Universidade de Santiago de Compostela

Facultade de Medicina

Departamento de Fisioloxía

Laboratorio de Endocrinoloxia Celular

Definition of phosphorylation acceptor sites in the ghrelin receptor (GHSR1a): key elements determining functionality

Mónica Bouzo Lorenzo

Santiago de Compostela, Novembro 2015

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Memoria para optar o Grao de Doutora en Química
pola Universidade de Santiago de Compostela presentada por:

Mónica Bouzo Lorenzo

Santiago de Compostela, Novembro 2015

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A memoria adxunta titulada “Definition of phosphorylation acceptor sites in the ghrelin receptor (GHSR1a): key elements determining functionality” que para optar ó Grao de Doutora en Química presenta Dna. Mónica Bouzo Lorenzo, foi realizada baixo a nosa dirección na Área de Endocrinoloxía Molecular e Celular do Instituto de Investigación Sanitaria de Santiago do Complexo Hospitalario Universitario de Santiago de Compostela.

Considerando que constitúe un traballo de Tesis Doutoral, autorizamos a súa presentación na Universidade de Santiago de Compostela.

E para que así conste, firmamos a presente en Santiago de Compostela a Novembro de 2015.

Dr. Jesús Pérez Camiña — Investigador SERGAS

Prof. Dr. Felipe Casanueva Freijo — Catedrático de Medicina

Santiago de Compostela, Novembro de 2015

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ABBREVIATIONS

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7TM: seven transmembane

AgRP: agouti-related peptide

Akt: serine /threonine kinase (Protein kinase B)

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ANOVA: analysis of variance

AP2: adaptor protein 2

ARC: arcuate nucleus

ATP:adenosine triphosphate

β2AR: β2 adrenergic receptor

bHLH: basic helix-loop-helix

BrdU: bromodesoxiuridina

BRET: bioluminescence resonance energy transfer

BSA: bovine serum albumin

CaMKK: calmodulin-dependent protein kinase kinase

cAMP: cyclic adenosine monophosphate

C/EBP: CCAAT/enhancers binding protein

cGMP: cyclic guanosine monophosphate

CNS: central nervous system

CoA: Coenzyme A

CPT1: carnitine palmitoyl transferase 1

CRISPR: clustered regularly interspaced short palindromic repeats

DAG: diacylglycerol

DEX: dexamethasone

DNA: deoxyribonucleic acid

DM: Doble mutant

DMEM: Dulbecco´s Modified Eagle´s Medium

DTT: dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EGFP: enhanced green fluorescent protein

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

ERK1/2: extracellular signal-regulated kinases 1/2

EYFP: enhanced yellow fluorescent protein

FBS: fetal bovine serum

GC: guanylyl cyclase

GDI: guanine dissociation inhibitor

GDP: Guanosine 5' diphosphate

GEF: guanine exchange factor

GFP: green fluorescent protein

GH: growth hormone

GHRL: The human ghrelin/obestatin prepropeptide gene

GHSR1a: growth hormone secretagogue receptor type 1a

GHSR1b: growth hormone secretagogue receptor type 1b

GOAT: ghrelin O-acyl-transferase

GPCR: G-protein-coupled receptor

GPR39: G-protein 39-coupled receptor

GRK: G-protein-coupled receptor kinase

GTP: Guanosine 5' triphosphate

HA: hemagglutinin

HDR: homology directed repair

HEK: human embrionary kidney cells

HEPES: hydroxyethyl piperazineethanesulfonic acid

IBMX: 3-isobutyl-1-methylxanthine

ICQ: immunocitochemistry

IGF1: insulin-like growth factor 1

IL: interleukins

IP1: inositol 1-monophosphate

IP3: inositol (1, 4, 5)-triphosphate

JNK: Jun amino-terminal kinases

KDa: KiloDaltons

KLF4: Krüppel-like factor 4

NAFLD: non-alcoholic fatty liver disease

NF-κβ: nuclear factor-κβ

MAPK: mitogen-activated protein kinase

mRNA: messenger RNA

MBOAT: membrane-bound O-acyl transferase

MEF: murine embryonic fibroblast

mTOR: mammalian target of rapamycin

mTORC2: mammalian target of rapamycin complex 2

NO: nitric oxide

NOS: nitric oxide synthase

NPY: neuropeptide Y

PAGE: polyacrilamide gel electrophoresis

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline tween

PCR: polymerase chain reaction

PDK1: 3-phosphoinositide-dependent kinase-1

PH: pleckstrin homology domain

PI3K: phosphatidylinositol 3´-kinase

PLC: phospholipase C

PIP3: phosphatidylinositol(3,4,5)-trisphosphate

PIPLC: phosphatidylinositol-specific phospholipase C

PKA: protein kinase A

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

POMC: proopiomelanocortin precursor protein

PP2A: protein phosphatase 2A

PPARγ: peroxisome proliferator-activated receptor γ

PRL: prolactin

PTH1R: parathyroid hormone type 1 receptor

PTP: protein tyrosine phosphatase

PTX: pertussis toxin

RFP: red fluorescent protein

RGS: regulator of G protein signaling

Rluc: Renilla luciferase

RNA: ribonucleic acid

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulfate

SE: status epilepticus

SHP1: Src homology-2 domain containing phosphatase-1

SIRT1: NAD-dependent protein deacetylase sirtuin-1

siRNA: small interfering RNA

SNP: single nucleotide polymorphism

SRE: serum response element

SREBP1: sterol-regulatory element binding protein-1

TK: tyrosin kinases

TNF: tumor necrosis factor

TM: Triple mutant

UCP2: uncoupling protein 2

USF: Upstream stimulatory factors

VEGF: vascular endothelial growth factor

VMH: ventromedial hypothalamic nucleus

WORT: wortmannin

WT: wild type

OBJECTIVES

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The growth hormone secretagogue receptor type 1a (GHSR1a) critically regulates the central and peripheral actions of ghrelin as a growth hormone (GH) secretagogue, an orexigenic peptide, and a long-term regulator of energy homeostasis. GHSR1a classically exerts its intracellular effects through G-protein activation, mainly via $G_{q/11}$ and $G_{i/o}$. However, recent evidence has demonstrated that β-arrestins act as molecular mediators of G-protein independent signaling by acting to scaffold of a variety of signaling proteins. Thus, it is becoming increasingly evident that β-arrestins, originally discovered as mere adaptor proteins for the GHSR1a endocytosis, have much broader signaling and physiological roles. What is not clear however is whose factors regulate GHSR1a coupling to β-arrestin-dependent signaling and the processes that regulate the relative contribution of G-protein versus β-arrestin-dependent signaling. It is now well established that GPCR phosphorylation plays a crucial role in the recruitment and activation of β-arrestin-dependent signaling. Indeed, recent studies have gone further and suggested that the phosphorylation pattern of a GPCR constitutes a barcode that determines, at least in part, the signaling outcomes. Thus, the main aim of this study was to determine the possibility that GHSR1a regulates β-arrestin-dependent signaling in a phosphorylation dependent manner and, thus, to establish the existence of a phosphorylation barcode that impacts on GHSR1a signaling. This global aim is divided into the following particular goals:

1. To determine the phosphorylation sites in the C terminus of the GHSR1a.

2. To analyze the impact that GHSR1a phosphorylation on various signaling and cellular responses.

3. To identify the protein kinases responsible for phosphorylation of the GHSR1a.

4. To determine the key structural elements of ghrelin which ensure its bioactivity in order to identify the minimum region of amino acids necessary to regulate the functionalities of the GHSR1a.

INTRODUCTION

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1.0 GROWTH HORMONE SECRETAGOGUE RECEPTOR (GHSR)

1.1 GHSR Gene

The human GHSR (hGHSR) is a single highly conserved gene located on chromosome 3q26.2 of approximately 4.3 kb. hGHSR gene is composed of two exons separated by one intron, whose alternative splicing generates two types of mRNA: GHSR1a and GHSR1b[1]. GHSR type 1a is encoded by both exons after splicing elimination at position 796/797 from the start codon, of 2152 nucleotides belonging to the intron sequence of the pre-mRNA. As a consequence, human GHSR1a is a 366-amino acid G protein-coupled receptor (GPCR) with seven transmembrane regions whose molecular mass is approximately 41 kDa. While exon 1 is composed of the 5'-untranslated region and the first 265 amino acids encoding for the transmembrane (TM) domains 1–5, exon 2 encodes the 101 amino acids of TM domains 6 and 7 and includes the 3'-untranslated region. On the other hand, GHSR type 1b is encoded by the exon 1 and retains the first 74 nucleotides of the GHSR1a intronic sequence, which generates a GPCR isoform of 289 amino acids containing only the first five transmembrane regions and 24 dissimilar amino acids at the C-terminal region comparing with the GHSR1a sequence, due to the use of an alternative stop codon[2,3] (Figure 1).

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[1] McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LH, Howard AD. Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol Endocrinol* 1997; 11: 415–423.

[2] Petersenn S, Rasch AC, Penshorn M, Beil FU, Schulte HM. Genomic structure and transcriptional regulation of the human growth hormone secretagogue receptor. *Endocrinology* 2001; 142: 2649–2659.

[3] Camiña JP. Cell Biology of the Ghrelin Receptor. *Journal of Neuroendocrinology*, 2006 18: 65–76

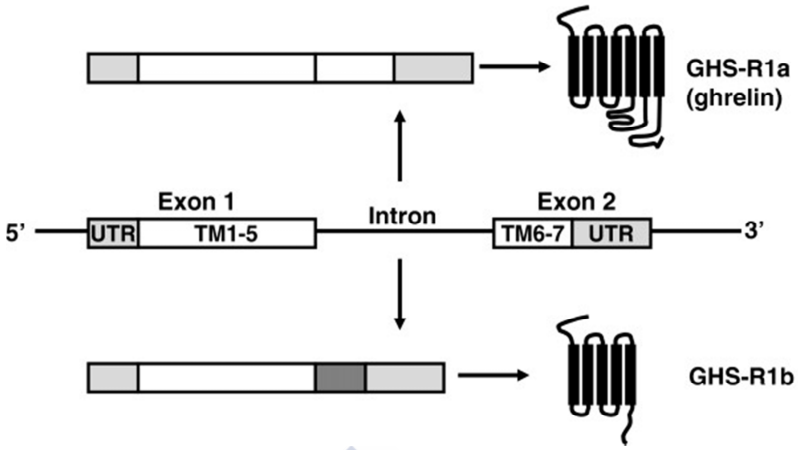

Figure 1. Alternative splicing of GHSR gene. An alternative splicing of GHSR gene generates GHSR1a and GHSR1b. Generation of the 5TM domains truncated ghrelin receptor results from failure to remove the intron between the two coding exons and the use of an alternative stop codon and a polyadenylation signal within the intron (dark box) (Extracted from J Mol Signal. 2012 Sep 1;7(1):13).

The expression of each GHSR subtype has been demonstrated to be different. While to GHSR1a mRNA is predominantly expressed in the pituitary and the hypothalamus, the GHSR1b mRNA is widespread in tissues as varied as liver, pancreas, breast, pituitary, myocardium, skin, thyroids or colon[4]. In addition, location studies have been shown that GHSR1a tagged with green fluorescent protein (GFP) was localized in the plasma membrane of HEK293 cells, whereas GHSR1b tagged with the same flag was confined to the nucleus. Moreover, GHSR1a homodimers were founded in all subcellular

[4] Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab*. 2002 Jun;87(6):2988.

fractions while GHSR1a/GHSR1b heterodimers were concentrated in the endoplasmic reticulum[5,6].

In terms of activation and associated functionality, massive differences have also been determined for both types. Contrary to GHSR1b receptor which fails to bind ghrelin, the type 1a has shown high affinity for the family of synthetic growth hormone (GH) secretagogues and specific binding to S3 acylated ghrelin. Indeed, this S3 acylation confers to ghrelin the high conformational flexibility necessary for the interaction with GHSR1a. In fact, ghrelin peptide functionality was reduced by experimental acylation of other S residues (S2, 6, and 18)[7]. The GHSR1a high binding affinity to acylated ghrelin is related with the modulation of a multiplicity of physiological functions such as energy homeostasis, GH secretion, food intake, adiposity, glucose metabolism, improvement of cardiovascular functions, modulation of stress and anxiety, protection against muscle atrophy or body weight gain[8,9]. On the contrary, the functional activity of GHSR1b remains to be

[5] Smith RG, Jiang H, Sun Y. Developments in ghrelin biology and potential clinical relevance. *Trends Endocrinol Metab*. 2005;16(9):436-42.

[6] Chow KB, Sun J, Chu KM, Tai Cheung W, Cheng CH, Wise H. The truncated ghrelin receptor polypeptide (GHS-R1b) is localized in the endoplasmic reticulum where it forms heterodimers with ghrelin receptors (GHS-R1a) to attenuate their cell surface expression. *Mol Cell Endocrinol*. 2012 Jan 2;348(1):247-54.

[7] Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH, Heck JV.Minimal Sequence of Ghrelin Necessary for Activation of Growth Hormone Secretagogue Receptor 1a *Journal of Medicinal Chemistry* 2000 *43* (23), 4370-4376.

[8] Sivertsen B, Holliday N, Madsen AN, Holst B. Functionally biased signalling properties of 7TM receptors - opportunities for drug development for the ghrelin receptor. *Br J Pharmacol*. 2013 Dec; 170(7):1349-62.

[9] Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, Batterham RL, Benoit SC, Bowers CY, Broglio F, Casanueva FF, D'Alessio D, Depoortere I, Geliebter A, Ghigo E, Cole PA, Cowley M, Cummings DE, Dagher A, Diano S, Dickson SL, Diéguez C, Granata R, Grill HJ, Grove K, Habegger KM, Heppner K, Heiman ML, Holsen L, Holst B, Inui A, Jansson JO, Kirchner H, Korbonits M, Laferrère B, LeRoux CW, Lopez M, Morin S, Nakazato M, Nass R, Perez-Tilve D, Pfluger PT, Schwartz TW, Seeley RJ, Sleeman M, Sun Y, Sussel L, Tong J, Thorner MO, van der Lely AJ, van der Ploeg LH, Zigman JM, Kojima M, Kangawa K, Smith RG, Horvath T, Tschöp MH. Ghrelin. *Mol Metab*. 2015 21;4(6):437-60.

unclear. Curiously, the concurrent expression of GHSR1b and GHSR1a attenuates the signal transduction capacity of GHSR1a, suggesting that both receptors may be interacting. Besides, further heterodimerization studies have also demonstrated that GHSR1b exerts a dominant negative inhibition of GHSR1a signaling[10,11].

The study of the promoter region of hGSR gen has shown a tissue-specific regulation. Sequencing analysis determined that the major transcription start site is an adenosine residue located 227 nucleotides upstream from the ATG translation initiation codon, as well as, it determines that the 5'-flanking region does not contain the usually required promoter regions, such as a TATA box, CAAT box or GC-rich region. Nonetheless, the sequencing study also revealed initiator elements located at -215 and -237 surrounding the transcription start site. In addition to the initiation elements in the 5'-flanking region of the hGHSR gen have also been identified a number of binding transcription factors among which should be highlighted the PUO-domain factors, Ptx1, Ptx2, Oct-1, Brn-2 and Pit-1 for their implication in the pituitary-specific expression of the gen. Furthermore, transient transfections of the hGHSR gene promoter shown significant activity in GH4 and GH3 cells (somatotroph pituitary cell lines), whereas no activity was detected in other tissues cell lines such as COS-7 (monkey kidney cells), Skut-1B (human endometrium cells), LHRH (mouse hypothalamic cells), GT1-7 (neuronal cells), AtT20 (mouse corticotroph pituitary cells), HeLa (human epithelioid cervix carcinoma cells) or EP1 (neuroblastoma cells) corroborating the tissue-specifity. The sequenced work also revealed the

[10] Chan CB and Cheng CH. Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream Acanthopagrus schlegeli. *Mol Cell Endocrinol*. 2004 Feb 12;214(1-2):81-95.

[11] Mary S, Fehrentz JA, Damian M, Gaibelet G, Orcel H, Verdié P, Mouillac B, Martinez J, Marie J, Banères JL. Heterodimerization with Its splice variant blocks the ghrelin receptor 1a in a non-signaling conformation: a study with a purified heterodimer assembled into lipid discs. *J Biol Chem*. 2013 Aug 23;288(34):24656-65.

presence of putative binding sites for activator proteins 1 and 2, basic helix-loop-helix factors, PEA-3, Myb and NF-IL6, some of which regulate the basal activity of the gene promoter. In addition, several systems suggest that hGHSR gen could be under regulatory control and the study of the hormonal regulation of the 5'-flanking region demonstrate on the one hand the stimulatory effect of the thyroid hormone and estrogen and on the other hand, the inhibitory effect of glucocorticoids in the promoter activity through the transcriptional mechanism involving nuclear factors2'[12,13]. However, to determine the specific transcription factors which regulate the promoter activity of the hGHSR gene, further studies need to be done.

The sequence of the hGHSR gen and the elucidation of its location and structure have been demonstrated to be crucial in the identification of mutations that could lead to better understanding of several diseases and even defining the GHSR gene as a possible epigenetic marker. It has been shown that a single nucleotide polymorphism (SNPs) and haplotypes within the hGHSR gene region might influence susceptibility to obesity and be involved on its pathogenesis due an increment on food intake[14], although exist other studies that have not been able to relate SNPs with obesity, weight regulation, food intake or GH secretion making these point controversial[15,16]. On the other hand, a link between epigenetic down-

[12] Kaji H, Tai S, Okimura Y, Iguchi G, Takahashi Y, Abe H, Chihara K. Cloning and characterization of the 5'-flanking region of the human growth hormone secretagogue receptor gene. *J Biol Chem* 1998; 273: 33885–33888.

[13] Kaji H, Kishimoto M, Kirimura T, Iguchi G, Murata M, Yoshioka S, Iida K, Okimura Y, Yoshimoto Y, Chihara K. Hormonal regulation of the human ghrelin receptor gene transcription. *Biochem Biophys Res Commun* 2001; 284: 660–666.

[14] Baessler A, Hasinoff MJ, Fischer M, Reinhard W, Sonnenberg GE, Olivier M, Erdmann J, Schunkert H, Doering A, Jacob HJ, Comuzzie AG, Kissebah AH,Kwitek AE. Genetic linkage and association of the growth hormone secretagogue receptor (ghrelin receptor) gene in human obesity. *Diabetes*. 2005 Jan;54(1):259-67.

[15] Hess O, Admoni O, Khayat M, Elias G, Almagor T, Shalev SA, Tenenbaum-Rakover Y. Ghrelin and growth hormone secretagogue receptor (GHSR) genes are not commonly involved in growth or weight abnormalities in an Israeli pediatric population. *J Pediatr Endocrinol Metab*. 2012;25(5-6):537-40.

regulation of hGHSR gen by DNA hypermethylation and breast cancer cell invasion has been reported[17,18] , as well as, has been describe that GHSR hypermethylation may have a functional role in tumorigenesis of other cancers. Bisulfite pyrosequencing studies has shown substantial hypermethylation at the promoter and first exon of the GHSR gen revealing GHSR hypermethylation as a common epigenetic mark that distinguishes cancers from non-cancer specimens regardless of tumor type. For this reason, GHSR methylation could be used as a signature with high specificity and sensitivity of cancer detection[19]. Moreover, common variants founded in the hGHSR region are associated with parameters of left ventricular mass and geometry independent of blood pressure and body mass in the general population and, thus, may be involved in the pathogenesis of left ventricular hypertrophy[20]. However, the results of all of these studies suggest that the effects of genetic variants in GHSR gen are modest, making necessary further studies of this gen in larger population groups with more individual diversity to investigate its direct effect on diseases.

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[16] Luglio HF, Inggriyani CG, Huriyati E, Julia M, Susilowati R.Association of SNPs in GHSR rs292216 and rs509035 on dietary intake in Indonesian obese female adolescents. *Int J Mol Epidemiol Genet*. 2014 Dec 15;5(4):195-9.

[17] Botla SK, Gholami AM, Malekpour M, Moskalev EA, Fallah M, Jandaghi P, Aghajani A, Bondar IS, Omranipour R, Malekpour F, Mohajeri A, Babadi AJ, Sahin Ö, Bubnov VV, Najmabadi H, Hoheisel JD, Riazalhosseini Y.Diagnostic values of GHSR DNA methylation pattern in breast cancer. *Breast Cancer Res Treat*.2012 Oct;135(3):705-13.

[18] Pabalan NA, Seim I, Jarjanazi H, Chopin LK. Associations between ghrelin and ghrelin receptor polymorphisms and cancer in Caucasian populations: a meta-analysis.*BMC Genet*.2014Nov 7;15:118.

[19] Moskalev EA, Jandaghi P, Fallah M, Manoochehri M, Botla SK, Kolychev OV, Nikitin EA, Bubnov VV, von Knebel Doeberitz M, Strobel O, Hackert T, Büchler MW, Giese N, Bauer A, Muley T, Warth A, Schirmacher P, Haller F, Hoheisel JD, Riazalhosseini Y. GHSR DNA hypermethylation is a common epigenetic alteration of high diagnostic value in a broad spectrum of cancers. *Oncotarget*. 2015 28;6(6):4418-27.

[20] Baessler A, Kwitek AE, Fischer M, Koehler M, Reinhard W, Erdmann J, Riegger G, Doering A, Schunkert H, Hengstenberg C. Association of the Ghrelin receptor gene region with left ventricular hypertrophy in the general population: results of the MONICA/KORA Augsburg Echocardiographic Substudy. *Hypertension*. 2006 May;47(5):920-7.

1.2. GHSR protein

The ghrelin receptor GHSR1a is a typical G protein-coupled receptor (GPCR) which belongs to a family of receptors with its own name. The ghrelin receptor family is a group of GPCRs type A which includes motilin receptor (~52% homology), neurotensin receptors 1 and 2 (~35% homology), GPR39 (~30% homology) and neuromedin receptors 1 and 2 (~30% homology)[1,21,22] (Figure 2).

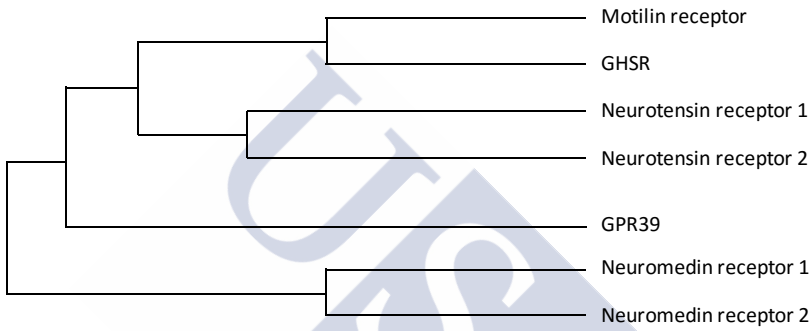

Figure 2. The ghrelin receptor family. Schematic phylogenic tree of the ghrelin receptor family indicating the relative relationship of the receptor (Modified from J Biol Chem. 2004 Dec 17;279(51):53806-17).

The receptors of this family are 7TM domains receptors which are localized in the plasma membrane and responsible for translating extracellular signals into intracellular responses. The critical role of 7TM receptors regulating several physiological processes makes them the targets of 30% of the drugs

[21] McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD, Van der Ploeg LH. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics*. 1997 Dec 15;46(3):426-34.

[22] Smith RG, Leonard R, Bailey AR, Palyha O, Feighner S, Tan C, Mckee KK, Pong SS, Griffin P, Howard A. Growth hormone secretagogue receptor family members and ligands. *Endocrine*. 2001;14(1):9-14.

currently on the market[23]. 7TM receptors are called so because they share a seven α-helix hydrophobic transmembrane domains structure which includes three intra- and extracellular loops, an N-terminal domain located on the extracellular side and a carboxy terminus on the intracellular side. Docking studies has shown that in the case of GHSR1a, the TM domains forms a round calyx-like structure with the transmembrane helices organized in an anticlockwise sense and the Pro residues in the center. In this organization, the third domain takes the most central position in transmembrane segment and the fifth occupy the most peripheral one. At the same time, domains 2, 3 and 7 forms the core of the transmembrane structure while domains 1 and 5 are in the external positions. The three extracellular loops mainly stabilized by polar contacts adopt hairpin geometries. The hairpin structure is also adopted by the N-terminal domain which involves residues between M1 and D32 and it is stabilized by hydrogenous bonds and electrostatic interaction between the D32 and the ammonium group that close the hairpin. Lastly, the docking studies shown the C-terminal tail as a helix–turn–helix structure with an eighth helix between S327and P342 perpendicular to seven helix and rich in residues like K328, K329, R331, and R336 which are positively charged and anchor the terminal domain to phospholipidic heads[24,25].

[23] Kenakin, T. and Christopoulos, A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov*. 2013; 12, 205–216

[24] Pedretti A, Villa M, Pallavicini M, Valoti E, Vistoli G. Construction of human ghrelin receptor (hGHS-R1a) model using a fragmental prediction approach and validation through docking analysis. *J Med Chem*. 2006 Jun 1;49(11):3077-85.

[25] Pedretti A, Vistoli G.Modeling of human ghrelin receptor(hGHS-R1a) in its close state and validation by molecular docking.*Bioorg Med Chem*.2007Apr15;15(8):3054-64.

