arthur[5], June Penman[1], Jennifer L Whistler[4], Evi Kostenis[3], Maria Waldhoer[2] and Andrew J Irving[1]

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Background and purpose: Although GPR55 is potently activated by the endogenous lysophospholipid, L-α-lysophosphatidylinositol (LPI), it is also thought to be sensitive to a number of cannabinoid ligands, including the prototypic CB1 receptor antagonists AM251 and SR141716A (Rimonabant®). In this study we have used a range of functional assays to compare the pharmacological activity of selected cannabinoid ligands, AM251, AM281 and SR141716A with LPI in a HEK293 cell line engineered to stably express recombinant, human GPR55.
Experimental approach: We evaluated Ca^{2+} signalling, stimulation of extracellular signal regulated kinase (ERK1/2) mitogen activated kinase MAP-kinases, induction of transcriptional regulators that are downstream of GPR55, including nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB) and cAMP response element binding protein (CREB), as well as receptor endocytosis. In addition, we assessed the suitability of a novel, label-free assay for GPR55 ligands that involves optical measurement of dynamic mass redistribution following receptor activation.
Key results: GPR55 linked to a range of downstream signalling events and that the activity of GPR55 ligands was influenced by the functional assay employed, with differences in potency and efficacy observed.
Conclusions and implications: Our data help to resolve some of the issues surrounding the pharmacology of cannabinoid ligands at GPR55 and highlight some differences in effector coupling associated with distinct GPR55 ligands.
British Journal of Pharmacology (2010) **160,** 604–614; doi:10.1111/j.1476-5381.2009.00625.x; published online 5 February 2010
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Keywords: GPR55; GPCR; cannabinoid; LPI

Scientific CV

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Aug. 08	Graduation with Distinction
Feb. 08 – Aug. 08	Masters Thesis, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria
Aug. 07 – Dec. 07	Exchange Student at Agnes Scott College, Atlanta, USA
Jan. 07 – Aug. 08	Studies of Molecular Microbiology (Master), University of Graz, Austria
Oct. 03 – Jan. 07	Studies of Molecular Biology (Bacc.), University of Graz, Austria
Oct. 99 – June 03	Grammar school

List of scientific publications

Original Articles

Kargl J, Balenga NA, Platzer W, Martini L, Whistler JL, Waldhoer M. (2011). The GPCR - associated sorting protein 1 regulates ligand-induced downregulation of GPR55. *Br. J. Pharmacol*., doi: 10.1111/j.1476-5381.2011.01562.

Balenga NA, Aflaki E, **Kargl J**, Platzer W, Schröder R, Blättermann S, Kostenis E, Brown A, Heinemann A, Waldhoer M. (2011). GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. *Cell Research*. **21**, 1452-1469

Henstridge CM, Balenga NA, Schroder R, **Kargl JK**, Platzer W, Martini L, Arthur S, Penman J, Whistler JL, Kostenis E, Waldhoer M., Irving AJ. (2010). GPR55 ligands promote receptor coupling to multiple signalling pathways. *Br. J. Pharmacol.*, **160**, 604-614.

Reviews/ Book Chapters

Henstridge CM, Balenga NA, **Kargl J**, Andradas C, Brown AJ, Irving A, Sanchez C, Waldhoer M. (2011) Recent developments in the physiology and pathology of the lysophosphatidylinositol-sensitive receptor GPR55. *Mol Endocrinol*., (11):1835-48

Balenga NA, Henstridge CM, **Kargl J**, Waldhoer M. (2011). Pharmacology, signaling and physiological relevance of the G Protein-coupled Receptor 55. *Advances in Pharmacology*, **62**, 251-77

Moser E, **Kargl J**, Whistler JL, Waldhoer M., Tschische P. (2010). G protein-coupled receptor-associated sorting protein 1 regulates the postendocytic sorting of seven-transmembrane-spanning G protein-coupled receptors. *Pharmacology*, **86**, 22-29.

Awards and Scholarships

Nov. 2011	Prize for Best Oral Communication DocDay 2011, Medical University of Graz, Austria
Oct. 2011	START Funding Project
Sep. 2011	ÖFG Fellowship – International Communication – Travel Grant
Nov. 2010	Prize for Best Oral Communication APHAR Meeting 2010, Vienna, Austria
Sep. 2010	EMBO Short Term Fellowship – for a 3 months research stay at UCSF
Sep. 2010	Erasmus Staff Training – Travel Grant
July 2010	BACA Visiting Scientists Program – Travel Grant
Nov. 2008	Research fellowship from the Austrian Government – 1 year
Jan. 2007	ISEP (International Student Exchange Program) fellowship – 1 semester

Research visits

Jan. – Feb. 2011	University of California, San Francisco, Ernest Gallo Clinic and Research Center, USA, Prof. Jennifer Whistler, PhD
Sep. – Nov. 2010	University of California, San Francisco, Ernest Gallo Clinic and Research Center, USA, Prof. Jennifer Whistler, PhD
Aug. – Dec. 2007	Agnes Scott College, Atlanta, Department of Biology, USA, Prof. Tim Finco, PhD

Scientific Presentations

Nov. 2011	DocDay of the Medical University of Graz, Graz, **[Talk]**
July 2011	21st Symposium of the International Cannabinoid Research Society, St. Charles, USA **[Poster]**
Jan. 2011	Gordon Research Conference – Molecular Pharmacology, Ventura, USA **[Poster]**
Nov. 2010	16th Scientific Symposium of the Austrian Pharmacological Society, Vienna, Austria **[Talk]**
July 2010	16th World Congress of Basic and Clinical Pharmacology, Copenhangen, Denmark **[Poster]**
Nov. 2009	15th Scientific Symposium of the Austrian Pharmacological Society, Graz, Austria **[Poster]**
May 2009	4th European Workshop on Cannabinoid Research, Madrid, Spain **[Poster]**
Nov. 2008	14th Scientific Symposium of the Austrian Pharmacological Society, Innsbruck, Austria **[Poster]**
Sep. 2008	1st SFB35 Symposium, Vienna, Austria **[Poster]**
Aug. 2008	1st Minisymposium on GPR55, Graz, Austria **[Talk]**