Conformational changes of GHSR1a activation and basal activity

Since GHSR1a was discovered, the conformational changes associated to its activation have been studied using a number of biochemical and biophysical approaches. Docking and mutational studies was performance with the purpose of elucidate the physiological impact of these alterations as well as the pharmacological consequences of the corresponding mutations. Among the functional alterations discovered it was found shifts in basal signaling, differences in the response to ghrelin and other agonists, distinct inverse agonist function and variations in the level of receptor expression. Key residues for ghrelin binding are located on the opposing inner faces of the TM domains 3, 6 and 7 which is in line with the toggle-switch model described for the 7TM receptors activation. According to this model, when the receptor is activated by its agonist, TM domains 6 and 7 vertical fluctuate around a pivot corresponding to the conserved P amino acid in the middle of each of these helices. The extracellular region of TM domains 6 and 7 helices seems to move inward toward the third TM domain while the intracellular ends move in the opposite direction making accessible the epitopes recognized for instance by G proteins. It has been proposed additional contact points for ghrelin and the receptor located for example in the loop regions but E09 of TM domain 3 seems to be the major hit for ghrelin[26,27,28]. The interaction of other GHSR1a natural or synthetic agonist was also studied. These works reflect that all the agonist shown a high dependence of E124 in the third TM domain as well as ghrelin. However, it is remarkable the fact that they all were affected by mutation of

[26] Holst B, Schwartz TW. Ghrelin receptor mutations--too little height and too much hunger. *J Clin Invest*. 2006 Mar;116(3):637-41.

[27] Holst B, Lang M, Brandt E, Bach A, Howard A, Frimurer TM, Beck-Sickinger A, Schwartz TW.Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on thereceptor.*Mol Pharmacol*.2006Sep;70(3):936-46

[28] Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM, Elling CE. Molecular mechanism of 7TM receptor activation - a global toggle switch model. *Annu Rev Pharmacol Toxicol*. 2006;46:481-519.

D99 in the second TM domain, while ghrelin was not affected by this mutation. Although the ligands have shown different binding sites to the receptor, they share a common binding pocket with the endogenous agonist on the opposing faces of TM domains 3, 6 and 7 of the ghrelin receptor. Some of the agonists studied also demonstrate to be ago-allosteric modulators, i.e. peptides that modulate dose-response curve for ghrelin in terms of potency and efficacy. Mutational map for allosteric properties of these peptides overlaps with its agonism map but allosteric effect is different and includes fewer residues in the main ligand binding pocket[29] (Figure 3).

Worthy of note, it is the fact that the GHSR1a receptor apparently shows a high constitutive activity due to its ability to interchange active and inactive conformations itself. This ligand independent activity seems to be control by an aromatic cluster located in the ghrelin binding pocket form by F279 in the TM6, and F309 and F312 in the TM domain 7 which are capable of stabilize the active conformation of the receptor without any ligand present[30,31]. It was also describe the importance of W276 (TM domain 3) and V131 and I134 (TM domain 6) in the maintenance of the basal activity, but more interesting is the fact that the second extracellular loop was also reported to be crucial for the basal activity of the receptor despite being far from the ghrelin binding pocket. The constriction of the α-helix structure of the second loop affects the free movement of TM domain 5 relative to the third TM domain which is required to establish the aromatic interactions.

[29] Holst B, Frimurer TM, Mokrosinski J, Halkjaer T, Cullberg KB, Underwood CR, Schwartz TW. Overlapping binding site for the endogenous agonist, small-molecule agonists, and ago-allosteric modulators on the ghrelin receptor. *Mol Pharmacol*. 2009 Jan;75(1):44-59.

[30] Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW. Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem*. 2004 Dec 17;279(51):53806-17.

[31] Els S, Schild E, Petersen PS, Kilian TM, Mokrosinski J, Frimurer TM, Chollet C, Schwartz TW, Holst B, Beck-Sickinger AG.An aromatic region to induce a switch between agonism and inverse agonism at the ghrelin receptor. *J Med Chem*. 2012 Sep 13;55(17):7437-49.

Especially important to maintain the α-helix structure is the aliphatic A204 whose mutation reduces dramatically the basal activity of the receptor keeping the capacity for ghrelin binding. The mutation of this A204 has also shown a reduction in the surface receptor expression and it was described in patients who suffer from obesity and short stature (Figure 3)[32,33,34,35,36].

[32] Wang HJ, Geller F, Dempfle A, Schäuble N, Friedel S, Lichtner P, Fontenla-Horro F, Wudy S, Hagemann S, Gortner L, Huse K,Remschmidt H, Bettecken T, Meitinger T, Schäfer H, Hebebrand J, Hinney A. Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescent, healthy normal-weight and under-weight students with short normal stature.*J Clin Endocrinol Metab*. 2004 ; 89(1):157-62.

[33] Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest*. 2006 Mar;116(3):760-8.

[34] Liu G, Fortin JP, Beinborn M, Kopin AS.Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. *J Pharmacol Exp Ther*. 2007 Sep;322(3):1036-43.

[35] Gozé C, Bergé G, M'Kadmi C, Floquet N, Gagne D, Galleyrand JC, Fehrentz JA, Martinez J. Involvement of tryptophan W276 and of two surrounding amino acid residues in the high constitutive activity of the ghrelin receptor GHS-R1a.*Eur J Pharmacol*.2010Sep25;643(2-3):153-61

[36] Mokrosiński J, Frimurer TM, Sivertsen B, Schwartz TW, Holst B. Modulation of constitutive activity and signaling bias of the ghrelin receptor by conformational constraint in the second extracellular loop.*J Biol Chem*.2012Sep28;287(40):33488-502.

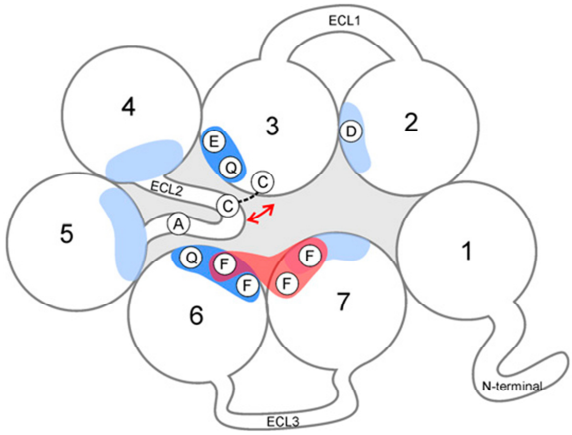

Figure 3. Key sites for agonist activation and constitutive activity of GHSR1a. The picture represents the external surface of the receptor, with TMs indicated by 1–7. The binding surfaces for ghrelin are in dark blue and the sites involve in others agonist interactions in light blue. The red area represents the pocket bordered by aromatic amino acids that determines constitutive activity. The crucial interaction for constitutive activity between ECL2 and TM3 is also represented (Extracted from Pharmacol Rev. 2014 Oct;66(4):984-1001).

Thus, blocking the basal activity of the GHSR1a by means agonists and/or antagonists could be a challenge point for effective treatment of obesity. Contrary to the agonists, the inverse agonist peptides bind in an extended pocket which involves D99, the last amino acid of the second TM domain and TM domains 3, 4, 5, 6 and 7. It is proposed that the inverse agonists prevent the spontaneous receptor activation by inserting relatively deeply across the main ligand-binding pocket and sterically blocking the movement of TM domains 6 and 7 into active conformation interacting with the aromatic cluster essential for ghrelin action (F279, F309 and F312). Unlike agonists, the invert agonist does not shown dependence of E124 of TM domain 3, but all the inverse agonists share a high dependence of the D99. It is noteworthy that minor changes in the peptides sequence determine between agonism and antagonism. Furthermore, the S123 of the receptor could be tricky for switch between agonism and inverse agonism[26,27,34,36].

2.0 GHRELIN

2.1 From gene to peptide

Ghrelin is a peptide hormone originally isolated from rat stomach by reverse pharmacology and identified as the endogenous ligand for GHSR1a. The name indicates its ability to stimulate GH release because "ghre" is a word root for "grow" in Proto-Indo-European languages[37].

The human ghrelin/obestatin prepropeptide gene (GHRL) is located on the short arm of chromosome 3 in the position 3q 25-26. The first studies of the ghrelin gene describe four exons and another short exon of 20 base pairs, which encodes part of the 5'-untranslated region and is transcribed as a minor component of ghrelin mRNA[38,39]. However, more recent studies have revealed the existence of a number of alternative upstream exons in the ghrelin gene (exon -1, exon 0, and extended exon 1), increasing the complexity of this gene[40] (Figure 4). The 5'-flanking region of the human GHRL gene contains a TATA box-like sequence (TATATAA), but is not able to affect the promoter activity, suggesting that this element is not used. Moreover neither a typical GC nor a CAAT box was found in the promoter region. However, different activating sequences was describe in TT cells, a human medullary carcinoma cell line, and ECC10 cells, a human stomach-derived cell line, suggesting that ghrelin gene expression may be cell-type

[37] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach.*Nature* 1999;402:656–660.

[38] Tanaka M, Hayashida Y, Iguchi T, Nakao N, Nakai N, Nakashima K.Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon. *Endocrinology*. 2001 Aug;142(8):3697-700.

[39] Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, Hosoda H, Kojima M, Kangawa K, Nakao K.Genomic structure and characterization of the 5'-flanking region of the human ghrelin gene. *Endocrinology*. 2004 Sep;145(9):4144-53.

[40] Seim I, Herington AC, Chopin LK.New insights into the molecular complexity of the ghrelin gene locus. *Cytokine Growth Factor Rev*. 2009 Aug;20(4):297-304.

specific[39,41,42]. The 5'-flanking region of the human GHRL gene has also exhibit several E-box consensus sequences and putative binding sites for several transcription factors, such as AP2, basic helix-loop-helix (bHLH), PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5, and nuclear factor-κβ(NF-κβ), as well as, half-sites for estrogen and glucocorticoid response elements[38,39,41]. Ablation or site-directed mutagenesis of E-box consensus sequences decreased the promoter activity in TT cells, implicating them in promoter activation. Upstream stimulatory factors (USF), a member of the bHLH family of transcription factors, bind to these E-box elements and may thus regulate human ghrelin gene expression. Other report also indicates that KLF4 (Krüppel-like factor 4) positively regulates human ghrelin gene expression by binding to a KLF-responsive element[39,43].

As its own name indicates ghrelin/obestatin prepropeptide gen, in addition to ghrelin, also encodes the 23 amino acid peptide obestatin as well as small signal peptides. The 28 amino acids of the functional ghrelin peptide are encoded only by the exons 1 and 2 of the GHRL gene. The active ghrelin sequence immediately follows the signal peptide in human GHRL gen (Figure 4). Although propeptides are usually processed at dibasic amino acid sites by prohormone convertases, the C-terminus of the human ghrelin peptide is processed at an uncommon P-R recognition site. PC1/3 was identified as the processing protease involve in the of proghrelin cleveage to yield the 28-amino acid ghrelin peptide in the stomach[44]. However, in pancreas,

[41] Kishimoto M, Okimura Y, Nakata H, Kudo T, Iguchi G, Takahashi Y, Kaji H, Chihara K.Cloning and characterization of the 5(')-flanking region of the human ghrelin gene. *Biochem Biophys Res Commun*. 2003 May 23;305(1):186-92.

[42] Nakai N, Kaneko M, Nakao N, Fujikawa T, Nakashima K, Ogata M, Tanaka M Identification of promoterregion of ghrelin gene in human medullary thyroid carcinoma cell line. *Life Sci*. 2004 Sep 17;75(18):2193-201.

[43] Lee HJ, Kang YM, Moon CS, Joe MK, Lim JH, Suh YH, Song J, Jung MH. KLF4 positively regulates human ghrelin expression. *BiochemJ*. 2009 May 27;420(3):403-11.

[44] Zhu X, Cao Y, Voogd K, Steiner DF. On the processing of proghrelin to ghrelin. *J Biol Chem*. 2006 Dec 15;281(50):38867-70.

proghrelin is not processed exclusively by PC1/3, PC2 has also demonstrated to play some part in the processing of pancreatic proghrelin[45].

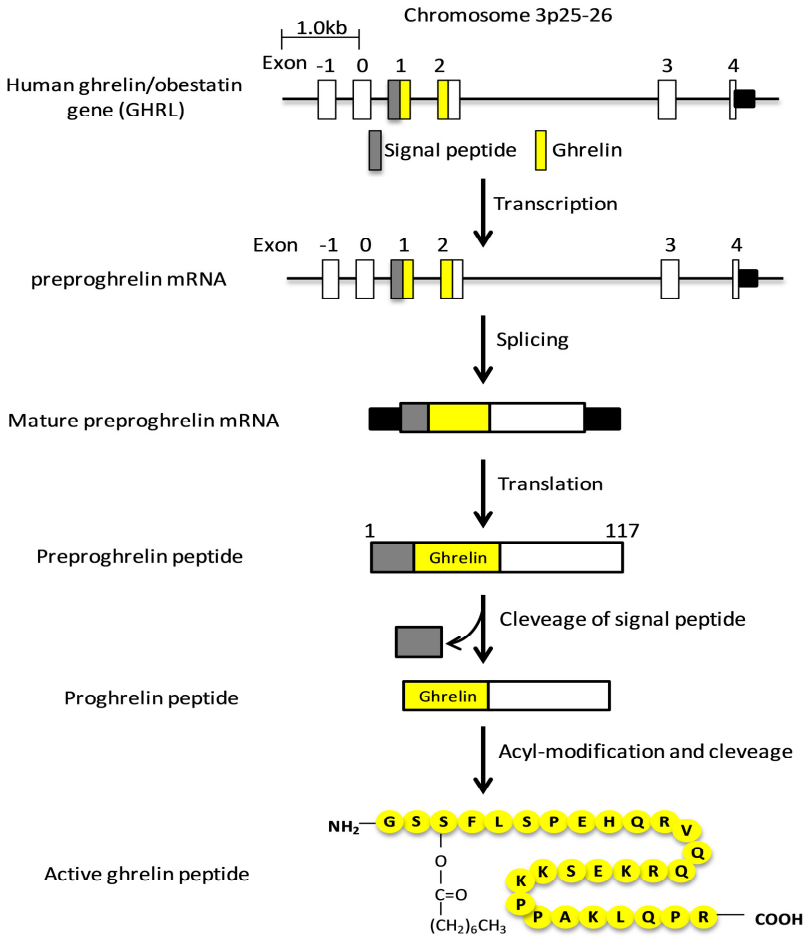

Figure 4. From the human ghrelin/obestatin gene (GHRL) to an active ghrelin peptide. The transcription of the human ghrelin/obestatin gene yields an mRNA which is translated into a 117 amino acid ghrelin precursor. Protease cleavage and acylation of the ghrelin precursor result in the 28 amino acid active ghrelin peptide (Modified from Results Probl Cell Differ. 2010; 50:185-205).

[45] Walia P, Asadi A, Kieffer TJ, Johnson JD, Chanoine JP. Ontogeny of ghrelin, obestatin, preproghrelin,and prohormoneconvertases in rat pancreas and stomach. *Pediatr Res*. 2009 Jan;65(1):39-44.

2.2 Ghrelin activation an its physiological consequences

As was previously said, to activate GHSR1a, ghrelin requires the attachment of a fatty acid side-chain to its serine 3 residue. This acylation is a rare post-translational modification; in fact ghrelin is the first case of a peptide hormone modified by a fatty acid[46,47]. Ghelin acylation is achieved by the ghrelin O-acyl-transferase (GOAT), an enzyme with multiple transmembrane domains which is a member of the membrane-bound O-acyltransferase (MBOAT) family. GOAT had not been identified until 2008 when two independent groups reported its identification[48,49]. GOAT distribution is similar to that of ghrelin, is predominantly found in gastrointestinal organs and particularly in the stomach[48,49,50]. GOAT specifically modifies the third S of human ghrelin. The replacement of the second, sixth, and 18th S of ghrelin by A, did not change the modified by *n*-octanoic acid[48], but when the third S was replaced by A, GOAT was not able to modify ghrelin. Something remarkable is that GOAT modifies not only S but also T residues. The third amino acid of frog ghrelin is T and it is also modified by *n*-octanoic acid as ghrelin's S3[51]. Moreover, when the third S of

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46 Matsumoto M Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y, Kangawa K. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochem Biophys Res Commun*. 2001 Sep 14;287(1):142-6.

47 Kojima M, Kangawa K. Ghrelin: from gene to physiological function. *Results Probl Cell Differ*. 2010;50:185-205.

48 Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL.Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell*. 2008 Feb 8;132(3):387-96.

49 Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A*. 2008 Apr 29;105(17):6320-5.

50 Sakata I, Yang J, Lee CE, Osborne-Lawrence S, Rovinsky SA, Elmquist JK, Zigman JM. Colocalization of ghrelin O-acyltransferase and ghrelin in gastric mucosal cells. *Am J Physiol Endocrinol Metab*. 2009 Jul;297(1):E134-41.

51 Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, Matsumoto M, Minamitake Y, Kikuyama S, Kangawa K. Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. *J Biol Chem*. 2001 Nov 2;276(44):40441-8.

rat ghrelin is replaced by T, GOAT modifies the replaced T by *n*-octanoic acid[48], indicating what is important is the position. Besides the third position of the S, the G1 and P4 are also critical components of the recognition sequence for GOAT. While the replacement of G1 and F4 by A inhibit the acyl modification of the S3, the change of S2, L5, S6, or P7 by A did not have an effect on the acyl modification of ghrelin. In addition, N-terminal addition of two amino acids (S-A) significantly suppressed the acylation of the third S of ghrelin[52].

Biochemically, GOAT appears to have two critical substrates, desacyl ghrelin (DAG) and short- to mid-chain fatty acids thioesterified with Coenzyme A (CoA). The ghrelin modification reaction does not occur with free acyl acid or a mixture of free acyl acid and CoA[53]. Cells expressing both ghrelin and GOAT synthesize S3 acylghrelin, with the acyl moiety precursors derived from fatty acids ranging from acetate (C2) to tetradecanoic acid (C14)[49]. However, the main molecular form of gastric ghrelin is modified by *n*-octanoic acid and other acyl-modified forms of ghrelin are only found at low levels[54,55]. Interestingly different ghrelin truncates and derivates (GSSFL-NH2, GSAFL-NH2, GSSFL-COOH, GSS(C8:0)FL-NH2 and GSS (diaminopropionic acid) FL-NH_2) has exhibit the ability to inhibit GOAT activity and subsequently the *n*-octanoyl modification of ghrelin[52]. On the other hand, the optimal pH for GOAT activity is between 7 and 8[53]. However, the optimal pH of the

[52] Yang J, Zhao TJ, Goldstein JL, Brown MS. Inhibition of ghrelin O-acyltransferase (GOAT) by octanoylated pentapeptides *Proc Natl Acad Sci U S A*. 2008 Aug 5;105(31):10750-5.

[53] Ohgusu H, Shirouzu K, Nakamura Y, Nakashima Y, Ida T, Sato T, Kojima M. Ghrelin O-acyltransferase (GOAT) has a preference for n-hexanoyl-CoA over n-octanoyl-CoA as an acyl donor. *Biochem Biophys Res Commun*. 2009 Aug 14;386(1):153-8.

[54] Hosoda H, Kojima M, Matsuo H, Kangawa K. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J Biol Chem*. 2000 Jul 21;275(29):21995-2000.

[55] Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *J Biol Chem*. 2003 Jan 3;278(1):64-70.

processing protease PC1/3 is rather acidic as well as the pH in the secretary granules (pH5–6). This suggests that the acyl-modification of ghrelin should occur before protease cleavage. In other words, ghrelin seems to be modified in the forms of preproghrelin, a 117 amino acids peptide of 13,3 kDa which yields ghrelin and obestatin and it is also able to produce only proghrelin. In fact, proghrelin has been shown to be modified by *n*-octanoic acid[44] (Figure 4).

When is acylated, ghrelin is capable of stimulate GH release from the anterior pituitary gland. However, in response to fasting or prolonged food restriction ghrelin is also produced from the stomach increasing its circulating levels and activating the orexigenic neural circuits. Subsequently, ghrelin has also shown the capacity of act in the brain to regulate numerous central and peripheral actions, including stimulation of gut motility and gastric acid secretion, modulation of sleep, taste sensation and reward seeking behavior, regulation of glucose metabolism, suppression of brown fat thermogenesis, modulation of stress and anxiety, protection against muscle atrophy, and improvement of cardiovascular functions9 (Figure 5).

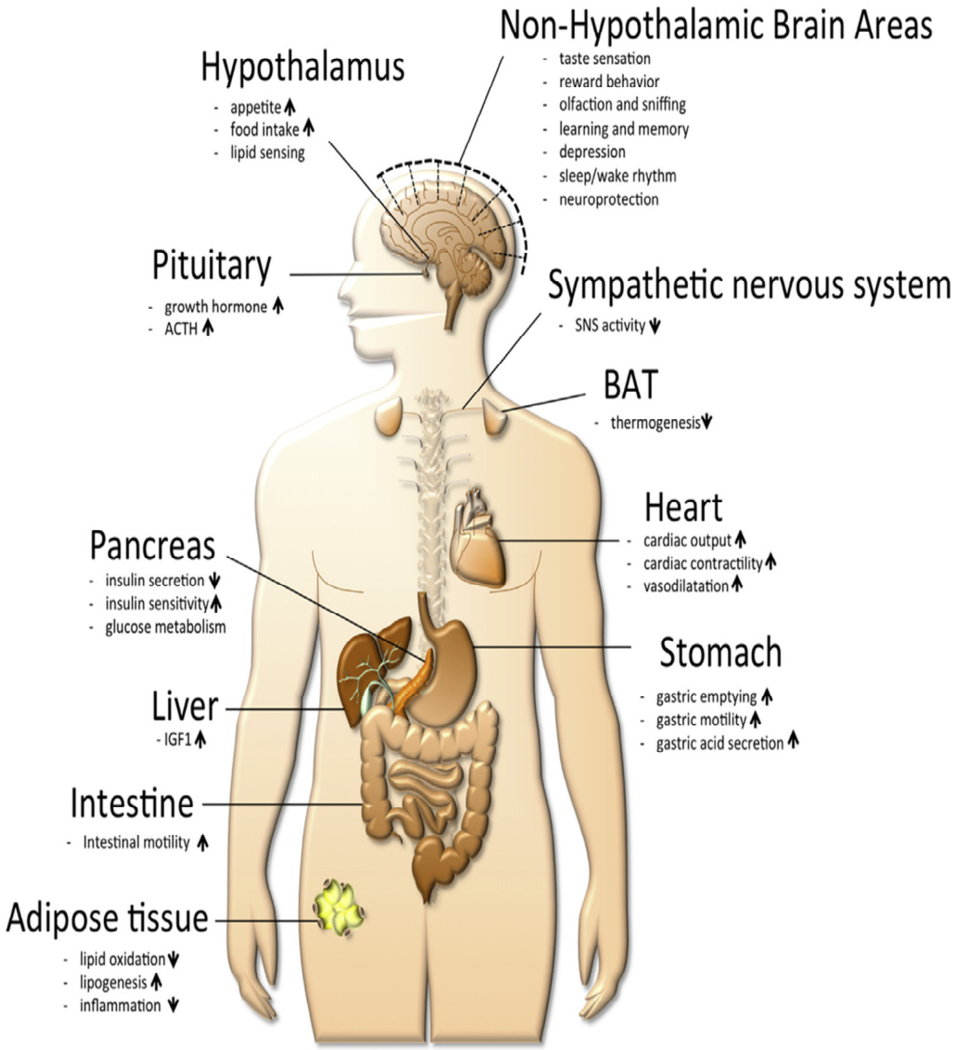

Figure 5: Summary of ghrelin-mediated physiological effects in humans (Extracted from Mol Metab. 2015 Mar 21;4(6):437-60).

3.0 ASSOCIATED SIGNALING TO THE SYSTEM GHRELIN/GHSR1A

3.1 G-proteins dependent signaling

GPCRs interact with heterotrimeric G proteins composed of α, β and γ subunits that are GDP bound in the resting state. Agonist binding triggers a conformational change in the receptor, which catalyses the dissociation of GDP from the alpha subunit followed by GTP-binding to G_α and the dissociation of G_α from $G_{\beta\gamma}$ subunits[56]. In this process three cellular regulators are involved. First, the RGS (regulator of G protein signaling) proteins stimulate the GTPase activity of the Gα subunit to hydrolyze GTP to GDP more quickly and terminate the signaling event. Second, GDI (guanine dissociation inhibitor) prevents the release of GDP from the G protein and stabilizes the Gα subunit in the inactive GDP-bound conformation. Third, GEF (guanine exchange factor) interacts with G_α and stimulates the exchange of GDP for GTP to accelerate the generation of the active form of the receptor[57,58,59]. Following the receptor activation, the GEF domain allosterically induces the G protein's GTPase activity, facilitating the hydrolysis of GTP to GDP at the Gα subunit. In the cycle of G protein activation, the GDP/GTP exchange and the GTP hydrolysis are two limiting steps. Once activated, the G protein subunits dissociate from the receptor, as well as each other, to yield a G_α-GTP monomer and a $G_{\beta\gamma}$ dimer, which

[56] Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer*. 2007 Feb;7(2):79-94.

[57] Siderovski DP, Willard FS. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *Int J Biol Sci*. 2005;1(2):51-66.

[58] Sato M, Blumer JB, Simon V, Lanier SM. Accessory proteins for G proteins: partners in signaling. *Annu Rev Pharmacol Toxicol*. 2006;46:151-87.

[59] Urano D, Jones JC, Wang H, Matthews M, Bradford W, Bennetzen JL, Jones AM.G protein activation without a GEF in the plant kingdom. *PLoS Genet*. 2012 Jun;8(6):e1002756.

activate intracellular signaling proteins[60,61]. The target functional proteins directly depend on the subunit type which is activated. $G_{\alpha s}$ stimulates adenylate cyclase, leading to cAMP (cyclic adenosine monophosphate)-mediated responses whereas $G_{\alpha i/o}$ inhibits adenylate cyclase and the subsequent cAMP accumulation. On the other hand, $G_{\alpha q/11}$ activates phospholypase C (PLC) leading to inositol tris-1,4,5-phosphate (IP3) and dyacilglycerol (DAG), while $G_{\alpha 12/13}$ activates Rho guanine exchange factors, triggering the kinases associated to Rho and cytoskeletal rearrangement. The $G_{\beta\gamma}$ subunits have also been shown to regulate signaling pathways such as calcium release, adenylate cyclase or MAPK[62,63,64].

In the case of the GHSR1a, $G_{\alpha q/11}$, $G_{\alpha i/o}$, $G_{\alpha s}$ and $G_{\alpha 12/13}$ are involved in the ghrelin-associated signaling. The first interaction of GHSR1a with G proteins was described in somatotrophs where through $G_{\alpha q/11}$ the ghrelin receptor activates PLC with the subsequent inositol phosphate activation and Ca^{2+} mobilization1,[65]. Evidence of activation of $G_{\alpha s}$, and thus, activation of adenylate cyclase and protein kinase A (PKA), has been reported for neurons

[60] Johnston CA, Siderovski DP Structural basis for nucleotide exchange on G alpha i subunits and receptor coupling specificity. *Proc Natl Acad Sci U S A*. 2007 Feb 6;104(6):2001-6.

[61] Denis C, Saulière A, Galandrin S, Sénard JM, Galés C. Probing heterotrimeric G protein activation: applications to biased ligands. *Curr Pharm Des*. 2012;18(2):128-44.

[62] Tang WJ, Gilman AGType-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science*. 1991 Dec 6;254(5037):1500-3.

[63] Crespo P, Xu N, Simonds WF, Gutkind JS. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature*. 1994 Jun 2;369(6479):418-20.

[64] Stehno-Bittel L, Krapivinsky G, Krapivinsky L, Perez-Terzic C, Clapham DE.The G protein beta gamma subunit transduces the muscarinic receptor signal for Ca2+ release in Xenopus oocytes. *J Biol Chem*. 1995 Dec 15;270(50):30068-74.

[65] Adams EF, Petersen B, Lei T, Buchfelder M, and Fahlbusch R. The growthhormone secretagogue, L-692,429, induces phosphatidylinositol hydrolysis and hormone secretion by human pituitary tumors.*Biochem Biophys Res Commun* 1995;208:555–561.

in the arcuate nucleus[66]. In addition, $G_{\alpha i/o}$ coupling has been demonstrated in guanosine 59-O-(3-thiotriphosphate) assays in model systems[67], as well as, in isolated lipid discs[68,69]. Moreover, in pancreatic islets, interaction of GHSR1a with the somatostatin receptor SST5 results in coupling through $G_{\alpha i/o}$[70]. Finally, the activation of RhoA kinase via $G_{\alpha 12/13}$ in response to ghrelin is observed in transfected cells. The combined actions of $G_{\alpha q}$ and $G_{\alpha 12/13}$ are responsible for the majority of the ghrelin-induced activation of serum response element (SRE)[8,34]. However, it still has not been described any GHSR1a associated signaling pathway mediated by $G_{\beta\gamma}$ subunits.

3.2 β-arrestins dependent signaling

Arrestins were initially discovered in the visual system and include four mammalian members divided into two groups, the visual arrestins (arrestin-1 in rod cells and arrestin-4 in cone cells) and the non-visual arrestins (β-arrestin1 and β-arrestin2, also called arrestin-2 and arrestin-3, respectively). Arrestins were named on the basis of their ability to arrest or

[66] Kohno D, Gao HZ, Muroya S, Kikuyama S, Yada T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes*. 2003 Apr;52(4):948-56.

[67] Bennett KA, Langmead CJ, Wise A, Milligan GGrowth hormone secretagogues and growth hormone releasing peptides act as orthosteric super-agonists but not allosteric regulators for activation of the G protein Galpha(o1) by the Ghrelin receptor. *Mol Pharmacol*. 2009 Oct;76(4):802-11.

[68] Damian M, Marie J, Leyris JP, Fehrentz JA, Verdié P, Martinez J, Banères JL, Mary S.High constitutive activity is an intrinsic feature of ghrelin receptor protein: a study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. *J Biol Chem*. 2012 Feb 3;287(6):3630-41

[69] Mary S, Damian M, Louet M, Floquet N, Fehrentz JA, Marie J, Martinez J, Banères JL.Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. *Proc Natl Acad Sci U S A*. 2012 May 22;109(21):8304-9.

[70] Park S, Jiang H, Zhang H, Smith RG. Modification of ghrelin receptor signaling by somatostatin receptor-5 regulates insulin release. *Proc Natl Acad Sci U S A*. 2012 Nov 13;109(46):19003-8.

switch-off the coupling of GPCRs to G proteins and its subsequent signaling. However, while all four arrestins terminate G protein signaling, it is now evident that the two β-arrestins which are ubiquitously express, are also able to function as scaffold proteins promoting GPCR internalization, degradation and recycling, as well as interact with many protein partners and protein kinases, leading to the phosphorylation of numerous intracellular targets in an alternative signaling independent of G proteins[71,72]. Among the β-arrestin-mediated signaling mechanisms could be found stress fiber formation via RhoA; inhibition of NF-κB gene expression through IκB stabilization; protein phosphatase 2A (PP2A)- mediated dephosphorylation of Akt with the consequent activation of glycogen synthase kinase3 and dopaminergic behavior control; phosphatidylinositol 3-kinase (PI3K)-mediated phospholipase A2 activation and increased vasodilation through GPR109A; induction of protein translation and antiapoptotic effects by extracellular signal-regulated kinases (ERKs) regulation; Kif3A-dependent trafficking and activation of the protein smoothened in the primary cilium[73] (Figure 6).

[71] Ostermaier MK, Schertler GF, Standfuss J. Molecular mechanism of phosphorylation-dependent arrestin activation. *Curr Opin Struct Biol*. 2014 Dec;29:143-51.

[72] Kang DS, Tian X, Benovic JL.Role of β-arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. *Curr Opin Cell Biol*.2014 Apr;27:63-71.

[73] Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors.*Annu Rev Pharmacol Toxicol*. 2012;52:179-97.

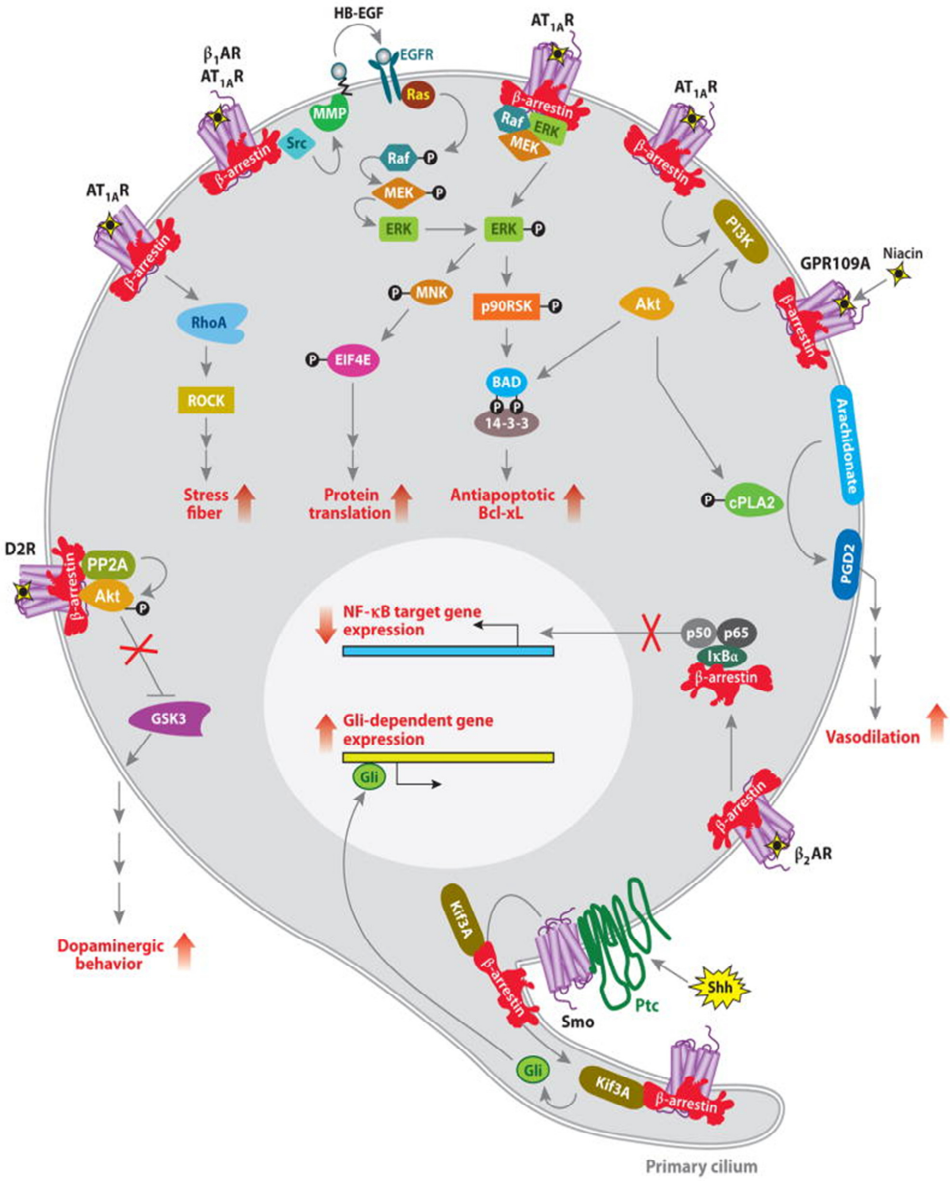

Figure 6. Summary of β-arrestin-dependent signaling at GPCRs (Extracted from Annu Rev Pharmacol Toxicol. 2012;52:179-97.)

The structure of all arrestins family members has been solved using X-ray techniques. All arrestins share two major domains, the N-terminal and the C-terminal, each composed of a seven anti-parallel β-sheets connected by short flexible loops. Both domains are connected by a region which acts like a hinge via loops at the pseudo two-fold rotation axis of the molecule. Arrestins also have a C-tail which is connected by a flexible linker to the C-domain and contains a short β strand that interacts with a lateral β strand of the N-domain. The overall structure is stabilized by a polar core of buried salt bridges and by a three-element interaction involving the first β strand, an α-helix in the N-domain and the C-terminal tail[74,75,76,77]. The interaction of charged residues from the amino terminus, N-domain, C-domain, and C-terminal tail maintain the different parts of the molecule together to preserve the basal conformation. The polar core is highly conserved within all four arrestins, suggesting that this structural element is critical for arrestins function[78,79,80]. During its interaction with a GPCR, arrestins adopts several conformations (Figure 7). Firstly, arrestins are mobilized from dimeric

[74] Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB, Schubert C: Crystal structure of beta-arrestin at 1.9 A: possible mechanism of receptor binding and membrane translocation. *Structure/Fold Design* 2001, 9:869-880.

[75] Milano SK, Kim YM, Stefano FP, Benovic JL, Brenner C: Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding. *J Biol Chem* 2006, 281:9812-9823.

[76] Milano SK, Pace HC, Kim Y-M, Brenner C, Benovic JL: Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis. *Biochemistry* 2002, 41:3321-3328.

[77] Zhan X, Gimenez LE, Gurevich VV, Spiller BW: Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. *J Mol Biol* 2011,406:467-478

[78] Vishnivetskiy SA, Francis D, Van Eps N, Kim M, Hanson SM, Klug CS, Hubbell WL, Gurevich VV: The role of arrestin a-helix i in receptor binding. *J Mol Biol* 2010,395:42-54.

[79] Palczewski K, Pulvermu¨ ller A, Buczyłko J, Hofmann KP: Phosphorylated rhodopsin and heparin induce similar conformational changes in arrestin. *J Biol Chem* 1991, 266:18649-18654.

[80] Tian X, Kang DS, Benovic JL.β-arrestins and G protein-coupled receptor trafficking. *Handb Exp Pharmacol.* 2014;219:173-86.

or tetrameric storage in its basal conformation. Then, arrestins are pre-activated by a C-tail exchange mechanism of C-termini from arrestin and phosphorylated C-terminus of its GPCR. In this pre-activated state, arrestins interacts with additional sites in the activated receptor and, potentially, induces further conformational changes to form a high affinity GPCR–arrestin complex. Loss of the agonist does not necessarily imply immediate complex dissociation and a low-affinity complex can be also formed on phosphorylation-independent pathways. Despite being associated or dissociated from different receptor states, arrestins have structurally similar conformations. However, monomeric arrestins are able to form low-affinity complexes with either activated or phosphorylated receptor adopting two distinct conformations[71].

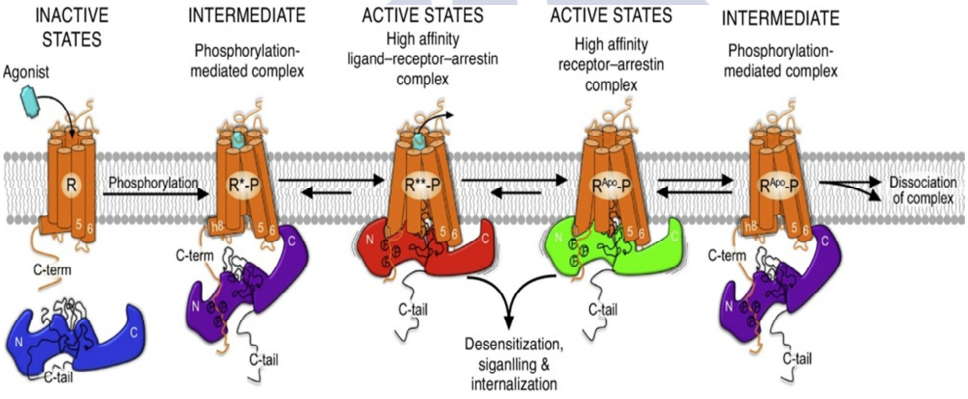

Figure 7. Arrestin activation mechanism (Extracted from Curr Opin Struct Biol. 2014 Dec;29:143-51)

Additionally to the classic G protein-mediated signaling, GHSR1a has also demonstrated be able to act through signaling pathways mediated by β-arrestins. Non-visual arrestins, β-arrestin1 and β-arrestin2, has shown the

ability of act as adaptor proteins that function to regulate GHSR1a signaling and trafficking. The interplay of G-protein and β-arrestin signaling largely determines the cellular consequences of the GHSR1a activation8[81,82].

β-arrestins activation

As was described above, the binding of arrestins to activated receptors, in order to establish high-affinity complexes, is determined by the phosphorylated conformation of GPCRs. Phosphorylation of GPCRs is one of the most important post-translation modifications mediated by agonist stimulation. Once GPCRs are activated, they are rapidly phosphorylated by G protein-coupled receptor kinases (GRKs). These kinases initiate the homologous desensitization of activated GPCRs through the phosphorylation of S or T residues located primarily in the carboxyl terminus of the receptors, but also in the intracellular loops. The covalent modifications produced mediate receptor desensitization as well as provide a mechanism by which receptors can engage with β-arrestins and trigger specific downstream signaling pathways[83,84].

In contrast to the large number of GPCRs, there are only seven members in the GRK family, and of those, only four GRKs (2, 3, 5 and 6) are ubiquitously expressed and thus, primarily responsible for the

[81] Camiña JP, Lodeiro M, Ischenko O, Martini AC, Casanueva FF. Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHS-R1a receptor: role of G-proteins and beta-arrestins. *J Cell Physiol*. 2007 Oct;213(1):187-200.

[82] Lodeiro M, Theodoropoulou M, Pardo M, Casanueva FF, Camiña JP. c-Src regulates Akt signaling in response to ghrelin via beta-arrestin signaling-independent and -dependent mechanisms. *PLoS One*. 2009;4(3):e4686.

[83] Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther*. 2012 Jan;133(1):40-69.

[84] Prihandoko R, Bradley SJ, Tobin AB, Butcher AJ.Determination of GPCR Phosphorylation Status: Establishing a Phosphorylation Barcode. *Curr Protoc Pharmacol*. 2015 Jun 1;69:2.13.1-2.13.26.

phosphorylation of most GPCRs. The remaining three GRKs are tissue specific, GRK1 and GRK7 expression is confined to the retina and GRK4 highest expression was found in the testes[85]. Among the ubiquitous GRKs, GRK2 and 3 share a pleckstrin homology domain and need interactions with $G_{\beta\gamma}$ to be bounded to the membrane; on the contrary, GRK5 and 6 are constitutively localized to the membrane due to their own modifications like palmitoylation[86,87]. Despite the differences, all GRKs have a conserved structural core comprising a protein kinase domain inserted into a loop of the regulator of G protein signaling homology (RH) domain. The RH domain act as an intramolecular scaffold maintaining the small lobe of the kinase domain in a competent state to phosphorylate activated GPCRs. Consequently, the kinase domain does not require phosphorylation on its activation loop for full activity. The first ~20 amino acids of GRKs are also highly conserved and seem to be critical for GPCR and phospholipid-stimulated autophosphorylation, but the disorganization of this region in the known GRKs structures keeps its molecular role unclear. GRKs have also demonstrated to share a C-terminal extension of the kinase domain characteristic of the AGC kinase family, which contributes residues to the active site cleft. Although this extension is not fully ordered in most GRKs structures, when this region was mutated in GRK2 and GRK1 the receptor phosphorylation was dramatically inhibit, consistent with the idea that this element may be regulating kinase activity as it does in other AGC kinases[88,89,90]. GRKs probably recognize certain structural determinants in the

[85] Penela P, Ribas C, Mayor F Jr. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal*. 2003; 15:973–981.

[86] Penela P, Ribas C, Aymerich I, Mayor F Jr.New roles of G protein-coupled receptor kinase 2 (GRK2) in cell migration. *Cell Adh Migr*. 2009 Jan-Mar;3(1):19-23.

[87] Stoffel RH, Randall RR, Premont RT, Lefkowitz RJ, Inglese J. Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J Biol Chem*. 1994 Nov 11;269(45):27791-4.

[88] Tesmer JJ.Structure and function of regulator of G protein signaling homology domains. *Prog Mol Biol Transl Sci*. 2009;86:75-113.

activated receptors, but a consensus GRK target sequence motif in the GPCRs is not fully established because the primary sequences of GPCRs are poorly conserved. Furthermore, each GRK can phosphorylate different S and T residues of the same receptor, and this phosphorylation pattern can be biased by the receptor conformation induced by the bound ligand. Consequently, the action of different receptor kinases could provide a unique phosphorylation signature on the receptor. The specific GRK phosphoacceptors activated on the receptors can act like a barcode determining the β-arrestin conformation and the subsequent β-arrestins action on downstream signaling pathways. The detail map of the phosphorylation residues of a GPCR targeted by individual GRKs and the understanding of how these sites regulate the specific functional consequences of β-arrestin engagement may aid in the discovery of therapeutic agents targeting individual β-arrestin functions[91,92]. Despite the importance of known the phosphorylation status of a GPCR to better understand its function and the associated signaling, until now, the activation of the GHSR1a and the key pieces involved on it, remain to be discover.

[89] Sterne-Marr R, Leahey PA, Bresee JE, Dickson HM, Ho W, Ragusa MJ, Donnelly RM, Amie SM, Krywy JA, Brookins-Danz ED, Orakwue SC, Carr MJ, Yoshino-Koh K, Li Q, Tesmer JJ.GRK2 activation by receptors: role of the kinase large lobe and carboxyl-terminal tail. *Biochemistry*. 2009 May 26;48(20):4285-93.

[90] Huang CC, Yoshino-Koh K, Tesmer JJ.A surface of the kinase domain critical for the allosteric activation of G protein-coupled receptor kinases. *J Biol Chem*. 2009 Jun 19;284(25):17206-15.

[91] Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ.Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci Signal*. 2011 Aug 9;4(185):ra51.

[92] Liggett SB.Phosphorylation barcoding as a mechanism of directing GPCR signaling. *Sci Signal*. 2011 Aug 9;4(185):pe36.

Desensitization and Endocytosis

GHSR1a without ghrelin is mainly localized at the plasma membrane but it is rapidly desensitizes after stimulation. The ghrelin/GHSR1a complex progressively disappears from the plasma membrane after 20min. Ghrelin-dependent GHSR1a desensitization is not mediated by protein kinase C, suggesting that the major cellular mechanism mediating desensitization might be phosphorylation by GRKs. Moreover, activation of the GHSR1a leads to recruitment of β-arrestins, in a G-protein coupling independent manner, hinting that GHSR1a endocytosis and internalization should be mediated by β-arrestins. However, internalization of the ghrelin receptor can occur in a ghrelin-independent manner, which is dependent on receptor constitutive activity and domains within the C-terminal tail but not β-arrestin dependent3[,36,68,93,94].

For GPCRs internalization two main pathways via clathrin-coated pits and through caveolae have been described. However, a number of studies reported that GHSR1a seems to internalize only via clathrin-coated pits. Firstly, potassium depletion decreases the number of surface clathrin-coated pits and inhibits GHSR1a internalization. Secondly, activation of the ghrelin receptor leads to the recruitment of the clathrin adaptor-related protein complex 2 (AP2). Thirdly, the mutation of a clathrin-binding domain found on β-arrestins [β-arrestin (319-418)] increases significantly the expression of GHSR1a on the cell surface, indicating that internalization is dependent on the interaction between β-arrestin and clathrin[68,95,96]. After

[93] Evron T, Peterson SM, Urs NM, Bai Y, Rochelle LK, Caron MG, Barak LS. G Protein and β-arrestin signaling bias at the ghrelin receptor. *J Biol Chem*. 2014 Nov 28;289(48):33442-55.

[94] Holliday ND, Holst B, Rodionova EA, Schwartz TW, Cox HM. Importance of constitutive activity and arrestin-independent mechanisms for intracellular trafficking of the ghrelin receptor. *Mol Endocrinol*. 2007 Dec;21(12):3100-12.

[95] Chu KM, Chow KB, Leung PK, Lau PN, Chan CB, Cheng CH, Wise H. Over-expression of the truncated ghrelin receptor polypeptide attenuates the constitutive activation of phosphatidylinositol-specific phospholipase C by ghrelin receptors but has no

internalize via clathrin pathway into vesicles, GHSR1a could either be sorted into endosomes to be recycled back to plasma membrane or, may be degraded within lysosomes. The evidence indicates that GHSR1a dissociates from ghrelin in the acidified endosomal compartments and it is recycled to the plasma membrane. After 20min exposure to ghrelin, GHSR-1a colocalize with the early endosome marker EEA1 but it fails to colocalize with the lysosomal marker cathepsin D at 60min despite being accumulate in the perinuclear region. Furthermore, GHSR1a reappears in the membrane after 2 hours of ghrelin treatment and the recycling is prevented using inhibitors of endosomal acidification, NH_4Cl and concanamycin A, which retain the ghrelin/GHSR1a complex inhibiting ghrelin degradation. A remarkable point is that the GHSR1a recycled from the endosomes has shown a complete restoration of binding capacity and functionality on the cell surface[96].

The desensitization of the GHSR1a can be prevented by unsaturated fatty acids which block its internalization, highlighting the importance of membrane composition for GHSR activation and desensitization. Free fatty acids improve GHSR1a sensitivity suppressing ghrelin-induced internalization by uncoupling the receptor from the endocytic pathway. Consequently, GHSR1a is retained on the cell surface, where it can continue to signal in response to ghrelin[96].

Kinases activation

Like other GPCRs, the GHSR1a shows an alternative signaling mediated by β-arrestins independently of G proteins. β-Arrestins, through ghrelin-induced GHSR1a activation, are able to activate various kinases such

effect on ghrelin-stimulated extracellular signal-regulated kinase 1/2 activity. *Int J Biochem Cell Biol*. 2007;39(4):752-64.

[96] Camiña JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG, Casanueva FF.Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. Endocrinology. 2004 Feb;145(2):930-40.

as Akt and MAPKs. MAPKs are protein kinases specific of serine, threonine and tyrosine amino acids which belong to the CMGC family (named after the initials of some members). MAPKs are involved in directing cellular responses regulating cell functions such as proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis. Among the major MAPKs can be found: the extracellular signal-regulated kinases (ERKs), Jun amino-terminal kinases (JNKs) and p38 kinases. On the other hand, Akt is a S/T-specific protein kinase that plays a key role in multiple cellular processes like glucose metabolism, apoptosis, cell proliferation, transcription and cell migration[97].

Ghrelin mediated GHSR1a activation trigger Akt activation with cSrc functioning as a switch that initiates both pathways associated to Akt, the $G_{i/o}$ protein–dependent pathway and the β-arrestin–scaffolded complex. The $G_{i/o}$-protein activates PI3K which translocates Akt to the plasma membrane through the binding of its pleckstrin homology domain to the second messenger PtdIns(3,4,5)P3 (PIP3). In the membrane, Akt is phosphorylated at Y by cSrc with the subsequent phosphorylation of Akt A loop in T308 by PDK1 and at S473 of the hydrophobic motif by mTORC2. The second signaling pathway mediated by β-arrestins starts after receptor activation and it involves the recruitment of at least β-arrestin 1, β-arrestin 2, c-Src and Akt. cSrc also regulates the phosphorylation and activation of the tyrosine phosphatase of SHP1 in both signaling pathways. cSrc phosphorylates the SHP1 C-terminus (Y536), which exerts an inhibitory effect on PI3K and Akt giving to cSrc its switch ability (Figure 8)[82,98].

[97] Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine* Reviews. 2001 Apr 22 (2): 153–83.

[98] Lodeiro M, Alén BO, Mosteiro CS, Beiroa D, Nogueiras R, Theodoropoulou M, Pardo M, Gallego R, Pazos Y, Casanueva FF, Camiña JP. The SHP-1 protein tyrosine phosphatase negatively modulates Akt signaling in the ghrelin/GHSR1a system. *Mol Biol Cell*. 2011 Nov;22(21):4182-91.

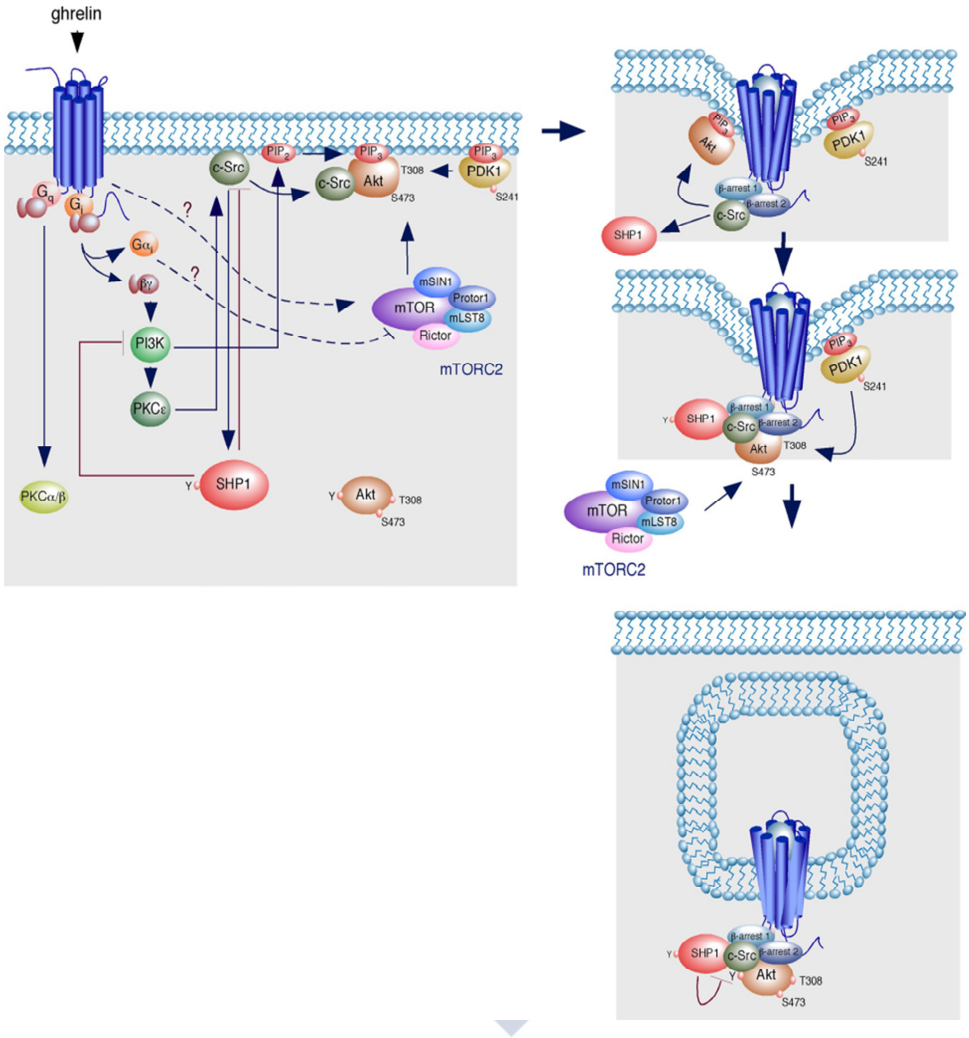

Figura 8. Ghrelin-mediated signaling to Akt. Ghrelin binding to GHSR1a, through $G_{i/o}$ protein, activates PI3K and consequently, the second messenger PIP3. Akt translocates to the plasma membrane by binding to PIP3, where it is phosphorylated at Y by the membrane-bound cSrc. cSrc activation induced phosphorylation of SHP1, attenuation of p85 subunit from PI3K and PDK1, and phosphorylation of Akt. SHP1 activation regulates cSrc activity by attenuation of Y416 phosphorylation. Once the receptor is activated, a second signaling pathway is mediated by β-arrestins 1 and 2, involving the recruitment of GHSR1a, cSrc, SHP1, and Akt into a β-arrestins complex (Extracted from Mol Biol Cell. 2011 Nov;22(21):4182-91).

On the other hand, ghrelin has also shown the ability to activate the MAPK kinase ERK1/2 through GHSR1a. ERK1/2 phosphorylation from GHSR1a activation is interplay of three signaling pathways which combine G protein and β-arrestins signaling. The pathway mediated by β-arrestins 1 and 2 requires the entry of the receptor into a multiprotein complex that at least compromises β-arrestins, Src, Raf-1 and ERK1/2. The $G_{q/11}$-dependent cascade involves PKCα/β and Src and the third pathway $G_{i/o}$-dependent implicates PI3K, PKCε, and Src. Moreover, G protein dependent signaling pathways are involved in the β-arrestin-mediated ERK1/2 activation and this activation differs in their temporal and spatial distributions. Upon ghrelin stimulation of GHSR1a receptor, $G_{q/11}$- and $G_{i/0}$- protein mediated ERK1/2 activation is immediately, while β-arrestin-dependent ERK1/2 activation is relatively slow but persistent (Figure 9)[81,94].

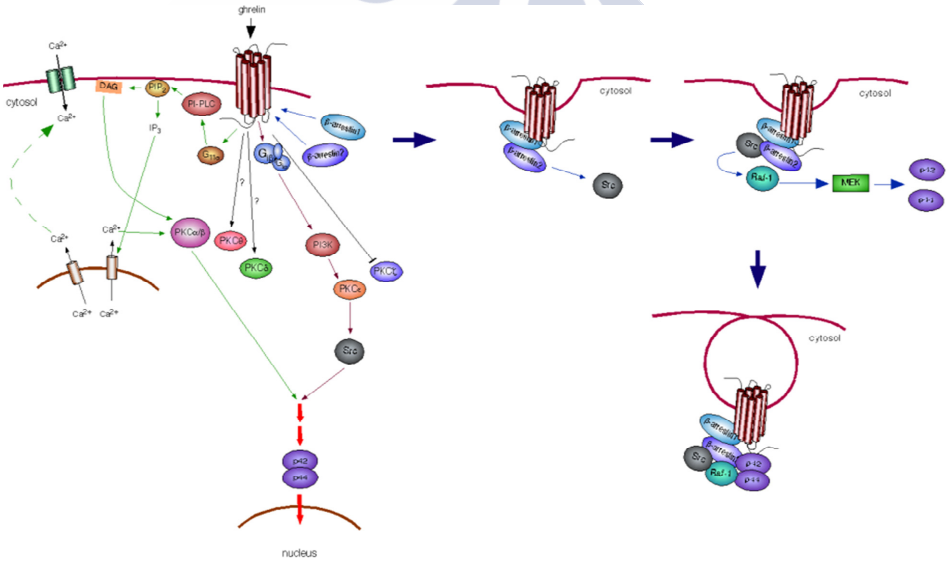

Figure 9. Proposed model for ghrelin-induced ERK1/2 activation. In HEK293 cells ghrelin, through GHSR1a, activates three pathways. First, the $G_{q/11}$-dependent pathway involves the consecutive activation of PIPLC, PKCα/β isoforms, and Src. Second, the $G_{i/0}$ dependent-pathway is mediated by PI3K, PKCε, and Src. Finally, the β-arrestins-mediated pathway involves recruitment of Src, Raf-1, and ERK1/2 to a β-arrestin complex with GHSR1a. This pathway is mediated by G-protein-regulated effectors such as PKCα/β (dotted lines) (Extracted from J. Cell. Physiol. 2007; 213: 187–200).

4.0 ASSOCIATED FUNCTIONALITY TO GHSR1a SIGNALING

4.1 G proteins-mediated Ca^{+2} release and GH secretion

Ghrelin feeding control is mediated in its first step by Ca^{+2} releases. Two calcium signaling pathways have been described for GHSR1a activation. In the first cascade, the $G_{\alpha q}$ subunit releases upon ghrelin administration activates cyclic adenosine monophosphate (cAMP) in the in the hypothalamic arcuate nucleus (ARC) and subsequently protein kinase A (PKA). The activated PKA induces CREB phosphorylation, which inhibits potassium channels. When the potassium channels are closed, the cell depolarizes and N-type calcium channels releases Ca^{+2} from intracellular stores[66]. The second pathway is $G_{\alpha s}$-dependent and it is dependent on calcium influx through the N-type calcium channel. In the neuropeptide Y (NPY)-containing neurons calcium induced phospholipase C (PLC) promotes phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis in the cell membrane. This process increases intracellular level of calcium in two ways. Firstly, PIP2 is able to activate diacylglycerol (DAG), which phosphorylates protein kinase C (PKC) causing the opening of L-type calcium channels. Secondly, PIP2 has also shown the capacity to activate inositol trisphosphate (IP3) inducing calcium release from the endoplasmic reticulum[99].

After being release, Ca^{+2} mediates the production of nitric oxide (NO) which is an important regulator of energetic homeostasis and a key piece in appetite control. Ca^{+2}-dependent nitric oxide synthase (NOS) generates NO from arginine which stimulates guanylyl cyclase (GC) with the subsequent cyclic guanosine monophosphate (cGMP) activation. Nitric oxide action in the increase in appetite induced by ghrelin is supported for several discoveries. Firstly, feeling of satiety and hunger was correlated with changes

[99] Van der Lely AJ, Tschop M, Heiman ML, Ghigo E.Biological,physiological, pathophysiological,and pharmacological aspects of ghrelin. *Endocr Rev* 2004;25:426–57.

in hypothalamic NOS concentration and inhibition of NOS activity resulted in an increase in food intake in rodents. Secondly, ablation of ghrelin increases NOS concentration and rise hypothalamic activity of NOS after intraventricular injections of NPY. Thirdly, central injections of ghrelin increase the concentration of NOS in the hypothalamus and exogenous ghrelin effect on feed intake was neutralized by the NOS inhibitor l-NG-nitroarginine methyl ester (l-NAME). Due to NO is a gas, it easily leaves the neurons and stimulate cGMP formation in other cells connecting CNS with peripheral tissues. Moreover, a high level of cytosolic calcium in the somatotropic cells of the hypophysis causes GH release[37,100,101,102,103,104].

4.2 AMPK: a master enzyme of metabolic regulation

Regulation of appetite, energy homeostasis, growth hormone (GH) secretion and body weight is due to a coordinated negative feedback between central neural circuits and peripheral target tissues. The synthesis and release of peripheral metabolic hormones is a key part of this negative feedback action. The main regulating appetite hormones are leptin from adipose tissue and ghrelin from the stomach.

[100] Morley JE, Alshaher MM, Farr SA, Flood JF, Kumar VB. Leptin and neuropeptide y (npy) modulate nitric oxide synthase: further evidence for a role of nitric oxide in feeding. *Peptides* 1999;20:595–600.

[101] Morley JE, Kumar VB, Mattammal M, Villareal DT. Measurement of nitric oxide synthase and its mrna in genetically obese (ob/ob) mice. *Life Sci* 1995; 57:1327–31.

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[103] Anderson LL, Jeftinija S, Scanes CG.Growth hormone secretion: molecular and cellular mechanisms and in vivo approaches. *Exp Biol Med (Maywood).* 2004 Apr;229(4):291-302.

[104] Gomperts BD, Tatham PER, Kramer IM. Nitric oxide synthase. In: Signal transduction. *London: Elsevier Inc.*; 2009.

In the hypothalamic arcuate nucleus (ARC) GHSR1a is activated by ghrelin increasing the coexpressed orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP). But also in the ARC, leptin is able to activate the proopiomelanocortin precursor protein (POMC) which is cleaved into the potent anorexigenic melanocyte-stimulating hormone[105,106]. For this reason, negative energy balance increases ghrelin amount in plasma releasing hypothalamic AMP-activated protein kinase (AMPK) and increasing appetite while leptin is secreted when adipose levels rise and suppress AMPK[107,108](Figure 10).

[105] Sun Y, Wang P, Zheng H, Smith RG. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A.* 2004 Mar 30;101(13):4679-84.

[106] Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol*. 2006 Jan 20;494(3):528-48.

[107] Briggs DI, Andrews ZB. Metabolic status regulates ghrelin function on energy homeostasis. *Neuroendocrinology*. 2011;93(1):48-57.

[108] Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature.* 2000 Oct 19;407(6806):908-13.

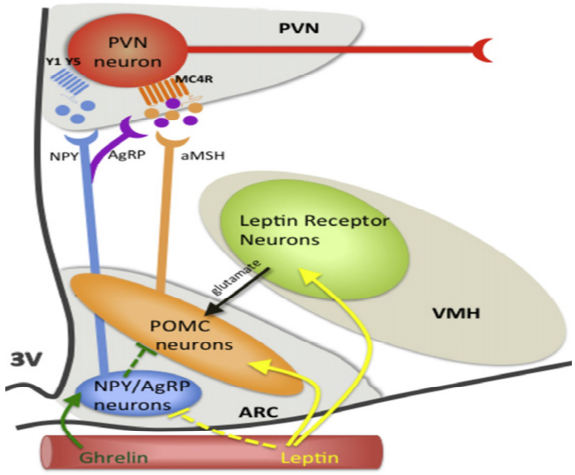

Figure 10. Hypothalamic circuits governing appetite. NPY/AgRP neurons in the ARC (blue) receive hormonal inputs from the plasma. While ghrelin activates NPY/AgRP (green arrow), leptin suppresses it (yellow dashed line). NPY/AgRP neurons inhibit POMC neurons (dashed green line). Leptin suppresses food intake by directly activating POMC neurons (yellow arrow) and indirectly by activating leptin receptor neurons in the ventromedial hypothalamic nucleus (yellow arrow) that send excitatory glutamatergic inputs onto POMC neurons. In the paraventricular nucleus, the anorexic POMC peptide, (aMSH, orange), activates the melanocortin 4 receptor (MC4R) to suppress food intake. NPY peptide release (blue) in the PVN acts on Y1 and Y5 receptors and AgRP (purple) acts as an antagonist at the MC4R to prevent the anorectic actions of aMSH. Together this results in increased food intake (Extracted from Mol Cell Endocrinol. 2013 Feb 25;366(2):215-23).

Due to its central position in the signaling pathways AMPK is a master enzyme of metabolic regulation. AMPK is a S/T protein kinase which acts as an intracellular energy sensor and whose activation requires phosphorylation of T172 within the T loop region of the catalytic α subunit. In response to an increase in AMP/ATP ratio AMPK is phosphorylated and switches off anabolic pathways which consume ATP (synthesis of lipids, carbohydrates and proteins) and switches on catabolic pathways which

produce ATP (glycolysis, glucose uptake, fatty-acid oxidation and mitochondrial biogenesis)[109].

AMPK action in the CNS

The ghrelin-induced elevation of Ca^{+2} concentration in the cytosol leads to the activation of calmodulin-dependent protein kinase kinase2 (CaMKK2), and subsequent AMPK phosphorylation in the ARC. However, ghrelin is able to activate AMPK not only in the ARC but also in the ventromedial hypothalamic nucleus (VMH) independently. Additionally, a second pathway for ghrelin-induced AMPK activation has been described where ghrelin specifically triggers a central SIRT1 (NAD-dependent protein deacetylase sirtuin-1)/p53 pathway that is essential for its orexigenic action, but not for the release of growth hormone[110,111,112,113].

The first downstream pathway describes in the ARC and VMH for ghrelin-mediated AMPK activation was a mitochondrial mechanism. In response to ghrelin, AMPK increases fatty acid oxidation in order to produce ATP. During this oxidation, hypothalamic mitochondrial respiration is enhanced generating an excess of reactive oxygen species (ROS) which is

[109] Steinberg GR, Kemp BE. AMPK in Health and Disease. *Physiol Rev*. 2009 Jul;89(3):1025-78.

[110] Kohno D, Sone H, Minokoshi Y, Yada T.Ghrelin raises [Ca2+]i via AMPK in hypothalamic arcuate nucleus NPY neurons. Biochem *Biophys Res Commun*. 2008 Feb 8;366(2):388-92.

[111] López M, Lage R, Saha AK, Pérez-Tilve D, Vázquez MJ, Varela L, Sangiao-Alvarellos S, Tovar S, Raghay K, Rodríguez-Cuenca S, Deoliveira RM, Castañeda T, Datta R, Dong JZ, Culler M, Sleeman MW, Alvarez CV, Gallego R, Lelliott CJ, Carling D, Tschöp MH, Diéguez C, Vidal-Puig A. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab*. 2008 May;7(5):389-99.

[112] Andrews ZB.Central mechanisms involved in the orexigenic actions of ghrelin. *Peptides*. 2011 Nov;32(11):2248-55.

[113] Velásquez DA, Martínez G, Romero A, Vázquez MJ, Boit KD, Dopeso-Reyes IG, López M, Vidal A, Nogueiras R, Diéguez C. The central Sirtuin 1/p53 pathway is essential for the orexigenic action of ghrelin. *Diabetes*. 2011 Apr;60(4):1177-85.

buffer or scavenge by uncoupling protein 2 (UCP2). Ghrelin activation of this mechanism involves after AMPK activation, acetyl CoA carboxylase phosphorylation, inhibition of malonyl CoA and increased carnitine palmitoyl transferase 1 (CPT1) activity in a UCP2 dependent way. This Ghrelin-AMPK–CPT1–UCP2 action is critical for mitochondrial biogenesis and electrical activation of NPY AgRP neurons, for trigger synaptic plasticity of POMC expressing neurons and for promote food intake. This signaling pathway connects neuronal function of G protein coupled receptor activity in mitochondria and the associated behavior[111,112]. Other candidate to mediate the effects of ghrelin downstream of AMPK is the mammalian target of rapamycin complex 1 (mTORC1), a multimolecular complex which is activated trough S6 kinases (S6K) and contains the serine/threonine protein kinase mTOR. Ghrelin-induced AMPK activation decreases mTORC1 activity. However, fasting or exogenous ghrelin administration increases mTORC1 activity in the ARC rather than inhibited it[114,115]. This may be due to mTORC1 activation is independent of AMPK to increase food intake or because AMPK exerts its metabolic effects through other molecular targets. The role of mTORC1 in the VMH remains to be unclear. Nonetheless, taking into account that fatty acid synthesis inhibitors decrease food intake by increasing hypothalamic mTORC1, the fact that fasting inhibit S6 phosphorylation in the VMH and that mTORC and AMPK action is the opposite, it would be expected that ghrelin will inhibit mTORC1 activity contrary to what happen in the ARC[114, 116]. Thereby, the role of mTORC1 regulation of food intake and energy

114 Villanueva EC, Münzberg H, Cota D, Leshan RL, Kopp K, Ishida-Takahashi R, Jones JC, Fingar DC, Seeley RJ, Myers MG Jr. Complex regulation of mammalian target of rapamycin complex 1 in the basomedial hypothalamus by leptin and nutritional status. *Endocrinology*. 2009 Oct;150(10):4541-51.

115 Watterson KR, Bestow D, Gallagher J, Hamilton DL, Ashford FB, Meakin PJ, Ashford ML. Anorexigenic and orexigenic hormone modulation of mammalian target of rapamycin complex 1 activity and the regulation of hypothalamic agouti-related protein mRNA expression. *Neurosignals*. 2013;21(1-2):28-41.

116 Proulx K, Cota D, Woods SC, Seeley RJ.Fatty acid synthase inhibitors modulate energy balance via mammalian target of rapamycin complex 1 signaling in the central nervous system. *Diabetes*. 2008 Dec;57(12):3231-8.

balance in the hypothalamus it would depend upon the neuronal populations that are specifically targeted by ghrelin[117].

Peripheral action of AMPK signaling

As was previously remarked, the central neural circuits are connected to peripheral target tissues. For this reason, ghrelin induced AMPK signaling pathways were studied in different tissues. In the pancreas for example, ghrelin decreases insulin secretion via AMPK-UCP2 signaling pathway as well as it has been demonstrated reduced the amount of pancreatic polypeptide secreted which inhibits appetite[118]. Ghrelin has also been related with the AMPK activity in the regulation of bone metabolism, bone nodule formation and maintenance of bone mass[119]. A recent study has demonstrated a potential protective role of ghrelin against myocardial injury and apoptosis induced by endoplasmic reticulum stress partially through a GHSR1a/CaMKK/AMPK pathway[120]. Ghrelin administration in rats attenuated NAFLD-induced hepatic injury, inflammation and apoptosis, partly through restoration of LKB1/AMPK and PI3K/Akt pathway[121]. Furthermore, ghrelin reduced tumor necrosis factor (TNFα) and IL-6 levels in obese mice attenuating lipotoxicity and regulating autophagy via AMPK/mTOR and

[117] Stevanovic D, Trajkovic V, Müller-Lühlhoff S, Brandt E, Abplanalp W, Bumke-Vogt C, Liehl B, Wiedmer P, Janjetovic K, Starcevic V, Pfeiffer AF, Al-Hasani H,Tschöp MH, Castañeda TR. Ghrelin-induced food intake and adiposity depend on central mTORC1/S6K1 signaling. Mol Cell Endocrinol. 2013 Dec 5;381(1-2):280-90.

[118] Chandra R, Liddle RA.Recent advances in pancreatic endocrine and exocrine secretion. Curr Opin Gastroenterol. 2011 Sep;27(5):439-43

[119] Shah M, Kola B, Bataveljic A, Arnett TR, Viollet B, Saxon L, Korbonits M, Chenu C. AMP-activated protein kinase (AMPK) activation regulates in vitro bone formation and bone mass. Bone. 2010 Aug;47(2):309-19.

[120] Zhang GG, Cai HQ, Li YH, Sui YB, Zhang JS, Chang JR, et al. Ghrelin protects heart against ERS-induced injury and apoptosis by activating AMP-activated protein kinase. *Peptides*.2013;48:156–65

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inhibiting NF-κB translocation into the nucleus[122]. Moreover, ghrelin contributes to the cytoprotection during hepatic ischaemia/reperfusion injury blocking the induced AMPK upregulation. However, it remains to clarify if the ghrelin action is direct or indirect on hepatocytes[123]. It is precisely the action in the inflammatory process the main peripheral action mediated by ghrelin via AMPK known to date.

Tissue inflammation mediated by immune cell in the adipose tissue, liver and skeletal muscle plays increase insulin resistance which may result in develop of obesity and diabetes[124]. Obesity associated inflammation causes inflammatory responses in the hypothalamus by a classic reaction pathway which involves the transcription factor NF-κB. Lipid accumulation in non-adipose tissue promotes the degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha and betta (IκB α and IκB β), resulting in the translocation of NF-κB into nucleus and subsequently secretion of TNFα, inflammatory cytokines such as interleukins(IL)- 6, 8, 10, 1β or VEGF120 and MCP1 adipokines. Ghrelin action in T cells, monocytes and microglia inhibit NF-κB decreasing the expression and production of proinflammatory cytokines and TNFα[125,126,127]. Despite this, it was described

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the cell specific action of ghrelin as a pro-inflammatory peptide inducing, rather than reducing, NF-κB expression and proinflammatory IL-8, VEGF120 and MCP1 secretion[128,129,130]. This controversial ghrelin action on inflammatory response was related with direct action of AMPK/mTOR signaling on the peripheral tissues and central nervous system.

AMPK was further described as point of convergence between the ghrelin and cannabinoid signaling pathways. Cannabinoids and ghrelin stimulate AMPK activity in the hypothalamus and heart, and both inhibit liver and adipose AMPK activity[131]. In addition, the central ghrelin treatment affects peripheral AMPK activity via the involvement of central CB1, suggesting a CNS-liver/adipose tissue neural link and a close interaction of the two systems in the regulation of metabolic pathways[132,133].

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4.3 Akt signaling, more than adipogenic effect

During the last decades obesity treatment is becoming a global concern because it is rising at an alarming rate in western countries while development of new efficient drugs is being much slower. Excessive adiposity is related to many chronic diseases as insulin resistance, type 2 diabetes mellitus, cardiovascular disease, liver steatosis or cancer. Ghrelin signaling has increasingly been recognized as a key regulator of obesity and its derivate pathologies9. Ghrelin is the only known circulating orexigenic hormone predominantly produced by the stomach in response to the negative energy balance. Circulating levels of ghrelin are augmented in response to fasting or prolonged food restriction. The total ghrelin levels are decreased in obesity, insulin resistance and diabetes type2[134]. However, ghrelin circulates in two distinct isoforms, acylated ghrelin and desacyl ghrelin (DAG) and a more detail study of circulating concentrations has shown that the acylated ghrelin, which is the only able to activate the GHSR1a, was increased in obesity, obesity-associated type2 diabetes. Ghrelin has also show the capacity of increases directly insulin stimulated glucose uptake in adipocytes[135, 136].

Ghrelin effect on adiposity is mediated by PI3K/Akt signaling. As was previously described, upon ghrelin stimulation, GHSR1a activates Akt through the $G_{i/o}$ protein-dependent pathway and the β-arrestins signaling with cSrc functioning as a switch between both. In addition, β-arrestins depletion leads to a significant reduction of the Akt downstream effectors

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mammalian target of rapamycin complex 1 (mTORC1) and the ribosomal protein S6 kinase beta-1 (S6K1), as well as inhibits lipid accumulation decreasing the adipogenic factors C/EBPβ, C/EBPδ, C/EBPα (CCAAT/enhancers binding protein α, β, δ) and PPARγ (peroxisome proliferator-activated receptor γ)[98,137.] Moreover, in differentiating omental adipocytes, stimulation with ghrelin has also produced a significantly increase in PPARγ and SREBP1 (sterol-regulatory element binding protein-1) mRNA levels, as well as several proteins related with fat storage, including acetyl-CoA carboxylase, fatty acid synthase, lipoprotein lipase and perilipin[138]. Consequently, all of these results together confirm that ghrelin stimulates intracytoplasmatic lipid accumulation via Akt activation.

Apart from the implication in the lipid storage in the adipose tissue, mitogenesis and apoptosis are also regulated by GHSR1a through AKT signaling. Ghrelin administration in rats attenuates NAFLD-induced hepatic injury, inflammation and apoptosis, partly through PI3K/Akt pathway. Isoproterenol-induced lipolysis is prevented by ghrelin triggered signaling pathways that involves PI3Kγ/AKT and PDE3B as key downstream effectors[121,139]. Neuroprotective mechanism of ghrelin after injury in rat hippocampus was associated with the promotion of PI3K/Akt signaling pathway and the inhibition of JNK signaling. Moreover, the activation of the JNK pathway might depend on the activation of PI3K/Akt and the activation of Akt has also been related with increased ratio of Bcl-2/Bax and inhibition of cytochrome c release from mitochondria with the subsequent prevention

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of caspase-3 activation. Accordingly, ghrelin promotes survival of hippocampus neurons through Akt pathway[140,141,142]. This antiapoptotic effect have also been demonstrated in cardiomyocytes, endothelial cells, hepatocytes, porcine ovarian follicular cells and pancreatic β-cells[143,144,145,146]. The proliferative effect in adult rat hippocampal cells and in neural progenitor cells (NPCs) involves multiple signaling pathways, such as MEK/ERK1/2, PI3K/Akt, and Jak2/STAT3. This mitogenic action was also related to PI3K/Akt signaling in osteoblast, intestinal epithelial cells, murine T cells, porcine ovarian follicular cells and cancer gastric cells[142,142,147,148,149,150].

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Besides, a recent study has reported that PI3K/Akt trough eNOS/NO induces endothelial progenitor cell (EPC) migration[151].

4.4 MAPKs: proliferation and differentiation

In addition to the PI3K/Akt signaling, ghrelin also regulates the proliferation and differentiation through MAP kinases (MAPKs) signaling in a wide variety of cell types. MAPKs pathway is implicated in the proliferation of rat hippocampal neural stem cells (NSCs) in combination with the PI3K/Akt, and Jak2/STAT3 signaling pathways[148]. Activation of GHSR1a stimulates the proliferation of human and rat adrenal zona glomerulosa cells through a mechanism involving ERK1/2[152]. MAPKs, especially ERK1/2, are rapidly activated by ghrelin in preadypocites; besides, ERK inhibition significantly attenuates the mitogenic and anti-apoptotic effect of ghrelin in these cells[153]. In intestinal epithelial cells GHSR1a regulates mitogenesis by cross-talk with epidermal growth factor receptor (EGFR) and activation of PI3K/Akt; both these pathways converge to stimulate ERK1/2 downstream[149]. Moreover, a positive effect of ghrelin in proliferation via MAPK pathway activation has

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been found in hepatoma, adipose, cardiomyocyte, prostate cell lines, and pituitary cell lines[154, 155,156].

Through MAPKS ghrelin has also show effect on apoptosis. For example, ghrelin inhibits oligodendrocytes H_2O_2-induced apoptosis in part through ERK1/2 activation and decreasing p38MAPK[157]. Moreover, PI3K/Akt and ERK1/2 pathways have shown to share the mediation of the neuroprotective protective effect of ghrelin against ischemic injury or status epilepticus (SE), as well as the ghrelin protective effect against the serum deprevation-induced apoptosis of osteoblastic MC3T3-E1 cells[142,147]. Furthermore, the protective effect of ghrelin on pancreatic β-cells from cytokine-induced toxicity during insulitis, was also mediated by ERK1/2 and Akt signaling pathways[158].

Nonetheless, ghrelin action in cardiovascular endothelial cells is open to discussion due a controversial effect. Some studies have reported the role of GHSR1a in the increase of proliferation, migration and angiogenesis of human dermal microvascular endothelial cells (HMVECs) and

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cardiac microvascular endothelial cells (CMECs) via MEK/ERK and PI3K/Akt signaling pathways[159,160]. Moreover, ghrelin has also improved H9c2 cardiomyocyte survival by suppressing the excessive autophagy through both ROS inhibition and mTOR induction via inhibition of AMPK and stimulation of p38-MAPK[161]. In addition, ghrelin has also demonstrated reduces aging deterioration of angiogenesis in HMVECs by MAPK/ ERK2 activation; induces endothelial progenitor cell (EPC) migration via PI3K/Akt/eNOS/NO; attenuates angiogenesis induced by oxLDL in HCAECs inhibiting NF-κB pathway; and inhibits cell apoptosis in human umbilical vein endothelial cells (HUVECs) via MEK/ERK1/2 and PI3K/Akt signaling pathways[151,162,163,164]. However, other in vitro studies have indicated that ghrelin can inhibit FGF-2-stimulated angiogenesis in rat brain microvascular endothelial cells (NECs) and HUVECs by inhibition of tyrosin kinases (TK)/MAPK cascades[165,166].

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Furthermore, in vivo studies have shown the same controversy. On the one hand, ghrelin stimulates retinal angiogenesis via GHSR1a in rats and induces angiogenesis in rats after myocardial infarction through the enhancement of vascular endothelial growth factor (VEGF) and its receptors Flk-1 and Flt-1 and inhibition of apoptosis[167,168]. On the other hand, a recent study reported that systemic administration of ghrelin did not restore angiogenesis in hindlimb ischemia in normal C57BL/6 mice and diet-induced obese mice[169].

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MATERIALS AND METHODS

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1.0 MATERIALS

Human ghrelin peptide and 1-5, 1-14 and 1-18 ghrelin was obtained from California Peptides (CA, US). Radioisotope [^{32}P]-orthophosphate (specific activity 8500–9120Ci/mmol), was from PerkinElmer Life Sciences. Secondary antibodies were purchased from Jackson immunoresearch laboratories (PA, US) and the primary antibodies are listed in the Table 1.

Antibody	Use	Dilution	Supplier	Reference
Actin	WB	1:5000	Abcam	ab1801
Akt	WB	1:1000	Cell Signaling	4685
EGFP	WB	1:1000	Abcam	ab111258
ERK1/2	ICQ	1:500	Cell Signaling	9102
ERK1/2	WB	1:1000	Cell Signaling	9102
GAPDH	WB	1:5000	Abcam	ab9485
GRK2	WB	1:1000	Novus	NB110-57020
GRK6	WB	1:1000	Abnova	H00002870-M10
HA	WB	1:5000	Sigma	H3663
pAkt (S473)	WB	1:1000	Novus	NB100-79891
pGRK2(S670)	WB	1:1000	Abcam	ab4473
PKCα	WB	1:1000	Cell Signaling	2056
pPDK1 (S241)	WB	1:1000	Cell Signaling	3061
pPKCε (S729)	WB	1:1000	Santa Cruz Biotechnology	sc-12355
pPKCγ (T514)	WB	1:1000	Cell Signaling	9379
pPKCδ(T507)	WB	1:1000	Santa Cruz Biotechnology	sc-11770
pPKCη (T655)	WB	1:1000	Abcam	ab5798
pPKCμ(PKD)(S916)	WB	1:1000	Cell Signaling	2051
pPKCζ(T410)	WB	1:500	Santa Cruz Biotechnology	sc-12894
pPKCθ(T538)	WB	1:1000	Cell Signaling	9377

pPKCα/β(T638/641)	WB	1:1000	Cell Signaling	9375
pERK(T202/Y204)	WB	1:1000	Cell Signaling	9101
β-arrestin 1	WB	1:1000	Abcam	ab31848
β-arrestin 2	WB	1:500	Cell Signaling	3857

Unless otherwise stated, all the rest biochemicals and reagents were from Sigma (MO, US).

2.0 METHODS

2.1 Cell culture

The human embryonic kidney (HEK) 293 wild-type (WT) cell line was obtained from the European Collection of Cell Cultures (ECACC) and was maintained in DMEM (Lonza; Basilea, CH) supplemented with fetal bovine serum (FBS) 10% (v/v), and 100U/mL penicillin-streptomycin solution. HEKs 293 stably transfected with GHSR1a-EGFP or GHSR1a-HA plasmids were maintained like WT adding 500µg/mL of G418 antibiotic.

The wild-type (WT), the β-arrestin 1 knockout (β-arrestin-$1^{-/-}$) and the β-arrestin 2 knockout (β-arrestin-$2^{-/-}$) murine embryonic fibroblast (MEF) cells, provided by Prof. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, US), were maintained in DMEM supplemented with 10% (v/v) FBS and 100U/mL penicillin-streptomycin solution.

The GC cell line was obtained from the American Type Culture Collection (ATCC) and was maintained routinely as a monolayer in DMEM medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) FBS and 100U/mL of penicillin-streptomycin solution. GCs stably expressing GHSR1a-EGF were maintained like the GC WT cells adding 500µg/mL of G418 antibiotic.

3T3-L1 preadipocytes were obtained from the ATCC and were maintained in DMEM containing 10%(v/v) FBS and 100U/mL of penicillin-streptomycin solution. For 3T3-L1 adipocyte differentiation, the 2-day-postconfluent cells (day 0) were treated with 0.5mM isobutylmethylxantine (IBMX), 25µM dexamethasone (DEX) and 861nM insulin, ghrelin or the corresponding truncate in DMEM containing 10%(v/v) FBS for 72h. After 72h, the differentiation medium was renewed every 48h with DMEM containing 10% (v/v) FBS and either insulin, ghrelin or the corresponding truncate (172nM) until the cells were used. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 Plasmids

The GHSR1a fused at its C terminus to enhanced green fluorescent protein (EGFP; GHSR1a-EGFP) in pEFGP-N1 (Clontech, CA, US) was provided by Prof. Catherine Llorens-Cortes (Institut National de la Sante et de la Recherche Medicale, College de France, Chaire de Medecine Experimentale, Paris, FR). The GHSR1a tagged at N-terminus with hemagglutinin (HA; GHSR1a-HA) in pcDNA3.1 was purchased from the cDNA resource center of the University of Missouri (MO, US).

β-arrestin 1 tagged at C-terminus with red fluorescent protein (RFP; β-arrestin 1-RFP) in pcDNA3.1, was provided by Prof. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, US) through Addgene (Cambridge, MA, US). β-arrestin 2 tagged with m-cherry at its C-terminus (β-arrestin 2-cherry) was provided by PROTEX laboratory of the University of Leicester.

Renilla luciferase-tagged β-arrestin 1 (Rluc-β-arrestin 1) was provided by Prof. Mark Scott (Institut Cochin, Paris, FR) and β-arrestin 2

tagged with Rluc (Rluc-β-arrestin 2) was provided by Prof. Graeme Milligan (University of Glasgow, Scotland, UK).

Human GRK2 KO CRISPR/Cas9 and human GRK2 homology directed repair (HDR), were purchased from Santa Cruz Biotechnology Inc (CA,US).

2.3 Site-directed mutagenesis

In order to generate the three GHSR1a mutants [Double mutant (GHSR1a-DM): S349A, T350A; Triple mutant (GHSR1a-TM): S362A, S363A, T366A; and, Total mutant (GHSR1a-Total): S349A, T350A, S362A, S363A, T366A], mutations to the GHSR1a wild type (GHSR1a-WT) sequence were incorporated using the in vitro site-directed mutagenesis QuikChange method (Stratagene, Cheshire, UK). The method used could be summarized in the following steps:

1. Using a temperature cycler the specifically design mutant oligonucleotide primers, each complementary to opposite strands of the base vector, was extended by PfuTurbo DNA polymerase. The cycle used was: denaturation 98° C 2min, 18 cycles of denaturation-anneling-elongation (98°C 30s - 55°C 1min - 68°C 8min) and a final elongation step of 68°C 8min.

2. The product was treated 1h at 37°C with DpnI, a specific endonuclease for methylated and hemimethylated DNA, to digest the parental DNA template and to select the synthesized DNA containing the desire mutation.

3. The DNA were transformed into DH5α supercompetent cells from Life technologies, Invitrogen (NY, US), ten or more colonies were growing overnight at 37°C and 220rpm and purified using the High Pure Plasmid Isolation Kit provided by Roche (Basel, CH).

4. The identity of the plasmids generated was verified by sequencing.

5. One Plasmid with the right mutation was used to introduce the next mutation or was transformed again into DH5α supercompetent cells if it included all the necessary mutations.

6. Finally, the GHSR1a mutants generated were grown in a large scale overnight at 37°C and 220rpm before being purified using QuiaFilter plasmid Maxi kit from QUIAGEN (Hilden, DE).

In this manner, GHSR1a mutants tagged at C-terminus with either EGFP or HA were generated.

2.4 Subcloning

Before being used to generate the GHSR1a mutants, the GHSR1a-HA plasmid tagged at C-terminus was generated from the N-terminus. The GHSR1a tagged at N-terminus was amplified by PCR using a forward primer which includes the XhoI restriction site and a reverse primer including KpnI restriction site and the 3xHA tag. PCR products were then subcloned into pcDNA3.1+.

To generate the GHSR1a WT and three mutants fused to enhanced yellow fluorescent protein (eYFP), GHSR1a-EGFP plasmids were amplified by PCR using primers which removed the stop codon and introduced a 5' HinDIII and 3' KpnI restriction sites. The resulting PCR products were then subcloned into pcDNA3.1 upstream of full length eYFP to generate C-terminal eYFP fusion constructs.

The DNA vectors containing the new constructions were transformed into DH5α supercompetent cells from Life technologies, Invitrogen (NY, US). Selected colonies of each plasmid were growing overnight at 37°C and 220rpm and purified using the High Pure Plasmid

Isolation Kit provided by Roche (Basel, CH) before verify the sequence of the plasmids generated. One of the plasmids including the right insert was then transformed again into DH5α supercompetent cells and grown overnight at 37°C and 220rpm. Finally, the DNA was purified using QuiaFilter plasmid Maxi kit purchased from QUIAGEN (Hilden, DE).

2.5 Cell transfections

The HEK293 and the MEF cells were transiently transfected with the GHSR1a-WT and its three mutants tagged with EGFP, using Lipofectamine 2000 (Life Technologies, Invitrogen; NY, US), according to the manufacturer's instructions to perform the immunoblot assays. All cellular assays involving transient transfections were only be carried out if the transfection efficiency was 80% or higher.

To perform the mass spectrometry, the silencing gene expression assays, the inhibition assays and the growth hormone secretion measurements, stably HEK293 and GC cell lines expressing GHSR1a WT-EGFP were generated by clone picking after 10 days under G418 selection. In both cases, the optimal selection antibiotic concentration, determined from the antibiotic kill curve previously done, was 500µg/mL. Likewise, GHSR1a cells lines stably expressing GHSR1a and the mutants tagged with HA at the C-terminus were generated. The GHSR1a expression of all clones was tested by immunoblot before use the stably cell lines.

In order to ensure the same amount of transfected cells in the ^{32}P labeling assays, HEK293 cell lines expressing GHSR1a-WT and the three mutants EGFP tagged were selected during 10 days on the basis of resistance to G418 (500µg/ml) and further selected using flow-assisted cell sorting.

2.6 CRISPR knocking down gene expression

To knockdown the expression of the GRK2 gene, HEK293 cells stably expressing GHSR1a-HA were cotransfected with human GRK2 KO CRISPR/Cas9 and human GRK2-HDR plasmids using Lipofectamine 2000 (Life Technologies, Invitrogen; NY, US),and following manufacturer's instructions. CRISPR/Cas9 GRK2 KO plasmids consist of a pool of three plasmids each encoding the Cas9 nuclease, a GRK2-specific guide RNA and green fluorescent protein (GFP). On the other hand, HDR plasmid consists of a pool of 2-3 plasmids, each containing a homology-directed DNA repair (HDR) template corresponding to the cut sites generated by the GRK2 CRISPR/Cas9 KO plasmids, a puromycin resistance gene to enable selection of stable knockout cells and, also contains RFP to visually confirm the transfection. Based on the puromycin resistance, the cells where selected during 10 days or more with 1µg/ml of puromycin before performed the assays. The puromycin concentration was determined from the antibiotic kill curve previously done.

In order to ensure the same amount of transfected cells in the ^{32}P labeling assays, HEK293 cells stably expressing the GHSR1a WT and the three mutants tagged with HA at the C-tail were transiently cotransfected with human GRK2 KO CRISPR/Cas9 and human GRK2-HDR plasmids using Lipofectamine 2000 (Life Technologies, Invitrogen; NY, US) following manufacture's instructions, selected during 10 days with puromycin (1µg/ml) and further selected using flow-assisted cell sorting based on the green fluorescence of the GFP insert included in the transfected GRK2 KO CRISPR/Cas9 plasmid.

2.7 [^{32}P] Orthophosphate labeling and GHSR1a immunoprecipitation

To perform GHSR1a and mutants labeling, 200,000 cells/well of GHSR1a-EGFP, GHSR1a-EGFP DM, GHSR1a-EGFP TM or GHSR1a-EGFP Total cell lines were plated in 6-well dishes 24h before experimentation and serum starved over night. For phosphorylation experiments, cells were washed three times with Krebs/HEPES buffer without phosphate [containing in mM: HEPES, 10 (pH 7.4); NaCl, 118; $CaCl_2$, 1.3; KCl, 4.3; $MgSO_4$, 1.17; $NaHCO_3$, 4.17; glucose, 11.7] and incubated in this buffer containing 100µCi/mL [^{32}P]orthophosphate for 1h at 37°C and 5% CO_2. Cells were then stimulated with ghrelin (100nM, 5min) and immediately lysed by addition of lysis buffer [containing in mM: Tris/HCl, 20 (pH 7.4); NaCl, 150; and EDTA, 3; and supplemented with 1% (v/v) Nonidet P-40, and 0.5% (w/v) sodium deoxycholate] supplemented with phosphatase and protease inhibitors. GHSR1a was then immunoprecipitated from the cleared lysates using GFP-trap (Chromotek; DE) following manufacturer's instructions. The washed immunoprecipitates were separated by SDS-PAGE on two 10% gels. The first gel was dried, and radioactive bands were revealed using autoradiography film. The second gel was transferred to nitrocellulose membrane as loading control. The analysis of the bands was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, US).

In the same way, to check the effect of the knock down GRK2 gene expression in the ^{32}P incorporation of the mutants, 200,000 cells/well of GHSR1a-HA, GHSR1a-HA DM, GHSR1a-HA TM or GHSR1a-HA Total cell lines stably expressing GRK2 KO CRISPR/Cas9 plasmid, were plated in 6-well dishes 24h before experimentation and serum starved over night. The next day, cells were washed three times with Krebs/HEPES buffer without phosphate and incubated in this buffer containing 100µCi/mL [^{32}P] orthophosphate for 1h at 37°C and 5% CO_2 before being stimulated for 5min with ghrelin 100nM. The cells where immediately lysed with lysis buffer supplemented with phosphatase and protease inhibitors. Using HA beads from Sigma Aldrich

(MO, US) and following manufacturer's instructions, GHSR1a was then immunoprecipitated from the cleared lysates. The washed immunoprecipitates were separated by SDS-PAGE on two 10% gels. One of the gels was dried, and radioactive bands were revealed using autoradiography film while the second gel was transferred to nitrocellulose membrane as loading control. The bands analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, US).

2.8 GHSR1a receptor purification and Mass Spectrometry

To purify the GHSR1a, the steps followed were:

1. Ten confluent T175 flasks of stably GHSR1a-EGFP cells were harvested, resuspended in Krebs/HEPES buffer and stimulated with ghrelin 100nM at 37°C for 5min.

2. Membranes were separated and solubilized by addition of 5 mL of TE buffer [containing in mM: Tris/HCl, 10 (pH 8.0); and EDTA, 1] plus a mixture of protease and phosphatase inhibitors.

3. After centrifugation at 20,000×g, the resulting supernatant was diluted 1:1 with phosphate buffered saline (PBS), and the receptor was then purified with GFP-trap (Chromotek, DE) following manufacturer's instructions.

4. The inmmunoprecipitates were extensive washed with solubilization buffer containing 0.5% Nonidet P-40 and the resin was resuspended in 2×SDS-PAGE before resolve the samples by SDS-PAGE on 10% gel.

5. Gel was stained with colloidal Coomassie Blue and the GHSR1a receptor was excised from the polyacrylamide.

6. Gel pieces were washed three times for 5min with 50mM ammonium bicarbonate [$(NH_4)HCO_3$].

7. Reduction and alkylation of cysteines were performed by addition of 10mM dithiothreitol (DTT) in 50mM $(NH_4)HCO_3$ at 55°C for 30min followed by addition of 100mM iodoacetamide in 50mM $(NH_4)HCO_3$ for 30min in the dark.

8. Gel slices were washed three times for 5min with 50mM $(NH_4)HCO_3$ containing 50% acetonitrile and incubated over night at 37°C in 50mM $(NH_4)HCO_3$ containing 10% (v/v) acetonitrile and 1µg of sequencing grade trypsin (Promega, Southampton, UK).

9. After tryptic digestion, phosphopeptides were enriched using PHOS-Select^{TM} iron affinity resin.

LC-MS/MS was carried out then on each sample using an LTQ OrbiTrap mass spectrometer (Applied Biosystems, Warrington, UK). Peptides resulting from in-gel digestion were loaded at a high flow rate onto a reverse-phase trapping column (0.3mm inner diameter × 1mm), containing 5µM of C18 300 Å Acclaim PepMap media (Dionex, UK) and eluted through a reverse-phase capillary column (75µm inner diameter × 150mm) containing Symmetry C18 100 Å media (Waters) that was self-packed using a high pressure packing device (Proxeon Biosystems, Odense, DK). The output from the column was sprayed directly into the nanospray ion source of an LTQ Orbital mass spectrometer. The resulting spectra were searched against the UniProtKB/SwissProt data base using MASCOT software (Matrix Science Ltd.) with peptide tolerance set to 5ppm and the MS/MS tolerance was set to 0.6Da. Fixed modifications were set as carbamidomethyl cysteine with variable modifications of phosphoserine, phosphothreonine, phosphotyrosine, and oxidized methionine. The enzyme was set to trypsin/proline, and up to two missed cleavages were allowed. Peptides with

a Mascot score greater than 20 and where the probability (*p*) that the observed match was a random event was <0.05 were included in the analysis. In Mascot, the ions score for an MS/MS match is -10Log(p), based on the calculated probability, *p*, that the observed match between the experimental data and the database sequence is a random event. The spectra of peptides reported as being phosphorylated were interrogated manually to confirm the precise sites of phosphorylation.

2.9 GHSR1a/β-arrestin interaction assays: BRET

A bioluminescence resonance energy transfer (BRET) assay was used to monitor interactions between GHSR1a mutants and β-arrestins. HEK293 cells were co-transfected with eYFP tagged-GHSR1a-WT (eYFP-GHSR1a-WT), eYFP-GHSR1a-DM, eYFP-GHSR1a-TM or eYFP-GHSR1a-Total plasmids and Rluc-β-arrestin 1 or Rluc-β-arrestin 2 at a ratio of 4:1 or the ratio indicated in the graph, using Lipofectamine 2000 (Life Technologies, Invitrogen; NY, US) and following manufacturer's indications. After 24h incubation, cells were subcultured into poly-D-lysine-coated white 96-well microplates, incubated for a further 24h prior to the assay and serum starved over night. Cells were then washed with Hanks' balanced salt solution (Gibco, CA, US) and incubated in this buffer for 30min prior to conducting the assay. To initiate the assay, the Rluc substrate coelenterazine (Life Technologies, Invitrogen; NY, US) was added to a final concentration of 2.5µM and incubated for 10min at 37°C. Following a further 5min incubation with ghrelin, luminescence emissions at 535nm and 475nm were measured using a CLARIOstar (BMG Labtech; Offenburg, DE), and the BRET signal was presented as the 535/475 ratio multiplied by 1000 to yield the arbitrary milli-BRET units.

2.10 Confocal assays

For analysis of the endocytosis time course, HEK293 cells on poly-D-lysine-coated coverslips were transfected with the EGFP-tagged GHSR1a-WT, GHSR1a-DM, GHSR1a-TM or GHSR1a-Total. The cells were serum starved overnight in a humidified atmosphere of 95% air and 5% CO_2 at 37°C prior to conduct the assay. To prevent de novo protein synthesis, the cells were preincubated for 2h at 37°C with 90µM cycloheximide. The cells were then preincubated for 30 min at 4°C in ice-cold Earle's buffer [containing (in mM): 140 NaCl, 5 KCl, 1.8 $CaCl_2$, and 3.6 $MgCl_2$ (pH 7.4); and, complemented with 0.2% BSA, 0.01% glucose, 90µM cycloheximide, and 0.8mm of 1–10 phenanthrolene] in the presence/absence of ghrelin (100nM). Internalization was promoted at the indicated time by placing the cells at 37°C. After rinsing the cells three times at 4°C with ice-cold Earle's buffer, they were fixed for 10min with 4% paraformaldehyde dissolved in 0.1mM PBS (pH 7.4). Before the coverslips were mounted using Vectashield medium with DAPI (Vector Laboratories, Compiègne, FR), the cells were rinsed again in cold Earle's buffer. To determine the interaction between the GHSR1a and β-arrestins, the HEK293 cells were cotransfected with the EGFP-tagged GHSR1a-WT, GHSR1a-DM, GHSR1a-TM or GHSR1a-Total and the corresponding RFP-tagged β-arrestin 1 or m-cherry-tagged β-arrestin 2 in a 1:1 ratio. To analyze the effect of ghrelin truncates in the endocytosis time course, HEK293 cells stably expressing GHSR1a-EGFP were stimulated with ghrelin or the corresponding ghrelin truncate (100nM).

For immunofluorescence analysis of ERK1/2 activation, the HEK293 cells were transfected and cultured as indicated above. After ghrelin stimulation for the indicated times, the cells were fixed with 4% buffered paraformaldehyde-PBS for 15min and washed with PBS three times. The cells where then permeabilized with PBST (1% (v/v) Tween 20 PBS) supplemented with 1% (v/v) Triton X-100 for 30min, blocked with PBST containing 1% (v/v) Triton X-100, 5% (v/v) heat-inactivated normal goat serum, and 0.2% (w/v)

BSA for 60min and incubated with anti-pERK1/2(T202/Y204) antibody diluted in PBST (1:500) over night at 4°C. After three washes with PBS, cells were incubated with the secondary antibody (Alexa Fluor 594-conjugate goat anti-rabbit antibody) in PBST (1:1000) for 60min at room temperature. Vectashield medium with DAPI (Vector Laboratories, Compiègne, FR) was used to mountain the coverslips.

Digital images of cells were acquired with a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems; Heidelberg, DE).

2.11 Small interfering RNA (siRNA) silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes for GRKs, β-arrestin 1, β-arrestin 2 and PKCα were selected from ON-TARGET plus SMART pool siRNA from Thermo Fisher Scientific (Dharmacon, Lafayette, CO, US).

Mouse β-arrestin 1 (5'→3'): ACGGGAAGCUCAAGCAUGA; UCAUAGAGCUUGACACCAA;GGAGAACCCAUCAGCGUUA; UGGAUAAGGAGAUCUAUUA.

Human β-arrestin 1 (5'→3'): UGGAUAAGGAGAUCUAUUA, AUGGAAAGCUCACCGUCUA,GAACUGCCCUUCACCCUAA, GAACGAGACGCCAGUAGAU.

Mouse β-arrestin 2 (5'→3'): GUGCCAAAACAAUAGAAGA, AUACCAACCUCAUCGAAUU,CUACUUGAAGGACCGGAAA, GGGCCUGUCUUUCCGCAAA.

Human β-arrestin 2 (5'→3'): CGAACAAGAUGACCAGGUA, CGGCGUAGACUUUGAGAUU,GGGCUUGUCCUUCCGCAAA, UAGAUCACCUGGACAAAGU.

Human PKCα (5'→3'): UAAGGAACCACAAGCAGUA, UUAUAGGGAUCUGAAGUUA,GAAGGGUUCUCGUAUGUCA, UCACUGCUCUAUGGACUUA

Human GRK2 (5'→3'): GGGACGUGUUCCAGAAAUU, GCUCGCAUCCCUUCUCGAA,GGAAUCAAGUUACUGGACA, GCGAUAAGUUCACACGGUU

Human GRK3 (5'→3'): GGUACCUAUGAACCUAGAA, CCAGAAUGGAAUUUAAAGA,CUGUGUAUAUUGACUUUCA, CUGCAUCCUGUCCUUGAUA

Human GRK5 (5'→3'): GAGAACCAUUCCACGAAUA, GAACGUGUUUGGACCUAAU, GGUGAACAUUGCAAUAGAA

Human GRK6 (5'→3'): CGAACACGGUGCUACUCAA, GAAAGUGAACAGUAGGUUU,GAGCUUGGCCUACGCCUAU, GGUAGAGAAUGAACGGUAC

Human PDK1 (5'→3'): CGAGGAGACAGAAACUGAA, GAGCUGUGUUGUUAGUUA,GUAGAGUUUGUAUGUUUGA

An ON-TARGET plus Non-targeting siRNA was used as a control for all siRNA experiments. The cells were transfected with Lipofectamine 2000 (Life Technologies, Invitrogen, NY, US) according to the manufacturer's instructions. Based on the short half-life of these siRNAs during adipocyte differentiation (~4 days), the confluent 3T3-L1 cells were transfected after the induction of adipogenesis with 0.5mM IBMX, 25µM DEX, and 861nM ghrelin in DMEM containing 10% (v/v) FBS for 3 days, and then after siRNA transfection maintenance in DMEM containing 10% (v/v) FBS supplemented with 172nM ghrelin for 3 days to finalize the differentiation.

2.12 Phosphoprotein gel stain

HEK293 cells stably expressing GHSR1a-EGFP were transfected with GRK2, GRK3, GRK5 and GRK6 SiRNA as indicated above. The cells where then immediately lysed with lysis buffer supplemented with phosphatase and protease inhibitors. After 18,000xg centrifugation, GHSR1a was immunoprecipitated from the cleared lysates using GFP-Trap (Chromotek; DE). The washed immunoprecipitates were separated by SDS-PAGE on two 10% gels. The first polyacrylamide gel was stained with Pro-Q Diamond phosphoprotein stain and the second one was stained with SYPRO Ruby protein gel stain as a control. Both labeling solutions were purchased from Life technologies, Invitrogen (NY, US).

For Pro Q staining the protocol followed was:

1. Gel fixation. The gel was immersed in 100mL of fix solution (50% methanol, 10% acetic acid) and incubated at room temperature with gentle agitation 30min. The fixation step was repeated to ensure that all of the SDS was washed out of the gel and leaved it over night.

2. Gel washing. The gel was washed three times in 100mL of ultrapure water with gentle agitation for 10min each time.

3. Gel Staining. The gel was incubated in 60mL of Pro-Q® Diamond phosphoprotein gel stain with gentle agitation in the dark for 90min.

4. Gel Destaining. The gel was incubated in 100mL of destain solution with gentle agitation for 30min at room temperature, protected from light. This procedure was repeated two more times.

5. Gel washing. The gel was washed three times with 100mL of ultrapure water at room temperature for 5min per wash.

For Sypro Ruby staining the protocol was:

1. Gel fixation. The gel was immersed in 100mL of fix solution (50% methanol, 7% acetic acid) and agitated on an orbital shaker for 30min. The fixation step was repeated one more time.

2. Gel washing. The gel was washed three times in 100mL of ultrapure water with gentle agitation for 10min each time.

3. Gel staining. The gel was incubated in 60mL of Pro-Q® Diamond phosphoprotein gel stain with gentle agitation over night.

4. Gel washing. The gel was washed in 100mL of wash solution (10% methanol, 7% acetic acid) for 30min. The gel was then rinsed in 100mL of ultrapure water twice for 5min each wash.

All the images were obtained scanning the gels with a Typhoon fluorescence scanner (GE, Uppsala, SE).

2.13 Immunoblot analysis

Serum-starved cells were stimulated with ghrelin or the corresponding ghrelin truncate for the indicated time period at 37°C and 5% CO_2. The medium was then aspirated and the cells were lysed with the lysis buffer described before supplemented with protease and phosphatase inhibitors. The solubilized lysates were transferred into centrifuge tubes and left at 4°C for 15min, before pre-cleared it by centrifugation at 18,000×g for 15min at 4°C. Protein concentration was evaluated with the QuantiPro BCA assay kit from Sigma-Aldrich (MO, US). Subsamples (same amount of protein) of each sample were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes. The immunoreactive bands were detected by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Pierce, Rockford, IL, US). The resulting protein

bands were scanned and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, US) and normalized for the corresponding loading controls.

In assays where HEK293 cells stably expressing GHSR1a-EGFP where pretreated with inhibitors, the concentrations and times employed were:

1. Pertussis toxin (PTX) from Gibco (CA, US), 100ng/ml 12h.
2. Wortmannin from Santa Cruz Biotechnology (CA, US), 1µM 30min.
3. Gö6850 (Bisindolylmeleimide I) from Santa Cruz Biotechnology (CA, US), 100nM 30 min.
4. Gö6983 [2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide] from Santa Cruz Biotechnology (CA, US), 100nM 30min.
5. Gö6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] from Santa Cruz Biotechnology (CA, US), 100nM 30min.

2.14 Proliferation assays

Cell proliferation of HEK293 expressing the GHSR1a-WT or the correspondent mutant was determined using the bromodesoxiuridina (BrdU) incorporation-ELISA assay (Roche, Swiss) following manufacturer's instructions. Briefly, 10,000 cells/well were plated in 96-well microplates and cultured in DMEM supplemented with ghrelin (100nM) or FBS 10% (v/v) as a positive control for 12h and then were incubated with 10mg/mL BrdU for another 12h before being fixed with FixDenat solution. The fixed cells were further treated with anti-BrdU-POD working solution, and rinsed with washing solution before substrate solution was added. The absorbance at

370nm (reference wavelength at 492nm) was measured using an ELISA plate reader (Reader VersaMaxPLUS). To measure the effect of the ghrelin truncates in HEK293 stably expressing GHSR1a-EGFP cell proliferation the cells were stimulated with 100nM ghrelin or the corresponding ghrelin truncate.

2.15 Quantification of lipid accumulation

Differentiated 3T3-L1 adipocytes were fixed for at least 2h with 4% buffered paraformaldehyde-PBS. Each well was rinsed three times with Milli-Q water and was incubated 5min at room temperature with 60% iso-propanol. Lipid droplets were then stained for 10min at room temperature with a working solution of 60% (v/v) Oil Red O. For quantification, cells were washed extensively with water to remove unbound dye, iso-propanol was added to the stained culture plates and the colour was analyzed by spectrophotometry at 520nm.

2.16 Inositol 1-phosphate accumulation

Inositol 1-phosphate (IP_1) accumulation was measured using IP-One HTRF® assay kit (Cisbio Assays, MA, US) based on FRET technology. GHSR1a-WT, GHSR1a-DM, GHSR1a-TM or GHSR1a-Total cell lines tagged with EGFP were cultured into poly-D-lysine-coated 96-well microplates, incubated for a further 24h prior to the assay and serum starved over night.

For IP-One HTRF® kit the protocol followed was:

1. Cells were then treated with stimulation buffer containing LiCl which inhibit IP_1 degradation plus ghrelin 100nM during 1h at 37°C.

2. Cells where lysed by addition of lysis buffer for 30min at room temperature with shaking at 500rpm.

3. To initiate the assay, 16µL of lysate was transferred to a white 384-well plate and incubated with IP1-d2 conjugate 30min at 37°C.

4. Anti-IP1 conjugated with Tb-cryptate was added during 1h at room temperature with shaking at 500rpm. This antibody competes with native IP1 produced by cells and IP1 coupled to the dye d2.

5. Fluorescence emissions were measured at 665nm and 620nm using a CLARIOstar (BMG Labtech; Offenburg, DE) and IP_1 acummulation was determined from the ratio 665nm/620nm.

2.17 GH assay

To measure murine GH secretion, GC-GHSR1a cells were plated in 6-well dishes 24h prior to induce the secretion during 30min with ghrelin or the corresponding truncate (100nM) diluted in fresh DMEM phenol red free. The medium collected from the stimulated GC-GHSR1a cells culture and the non-stimulated control was concentrated to 100µL by ultracentrifugation using Amicon Ultra 3kDa tubes (Millipore, Billerica, US) and following manufacturer's indications. The amount of total protein of each sample was then evaluated with the QuantiPro BCA assay kit from Sigma-Aldrich (MO, US). Equal amount of protein was loaded on each well of the microtiter plate coated by a pre-tittered amount of anti-Growth Hormone polyclonal antibodies of the rat/mouse GH ELISA kit from EMD Millipore (Billerica, Massachusetts, US). After incubation with the primary antibody, the unbound materials from samples were washed and the second biotinylated anti-Growth Hormone polyclonal antibody was added. Following three washes, the horseradish peroxidase conjugate was loaded before wash the plate again. The quantification of immobilized antibody-enzyme conjugates

was monitored following the horseradish peroxidase activity in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbency at 450nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured rat or mouse Growth Hormone in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Rat GH.To evaluate the effect of β-arrestins, before being stimulated with ghrelin 100nM GC-GHSR1a cells were transfected 48h prior to conduct the assay with the corresponding SiRNA. GH levels were analyzed in quadruplicate.

2.18 Data analysis

All values are presented as mean ± standard error of the mean (SEM). Student t test were performed to assess the statistical significance of 2-way analysis. For multiple comparisons, ANOVA was employed. $p<0.05$ was considered as statistically significant (*).

RESULTS

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CHAPTER 1

GHSR1a phosphorylation

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Identification of phosphorylation sites in GHSR1a

The GHSR1a following 5min stimulation with ghrelin (100nM) enhanced its phosphorylation ~2.8±0.2 fold compared to the unstimulated receptor as monitored by increased incorporation of ^{32}P after immunoprecipitate the GHSR1a into a protein with an apparent molecular mass ~100KDa (Figure 11).

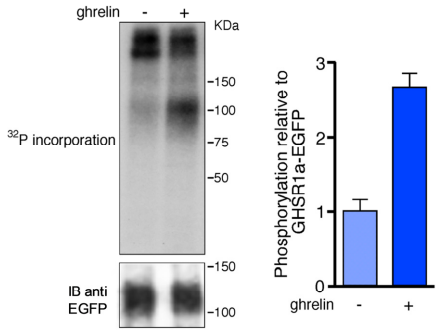


Figure 11. ^{32}P incorporation of GHSR1a. HEK293 cells transiently expressing C-terminally EGFP-tagged GHSR1a were treated with the agonist ghrelin (100 nM) or vehicle for 5min and then labeled with ^{32}P. *Left panel*, autoradiogram and loading control (EGFP immunoblot) is shown. *Right panel*, levels of ^{32}P were quantified by densitometry, normalized to GHSR1a-EGFP, and expressed as fold increase relative to the control cells. Immunoblot is representative of five independent experiments. The data are expressed as the mean ± SEM (*, $p < 0.05$).

To identify the precise phosphorylation sites, upon 5min of stimulation with ghrelin (100nM), the receptor was excised from a stained polyacrylamide gel to generate tryptic peptides which was analyzed by mass spectrometry-based proteomics (Figure 12 and 12'). The study of the spectra generated, revealed 3 serines (S^{349}, S^{362} and S^{363}) and 2 threonines (T^{350} and T^{366}) as phosphor-acceptor sites at the C-tail of the GHSR1a. Despite the tryptic peptides generated from the receptor revealed peptide coverage of the third intracellular loop, there was no detection of phosphorylation residues in this region.

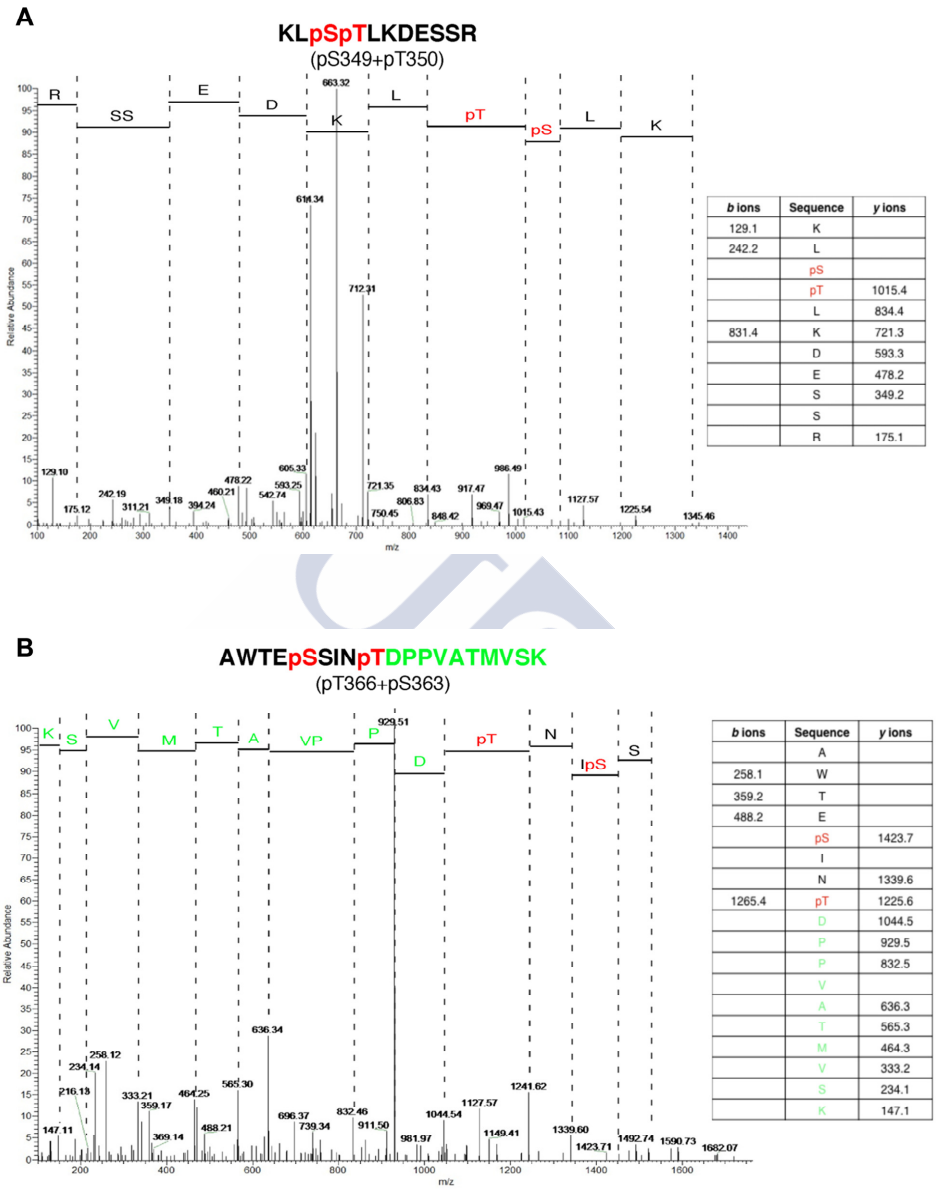
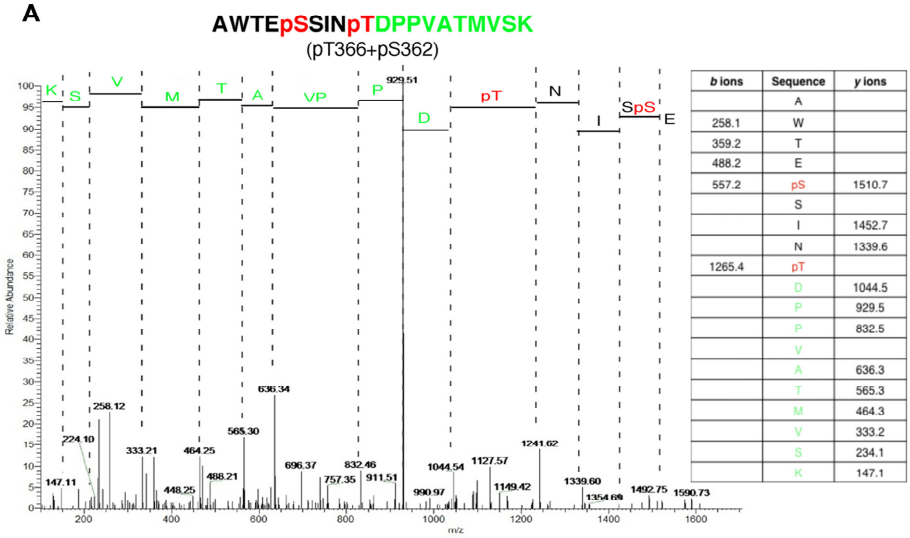


Figure 12. Mass spectrometry identification of phosphorylation sites in the GHSR1a. HEK293 cells transiently expressing C-terminally EGFP-tagged GHSR1a were immunoprecipitate and then digest for analysis using mass spectrometry. **A and B**, representative mass spectra and associated fragmentation tables that cover the four phosphorylated residues identified in various experiments, 2 serines (S^{349}, and S^{363}) and 2 threonines (T^{350} and T^{366}) (noted in green are the EGFP amino acids).

A



b ions	Sequence	*y* ions
	A	
258.1	W	
359.2	T	
488.2	E	
557.2	pS	1510.7
	S	
	I	1452.7
	N	1339.6
1265.4	pT	
	D	1044.5
	P	929.5
	P	832.5
	V	
	A	636.3
	T	565.3
	M	464.3
	V	333.2
	S	234.1
	K	147.1

B

Peptide sequence	Mascot ion score	Delta ppm	Residue number	N° of time observed
(R)KLSTLKDESSR(A)	79	0.0013	347-357	16
(R)KLSTLKDESSR(A)	56.7	-2.8	347-357	2
(R)KLSTLKDESSR(A)	62	0.82	347-357	4
(R)AWTESSINTDPPVATMVSK(G)	69.9	-3.1	358-376	2
(R)AWTESSINTDPPVATMVSK(G)	80. 9	-2.0	358-376	2
(R)AWTESSINTDPPVATMVSK(G)	80.9	-2.4	358-376	2

Figure 12'. Mass spectrometry identification of phosphorylation sites in the GHSR1a (*Continuance*). A, representative mass spectra and associated fragmentation table that cover the phosphorylated residues S^{362} and T^{366} identified in different experiments (in green are noted the EGFP amino acids). **D**, Summary of the mass spectrometry data.

To ensure that these amino acids are the key residues in GHSR1a phosphorylation, recombinant HEK cells were generated in which these phosphor-acceptor sites were mutated to A residues that are not able to be phosphorylated (Figure 3A). In a double mutant of GHSR1a, designated

GHSR1a-DM, in which T^{350} and S^{349} were mutated to A, phosphorylation in response to 5min of treatment with ghrelin was reduced by 57±3% (Figure 13B). Triple mutation of GHSR1a, designated GHSR1a-TM, in which S^{362}, $S^{363}$and T^{366} were mutated to A, phosphorylation was decreased by 58±2%. A further variant was generated where all of the residues identified by mass spectrometry to be phosphorylated were substituted by A (S^{349}, S^{362}, S^{363}, T^{350} and T^{366}) in a mutant, designated GHSR1a-Total. The phosphorylation status of GHSR1a-Total was significantly less than that of either GHSR1a-DM or GHSR1a-TM indicating that the sites of ghrelin-regulated phosphorylation in the GHSR1a were mainly S^{349}, S^{362}, S^{363}, T^{350} and T^{366} (Figure 13B).

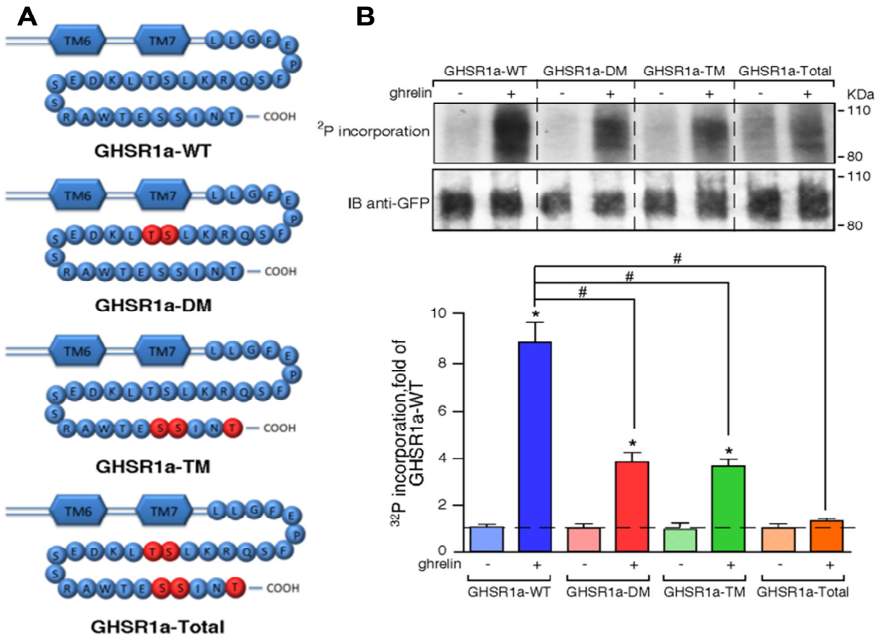


Figure 13. Analysis of GHSR1a mutations. A, Illustration of the S and T (red) phosphorylation sites that were mutated to A in the GHSR1a-WT C-tail to generate the GHSR1a-DM, GHSR1a-TM and GHSR1a-Total mutants. **B**, *Upper panel*, ^{32}P labeling of HEK293 cells expressing either the EGFP-tagged GHSR1a-WT or the mutants. *Bottom panel,* levels of ^{32}P were quantified by densitometry, normalized to GHSR1a-EGFP, and expressed as fold increase relative to the control cells expressing the GHSR1a-WT. Immunoblot is

representative of five independent experiments. The data are expressed as the mean ± SEM (*,# $p < 0.05$).

β-arrestin 1 and 2 recruitment are related to the phosphorylation of the GHSR1a C-tail.

To determine the importance of the GHSR1a C-terminus phosphorylation in directing specific signaling events, the receptor endocytosis induced by ghrelin (100nM) was studied by confocal microscopy in HEK293 cells expressing GHSR1a-WT or mutants tagged with EGFP. In the resting cells, the fluorescence associated with the receptor was predominantly localised to the plasma membrane. Despite being treated with cycloheximide to inhibit the *de novo* protein synthesis, the cells had also shown a slight fluorescent associated with the Golgi apparatus. After exposure to ghrelin for 20 and 60min, the GHSR1a-WT-associated fluorescence almost completely disappeared from the plasma membrane to be redistributed to a population of intracellular vesicles distributed throughout the cytoplasm (Figure 14A). In cells transfected with the GHSR1a-DM, the receptor was primarily spread throughout the cytoplasm after 20 and 60min of agonist treatment although the population of intracellular vesicles was relatively reduced compared with the WT receptor (Figure 14A). By contrast, ghrelin was not able to induce receptor internalization in HEK293 cells expressing GHSR1a-TM or GHSR1a-Total, consequently, the fluorescence associated to the receptor remained to be mainly localized in the plasma membrane and no redistribution of the fluorescent could be observed (Figure 14B).

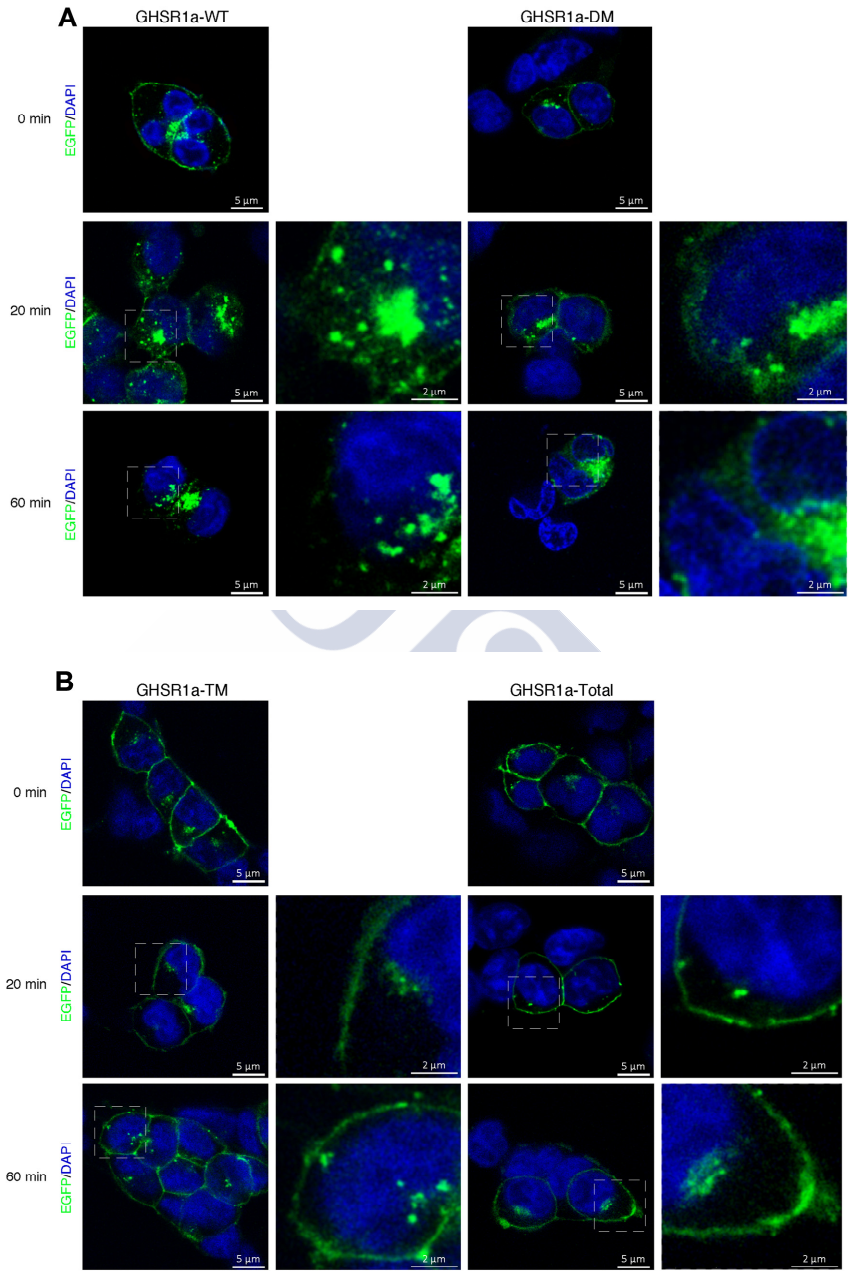


Figure 14. Analysis of the endocytosis time course by confocal microscopy of the EGFP-tagged GHSR1a-WT and the mutants. HEK293 cells transiently transfected were stimulated with ghrelin (100nM) for different times at 37℃. Confocal images are representative of three independent experiments.

In order to resolve whether this change in the patterns of endocytosis displayed by the mutant receptors was due to differences in their ability to interact with β-arrestins, cells were transiently co-transfected with the receptors tagged with EGFP and RFP-tagged β-arrestin 1 or m-cherry-tagged β-arrestin 2. As shown in Figures 15A and 15B, the receptors (shown in green) were located at the cell surface, whereas RFP-β-arrestin 1 or m-cherry-β-arrestin 2 was uniformly distributed in the cytoplasm in unstimulated cells (shown in red). After 20min of ghrelin (100nM) treatment, GHSR1a-WT appeared to colocalize with both β-arrestin 1 and 2 in endocytic vesicles (shown in yellow; Figure 5A and B). This colocalization reflected the assembly of a protein complex containing β-arrestin and the receptor being more robust at 60min of agonist stimulation. Similarly, ghrelin stimulation of GHSR1a-DM triggered the colocalization of the receptor with both β-arrestins at 20 and 60min (Figure 15A and 15B). However, this colocalization was rather evenly distributed in the cytoplasm in a diffuse granular pattern, with no apparent enhanced localization in endocytic vesicles. By contrast, in the case of GHSR1a-TM and GHSR1a-Total no colocalization events was observed, the green fluorescence associated to the receptors were seen at plasma membrane while the red fluorescence of the β-arrestin continued distributed throughout the cytoplasm after agonist treatment (Figure 15A and 15B).

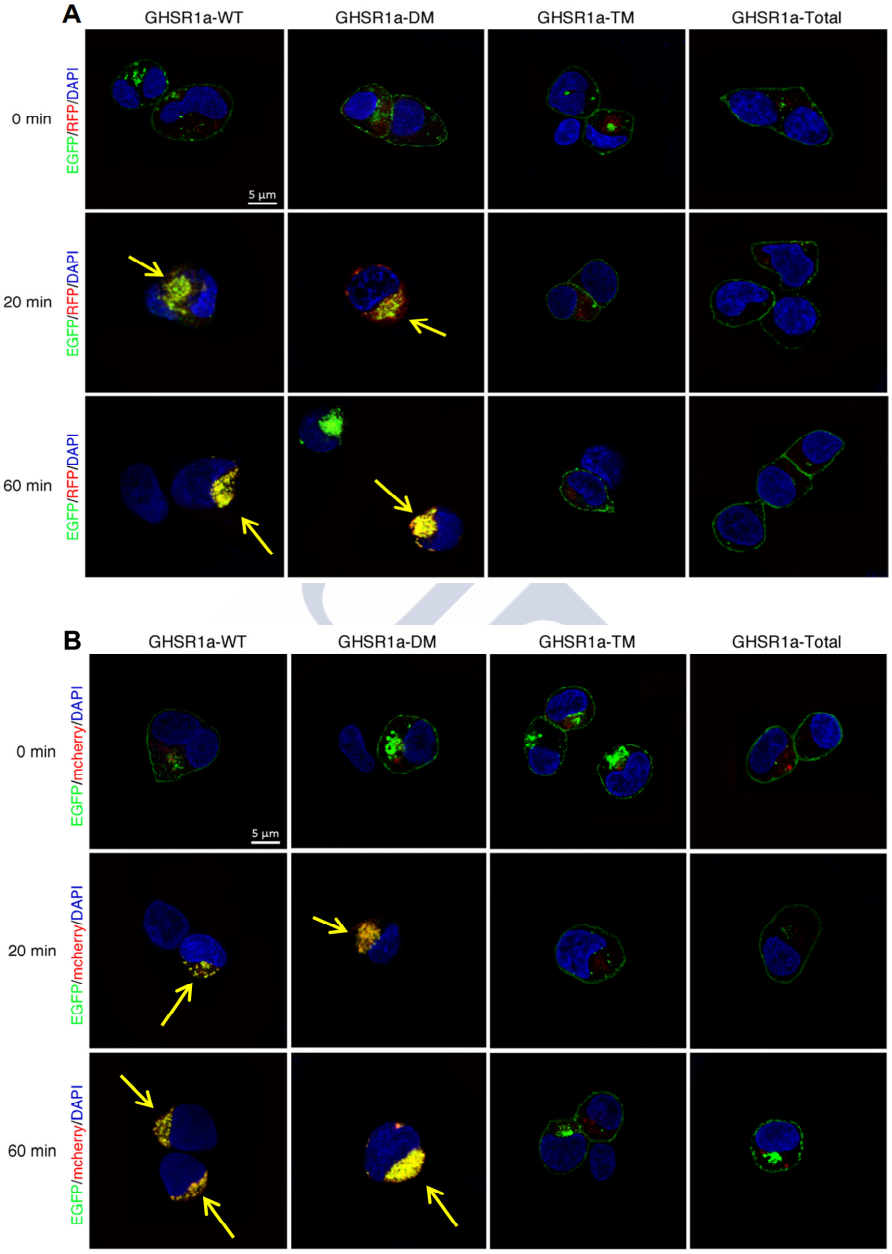


Figure 15. Phosphor-acceptor sites in GHSR1a-WT C-tail govern binding and trafficking patterns of β-arrestins. A, Trafficking of RFP-β-arrestin 1 and EGFP-tagged GHSR1a-WT or mutants in HEK293. **B**, Trafficking of m-cherry-β-arrestin 2 and EGFP-tagged GHSR1a-WT or mutants in HEK293. **A and B**, Confocal images are representative of three experiments. Colocalization of β-arrestins (red) and receptors (green) is pointed by yellow arrows.

The contributions of agonist dependent phosphorylation in the C-tail of GHSR1a to recruitment of β-arrestin 1 and 2 were further studied by BRET assays, which measured the capacity of association between eYFP-tagged GHSR1a or mutants with and Rluc-β-arrestin 1 or 2 in real time in HEK293 living cells (Figure 16A and 16B, respectively). These BRET assays revealed that the β-arrestins are recruited by GHSR1a in a ghrelin dependent manner. The concentration response curves of β-arrestin 1 for the double mutant receptor in comparison with the WT had shown a slight but significant decrease in both potency (pEC_{50}= 7.54 for GHSR1a-WT, and pEC_{50}= 7.10 for GHSR1a-DM; $p<0.05$) and efficacy (81.3% of GHSR1a-WT response; $p<0.05$). At the same time, β-arrestin 2 response curves had also exhibited that GHSR1a-DM significant decrease in both potency (pEC_{50}= 7.30 for GHSR1a-WT, and pEC_{50}= 7.14 for GHSR1a-DM; $p<0.05$) and efficacy (82.4% of GHSR1a-WT response; $p<0.05$) compared with the GHSR1a-WT. The fact that the BRET values for β-arrestin 2 recruitment were higher than those for β-arrestin 1 in both receptors, GHSR1a-WT and GHSR1a-DM, might be caused by a conformational difference in the configuration of the receptor/β-arrestin complex that results in a greater distance between the luciferase and eYFP tags or by a distinct affinity for β-arrestin 1 and 2. On the other hand, in comparison with the WT receptor, the triple and the total mutants exhibited a significant reduction in the efficacy to recruit β-arrestin 1 (36.8% and 31.8% of GHSR1a-WT, respectively) and β-arrestin 2 (24.6% and 18.0% of GHSR1a-WT, respectively) what is consistent with the lack of receptor/β-arrestin colocalization observed by confocal analysis.

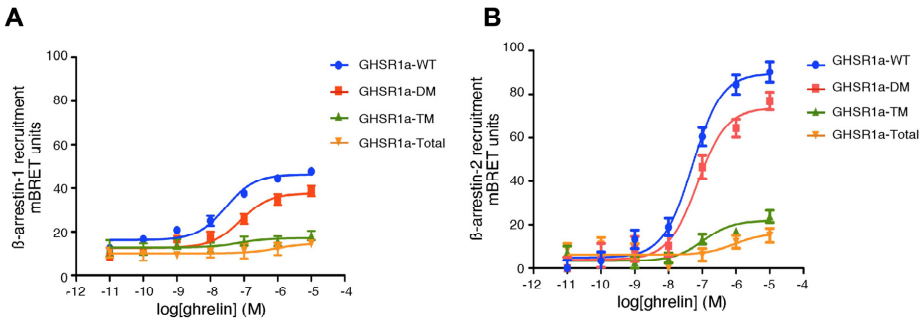


Figure 16. **BRET approach to monitor the β-arrestin interactions with the GHSR1a. A**, BRET ratio was estimated in response to incremental doses of ghrelin for the interactions between the YFP-GHSR1a-WT/GHSR1a mutants and Rluc-β-arrestin 1. **B**, BRET ratio was estimated in response to incremental doses of ghrelin for the interactions between the YFP-GHSR1a-WT/GHSR1a mutants and Rluc-β-arrestin 2.

An intriguing observation was that the potency and efficacy of β-arrestins binding was subtly reduced by the mutation of T^{350} and S^{349} in the GHSR1a-DM, which might suggest the implication of these phosphorylation sites in the fine-tune of their interactions with the GHSR1a. To gain further insight into this possibility, the BRET values of different acceptor/donor ratios was monitored (eYFP-receptor/Rluc-β-arrestin) to determine at which acceptor-donor ratio are observed the maximal BRET ($BRET_{max}$) and the half-maximal BRET ($BRET_{50}$) for each β-arrestin (β-arrestin 1 Figure 17A and β-arrestin 2 Figure 17B). $BRET_{50}$ values for GHSR1a-DM/β-arrestins interactions were higher than those for GHSR1a-WT, which advocate that the WT receptor has a higher relative affinity for both β-arrestins than the double mutant. However, the $BRET_{max}$ values were smaller for the GHSR1a-DM, which suggests that the nature of the receptor/β-arrestin interactions were different for both receptors; in such a way that the acceptor and donor tags were in closer proximity with the GHSR1a-WT.

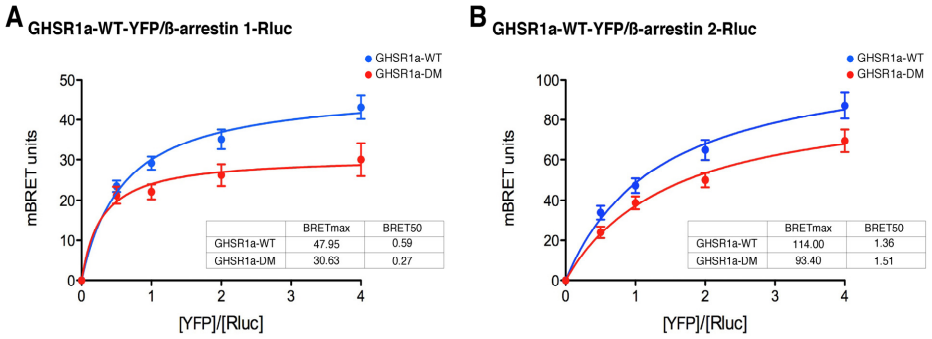


Figure 17. BRET curve acceptor/donor for WT and DM GHSR1a and β-arrestins A, Cells were transfected with a constant amount of β-arrestin 1 and increasing amounts of YFP-GHSR1a-WT or YFP-GHSR1a-DM (acceptors) and treated with ghrelin. Net BRET is graphed as a function of acceptor/donor. **B**, Titration curves monitoring net BRET in response to varying acceptor/donor ratios were performed as in A for β-arrestin 2. $BRET_{50}$ and BRETmax values calculated from three independent experiments.

All these results taken together indicate that the binding of β-arrestins with the phosphorylated C-tail of the GHSR1a involves two separate areas of interaction. The first interplay zone comprises the phosphorylation sites S^{362}, $S^{363}$and T^{366} that serve as essential phosphate recognition elements for the β-arrestin recruitment, and the second area contains the phosphorylation residues T^{350} and S^{349} that might stabilize active conformation of β-arrestins.

β-arrestin signaling is determined by the phosphorylation of the C-tail of the GHSR1a: ERK1/2 and Akt activation.

To determine the role of the two areas of phosphorylation founded in the GHSR1a C-tail (S^{362}, $S^{363}$and T^{366}, or S^{349} and T^{350}) the phosphorylation of ERK1/2 [pERK1/2(T202/Y204)] induced by ghrelin was analyzed in HEK293

cells transiently transfected with GHSR1a-WT, GHSR1a-DM, GHSR1a-TM or GHSR1a-Total. In GHSR1a-WT cells stimulated with ghrelin (100nM), pERK1/2(T202/Y204) was resolved into two components, mediated respectively, by G proteins or β-arrestins signalling, as was previously described[81]. Here the same was observed for the WT receptor, while the G protein-dependent activity was quick with a maximum peaking within ~5min, the β-arrestins-dependent ERK1/2 activation was slower in onset with a maximum peak ~20min, but sustained over time (Figure 18A). However, this sustained pERK1/2(T202/Y204) signal declined with C-terminal tail mutations GHSR1a-DM, GHSR1a-TM, and GHSR1a-Total keeping only the fast and transient G protein-dependent activation. The decrease in the ERK1/2 activation is consistent with the decline of the receptor to form complex with β-arrestins and subsequently act trough them (Figure 18A). Intriguingly, GHSR1a-Total had shown a slightly higher sustained ERK1/2 activation than the GHSR1a-TM despite had been exhibited less interaction with β-arrestins. To further study the role of the mutations in the sustained ERK1/2 activation we used MEF cells from wild-type mice (MEF WT), MEF cells from β-arrestin1 null mice (β-arrestin $1^{-/-}$) and MEF cells from β-arrestin 2 null mice (β-arrestin $2^{-/-}$). In the MEF WT cells, ghrelin led to the same pattern observed in HEK293 cells, where the GHSR1a-Total had shown higher sustained ERK1/2 activation than the GHSR1a-TM and even a little higher at 20min than the GHSR1a-DM (Figure 8B). In the MEF β-arrestin $2^{-/-}$ cells, there were no differences in the kinetic of the ERK1/2 activation among GHSR1a-WT, GHSR1a-DM, GHSR1a-TM and GHSR1a-Total in which the G protein-dependent pathway is the major mechanism (Figure 18D). Nonetheless, in the MEF β-arrestin $1^{-/-}$ the GHSR1a-Total had shown again a higher sustained ERK1/2 activation at 20min than the other receptors, suggesting that the lack of internalization allows the total mutant remain available to be activated by ghrelin in the plasma membrane indefinitely (Figure 18C). To clarify this hypothesis, a re-stimulation assay was performed with the GHSR1a-WT and the GHSR1a-Total mutant (Figure 18E). As was described in previous studies,

the maximum peak of internalization of the GHSR1a-WT is between 20 and 60min; moreover, after 2h of ghrelin treatment the GHSR1a starts to reappear in the membrane and the recycling is almost 100% after 3h[96]. For this reason, the ERK1/2 activation produced by the receptor WT and the total mutant was compared after re-stimulate with ghrelin 5min cells that was previously stimulated for 1h and 2h. While the phosphorylation of ERK1/2 at 5 and 65min was similar for both receptors, at 125min the phosphorylation induced by the total mutant was clearly higher, suggesting that the GHSR1a-Total could be reactivated by ghrelin because remains on the cell surface.

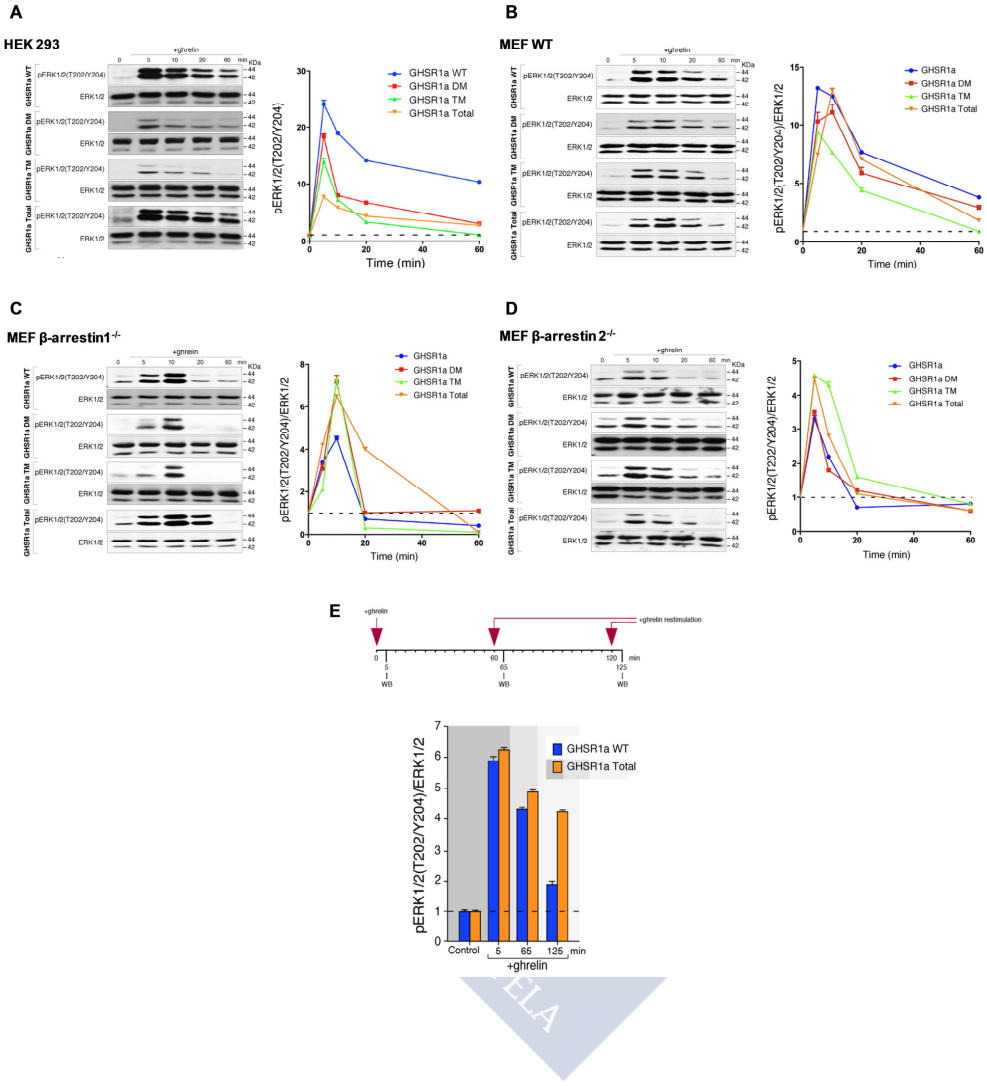


Figure 18. GHSR1a and mutants ERK1/2 activation. A-D, MEF WT, β-arrestin $1^{-/-}$, β-arrestin $2^{-/-}$ and HEK293 cells were transiently transfected with the indicated GHSR1a-WT or mutants and stimulated with ghrelin (100nM) for the indicated times. **E**, HEK293 cells were transiently transfected with the GHSR1a-WT or GHSR1a-Total, treated with ghrelin (100nM) for the indicated times and re-stimulated for 5min. **A-E,** The levels of pERK1/2(T202/Y204) were quantified by densitometry, normalized to total ERK1/2, and expressed as the fold change relative to the unstimulated cells. Results are representative of three independent experiments. The data are expressed as the mean ± SEM.

Going a step forward, to study the functional consequence of ERK1/2 activation associated to the C-terminal phosphor-acceptor sites of the GHSR1a, was determined the mitogenic activity associated to the GHSR1a mutants in HEK293 cells. Whereas the GHSR1a-WT cells treated with ghrelin 100nM incorporated BrdU at a ~2-fold over control, cells expressing GHSR1a-DM or GHSR1a-TM failed to incorporate BrdU and GHSR1a-Total exhibit a negligible incorporation (Figure 19A). The results obtained with the GHSR1a-TM suggest that ghrelin-induced proliferation is not dependent on the G protein mediated signaling. However, the lack of GHSR1a-DM mitogenic effect might be related to the spatial control over MAPKs that exerts the stabilization of the active conformation of β-arrestins. Accordingly, the subcellular location of pERK1/2(T202/Y204) was examined in HEK293 cells transfected with EGFP tagged GHSR1a-WT and GHSR1a-DM receptors upon ghrelin stimulation (100nM) by confocal microscopy. While, in cells expressing the double mutant, pERK1/2(T202/Y204) was mainly located in the cytoplasm after activation, in the cells expressing the WT receptor, pERK1/2(T202/Y204) was principally translocated into the nucleus (Figure 19B). As a result, the phosphor-acceptor residues located in the GHSR1a C-tail determine the latest cellular consequence of β-arrestins recruitment.

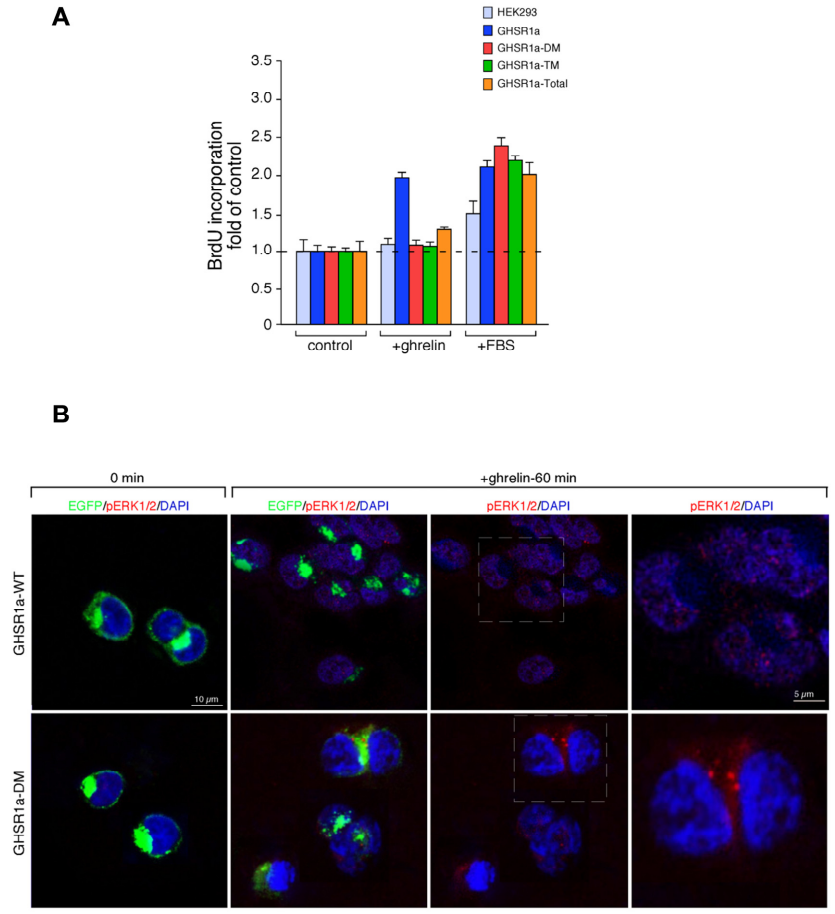


Figure 19. Functional relation between the GHSR1a-associated β-arrestin-scaffolded complex and the ERK1/2 activity. A, Mitogenic effect of ghrelin (100nM) on cells transiently transfected with the GHSR1a-WT or mutants (n=6). Results were expressed as a-fold of the BrdU incorporation relative to control cells. Results are representative of three independent experiments. The data are expressed as the mean ± SEM (*,$^{\#}$ $p < 0.05$). **B**, The HEK293 cells expressing GHSR1a-WT and GHSR1a-DM and stimulated with ghrelin for indicated time were stained for pERK1/2(T202/Y204) and images were taken by confocal microscopy. DAPI was used to identify nuclei. Confocal images are representative of three independent experiments.

The role of the five phosphor-acceptor residues founded on the GHSR1a was also study in the ghrelin-induced Akt activation. The model for the activation of Akt by ghrelin similarly to ERK1/2 activation, involves the interplay of an early $G_{i/o}$ protein-dependent pathway and a late pathway mediated by β-arrestins[82,98]. In fact, the GHSR1a-WT transiently transfected in HEK293 cells had shown after stimulation with ghrelin (100nM) a pAkt(S473) activation resolved into two components: a first G protein-dependent signaling with maximum activity at ~10 min, and a second part mediated by β-arrestins activation which was sustained over time (Figure 20A). This sustained pAkt(S473) signal decreased with C-tail mutations of GHSR1a-DM and GHSR1a-TM, reflecting the decline of the receptor/β-arrestin signaling complex formation, an effect confirmed in MEF WT , MEF β-arrestin $1^{-/-}$ and $2^{-/-}$ cells (Figure 20B-D). Notwithstanding, the GHSR1a-Total mutant had shown an Akt activation sustained in time comparable to that of GHSR1a-WT in HEK293, MEF WT and both β-arrestin knock out MEFs. According to what was observed in the activation of ERK1/2, the effect of the ghrelin re-stimulation on Akt phosphorylation (S473) was also studied (Figure 20E). While the phosphorylation of Akt at 5min was similar for both receptors, at 65min the activation induced by the total mutant is nearly double than that of WT and at 125min the phosphorylation induced by the total mutant is double than the control while GHSR1a-WT hardly induces Akt activation. All of these seem to confirm the idea of the GHSR1a-Total being reactivated by ghrelin due to its capacity of remain on the plasma membrane of the cell.

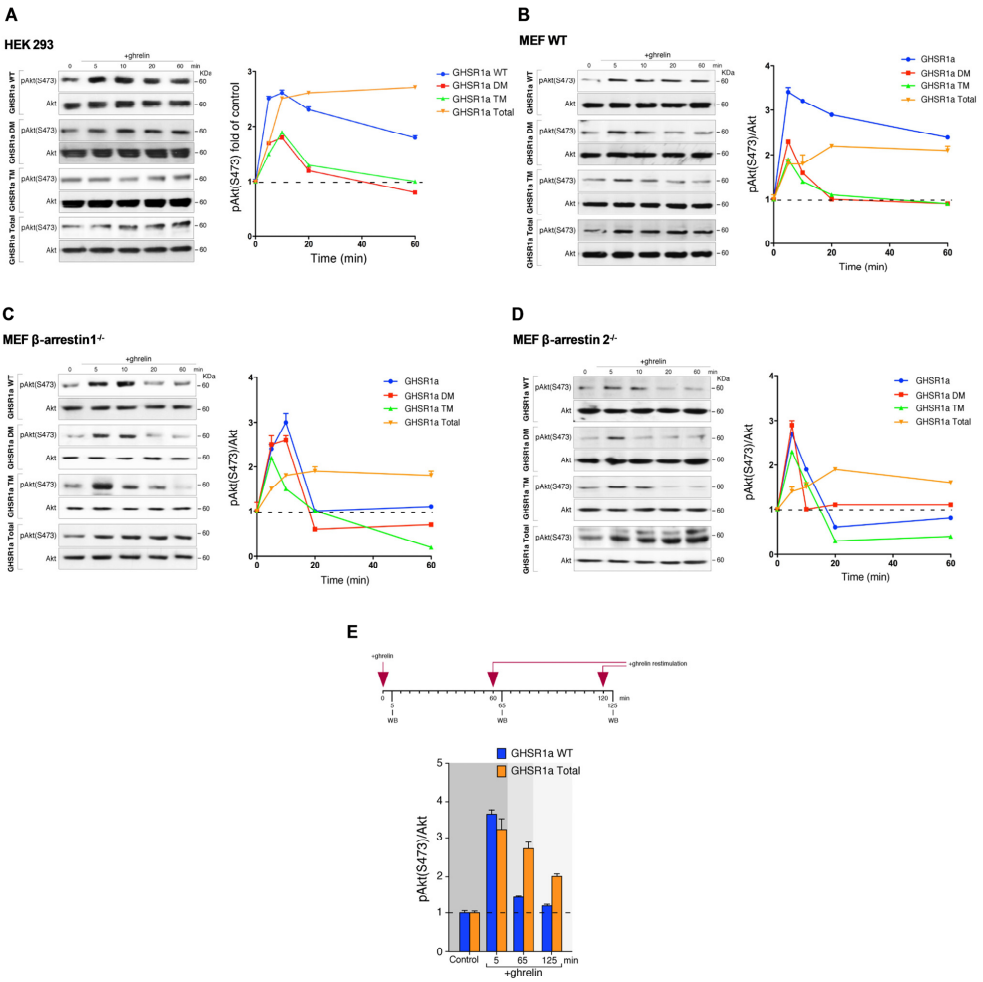


Figure 20. GHSR1a and mutants Akt activation. A-D, MEF WT, β-arrestin $1^{-/-}$, β-arrestin $2^{-/-}$ and HEK293 cells were transiently transfected with the indicated GHSR1a-WT or mutants and stimulated with ghrelin (100nM) for the indicated times. **E**, HEK293 cells were transiently transfected with the GHSR1a-WT or GHSR1a-Total, stimulated with ghrelin (100nM) for the indicated times and re-stimulated for 5min. **A-E,** The levels of pAkt(S473) were quantified by densitometry, normalized to total ERK1/2, and expressed as the fold change relative to the un-stimulated cells., Results are representative of three independent experiments. The data are expressed as the mean ± SEM.

To determine the importance of the GHSR1a C-tail in directing specific Akt signaling events, the effects of β-arrestins depletion by siRNA were examined on the intracellular lipid storage in 3T3-L1 cells mediated by ghrelin. This approach was necessary because both undifferentiated (preadipocytes) and differentiated 3T3-L1 (adipocytes) express GHSR1a endogenously, making it difficult discern the differences among GHSR1a mutants. Thus, using a standard adipogenic induction cocktail of IBMX, DEX and ghrelin, 3T3-L1 preadipocyte cells were induced to differentiate into adipocytes for 72h (early differentiation), followed by inhibition of β-arrestin 1 and 2 with specific siRNAs during terminal differentiation. At day 6 after the initiation of differentiation, Oil Red O staining was performed to monitor the intracellular lipid accumulation induced by ghrelin. Efficiency of β-arrestin 1 and 2 siRNA depletion was confirmed by immunoblot analysis after differentiation (65±5% and 69±2%, respectively). The depletion of β-arrestin 1 or 2 produced a substantial inhibition of the ghrelin-induced fat droplet storage in comparison to siRNA control (61±13% and 73±16%, respectively; Figure 21), which confirms that the β-arrestin signal complex determines the adipogenic functions of ghrelin and subsequently the importance of the phosphor-acceptor sites of the GHSR1a C-tail in the regulation of the ultimate signaling outcomes.

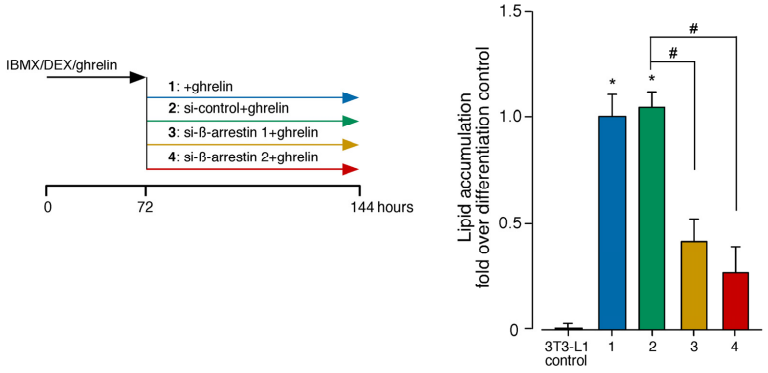


Figure 21. GHSR1a-mediated lipid accumulation associated to β-arrestin-scaffolded complex. Effect of siRNA depletion of β-arrestins on 3T3-L1 cells terminal adipogenesis. After the induction for 3 days under treatment with IBMX (0.5mM), DEX (25µM), and ghrelin (861nM) in DMEM/10% FBS, the cells were transfected with β-arrestin 1 or 2 siRNAs and then maintained for 3 days with ghrelin (172nM) in DMEM/10% FBS. The cells were stained with Oil red O and the lipid droplet accumulation were analyzed using the spectrophotometric absorbance at 520 nm. The results are expressed as the fold change of lipid accumulation relative to the differentiation control.

$G_{q/11}$ activity of ghrelin is not related to the phosphorylation of the C-tail of the GHSR1a.

Upon activation, GHSR1a carries information within the cell via a transient increase of intracellular Ca^{2+} through the generation of inositol 1,4,5-triphosphate (IP_3) triggered by protein subunit $G_{\alpha q/11}$[81]. Because the lifetime of IP_3 is extremely short, $G_{\alpha q/11}$-dependent GHSR1a activation can be followed by monitoring IP_3 degradation products, such as inositol 1-phosphate (IP_1), which accumulates in the cell in the presence of lithium chloride. The study of the IP_1 accumulation in response to ghrelin (100nM) performed in HEK293 cells transiently expressing GHSR1a-WT, GHSR1a-DM, GHSR1a-TM and GHSR1a-Total had revealed similar values among all the receptors (Figure 22A). On the other hand, it is also well established that

GHSR1a is able to stimulate GH release through intracellular Ca^{2+} concentration via IP_3. To further investigate the role of the GHSR1a phosphorylation sites and β-arrestin signaling, the effect siRNA knockdown of β-arrestins on the GH release in GC cells was tested. As with 3T3-L1 cells, this approach was was selected based on the stable GHSR1a expression in GC cells. The transfection of the cells with β-arrestin 1 or 2 siRNAs decreased the β-arrestin 1 and 2 expression by 50±1% and 80±3% respectively, but did not alter significantly the GH release induced by ghrelin in comparison to that in cells treated with control siRNA (Figure 22B). These results taken together demonstrate the independent contribution of G proteins and β-arrestins mediated pathways in the GHSR1a signaling. Thus, the GHSR1a display the capacity to tip the scale of functional selectivity in either direction: toward either β-arrestin or G-protein signaling.

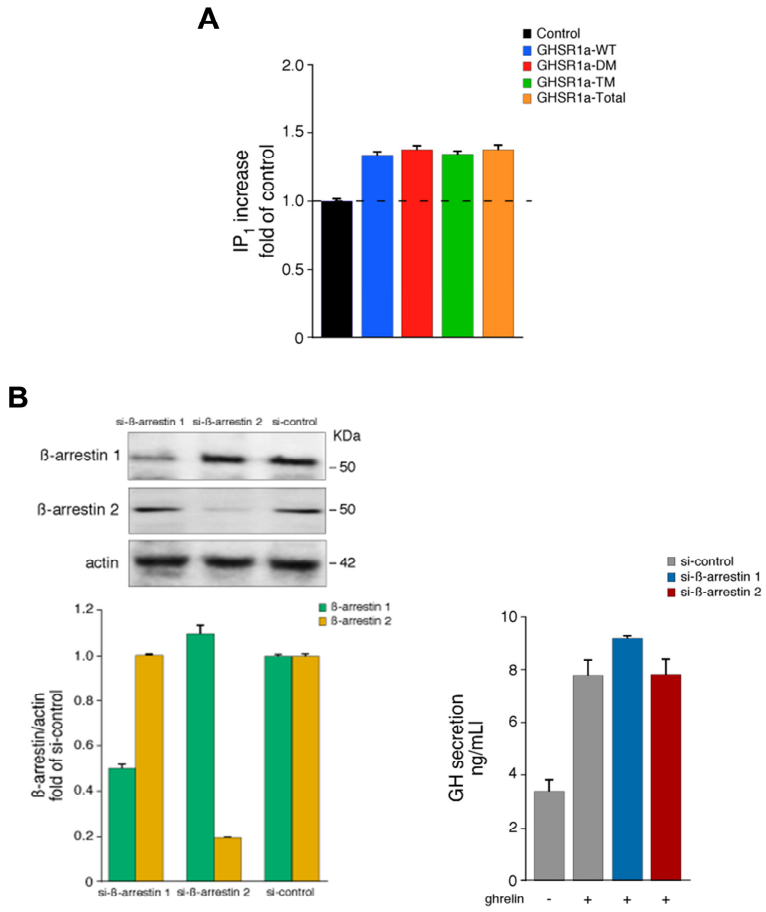


Figure 22. The functionality of $G_{q/11}$-protein mediated GHSR1a signaling is independent of receptor phosphorylation and β-arrestins binding. A, Determination of ghrelin-induced IP_1 accumulation in HEK293 cells transiently transfected with the GHSR1a-WT or mutants. The levels of IP_1 were expressed as the fold change relative to the unstimulated GHSR1a-WT cells. Data points correspond to means ± SEM of three experiments performed in triplicate. **B**, *Left panel*, The levels of β-arrestins and actin were quantified by densitometry, normalized to total actin, and expressed as fold relative to the siRNA control. Immunoblots are representative of three independent experiments. The data are expressed as the mean ± SEM (*, p< 0.05). *Right panel*, Analysis of the effect of ghrelin (100 nM) on the GH release in GC-GHSR1a cells in the absence or presence of β-arrestin siRNAs. Results are representative of three different experiments performed by triplicate.

CHAPTER 2

PKCs and GRKs: role in GHSR1a signaling

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Ghrelin induces PKCs activation through $G_{i/o}$ and $G_{q/11}$-dependent mechanism

Protein kinase C (PKC) is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of S and T amino acid residues. The PKC family is divided into three subfamilies, based on their second messenger requirements: conventional (or classical), novel, and atypical[170]. Conventional (c)PKCs contain the isoforms α, β, and γ. These PKCs depends on Ca^{2+}, diacylglycerol (DAG), and phospholipid such as phosphatidylserine for activation. Novel (n)PKCs include the δ, ε, η, and θ isoforms, and require DAG, but do not need Ca^{2+} for activation. Thus, conventional and novel PKCs are activated through the same signal transduction pathway as phospholipase C. On the other hand, atypical (a)PKCs (including protein kinase ζ and ι / λ isoforms) require neither Ca^{2+} nor DAG for activation[170]. Previous studies from our group demonstrated the activation of cPKC such as PKCα/β in the system ghrelin/GHSR1a[81] . To explore the activation of the rest of the PKCs induced by ghrelin through GHSR1a, the phosphorylation of the cPKC, PKCγ [pPKCγ (T514)]; the nPKCs, PKCε [pPKCε(S729)], PKCδ[pPKCδ(T507)], PKCη [pPKCη(T655)], PKCθ [pPKCθ(T538)]; and, the aPKC, PKCμ/PKD [pPKCμ(S916)], and PKCζ [pPKCζ(T410)], was evaluated in HEK293 cells stably expressing GHSR1a (HEK-GHSR1a). Maximum of pPKCε(S729) was observed at 5-10min post ghrelin stimulation (100nM). pPKCδ(T507), pPKCμ/PKD(S916), pPKCη(T655) and pPKCζ(T410) showed maximal phosphorylation at 10-20min of ghrelin activation, whereas pPKCθ(T538) increased during all the tested time. By contrast, PKCγ was not activated by ghrelin (Figure 23).

[170] Ron D, Kazanietz MG. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* 1999 Oct;13(13):1658-76.

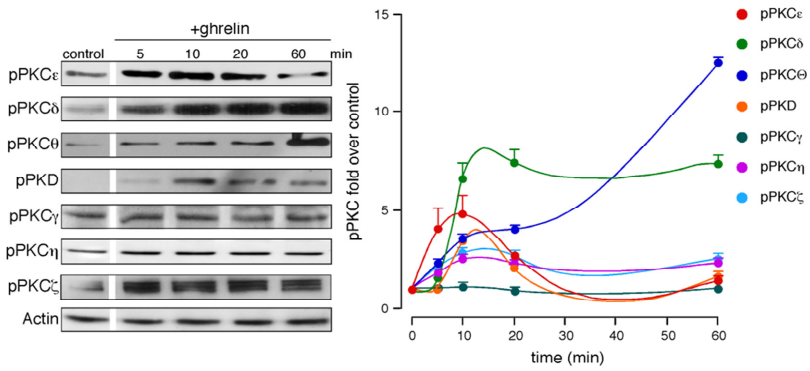

Figure 23. PKCs activation upon ghrelin stimulation. HEK-GHSR1a cells were stimulated with ghrelin (100nM) at the indicated times. The levels of pPKCγ(T514), pPKCε(S729), pPKCδ(T507), pPKCη(T655), pPKCθ(T538), pPKCμ(S916) and pPKCζ(T410), were quantified by densitometry, normalized to actin, and expressed as the fold change relative to the unstimulated cells. Immunoblots are representative of three independent experiments and the data are expressed as the mean ± SEM.

Because several signaling pathways are able to activate PKCs and these pathways are related to the action of G proteins, we first analyzed the implication of $G_{i/o}$ by PTX pretreatment (100ng/ml, 12h), which selectively inhibits the coupling of $G_{i/o}$ to the receptor. As shown in Figure 24A, ghrelin-induced activation of PKCδ, PKCθ, PKCμ and PKCζ was inhibited by PTX action. By contrast, PKCη phosphorylation was not affected by PTX. Moreover, the phosphoinositide-3-kinase (PI3K) is one of the intracellular targets of $G_{i/o}$ protein, which was described to be involved in the activation of PKCε[81]. For this reason, the effect of wortmannin, PI3K inhibitor was examined in the activation patterns of PKCs. After wortmanin pretreatment (1μM, 30min), PKCδ, PKCθ, PKCμ and PKCζ activation was abolished while PKCη activation was not affected (Figure 24B). Furthermore, the $G_{i/o}$/PI3K downstream signaling produces PIP3 and consequently allows the translocation to the membrane of phosphoinositide-dependent kinase-1 (PDK1), which triggers Akt

phosphorylation at T308 being involved in the activation of Src through PKCε signaling[98]. The cell transfection of PDK1 siRNA (~80% reduction related to siRNA control; Figure 24C) attenuated PKCδ, PKCθ, PKCμ, and PKCζ activation induced by ghrelin (100nM) whereas PKCη phosphorylation was not affected by PDK1 siRNA. Thus, the $G_{i/o}$/PI3K/PKD1 route regulates the PKCδ, PKCθ, PKCμ and PKCζ activation.

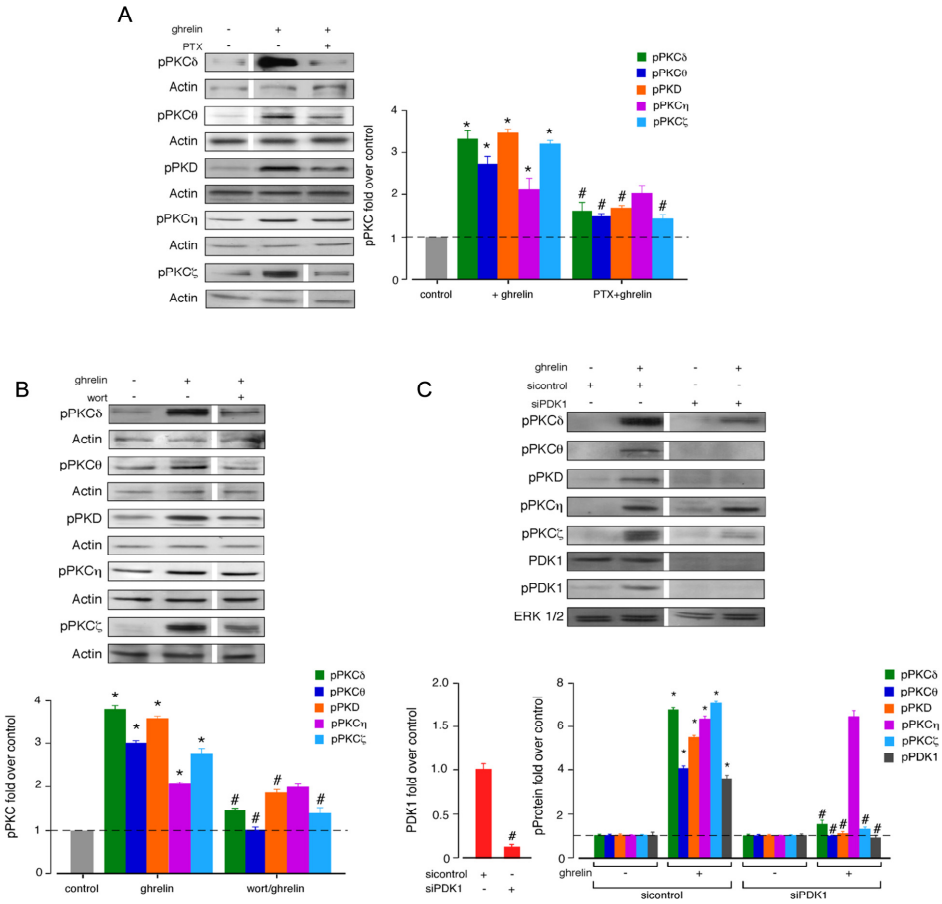

Figure 24. PKCs activation through $G_{i/o}$/PI3K route. A, HEK-GHSR1a cells were treated with PTX 100ng/ml 12h and stimulated 5min with ghrelin 100nM. **B**, HEK-GHSR1a cells were treated with wortmannin 1μM 30min and stimulated with ghrelin 100nM 5min. **A and B**, Levels of pPKCδ(T507), pPKCη(T655), pPKCθ(T538), pPKCμ(S916) , and pPKCζ(T410) were quantified and normalized to actin. **C**, HEK-GHSR1a cells were transfected with PDK1 or control siRNA and treated with ghrelin 100nM 5min. The levels of PDK1 and actin were quantified, normalized to actin, and expressed as fold relative to the siRNA control. The levels of pPKD1(S241), pPKCδ(T507), pPKCη(T655), pPKCθ(T538), pPKCμ(S916) ,pPKCζ(T410), were quantified by densitometry and normalized to actin. **A-C**, Immunoblots are representative of three independent experiments. The data are expressed as the mean of the fold change relative to the unstimulated cells ± SEM (*, $p < 0.05$).

To study if PKCη is acting trough $G_{q/11}$/PKCα pathway, the effect of PKCα knockdown by siRNA was explored. PKCα depletion by siRNA transfection caused a ~52% reduction and produced a similar attenuation of PKCη activation (~53%) (Figure 25). In addition, the phosphorylation of PKCμ was unaffected by PKCα knockdown. Human PKCμ was firstly catalogued as a PKC but deeper studies made it the founding member of a new family of S/T kinases known as protein kinase D (PKD) family and changed its name by PKD or PKD1[171]. It is believed that PKD is a direct substrate of PKCs and for this reason was study in both, $G_{i/o}$/PI3K route and $G_{q/11}$.

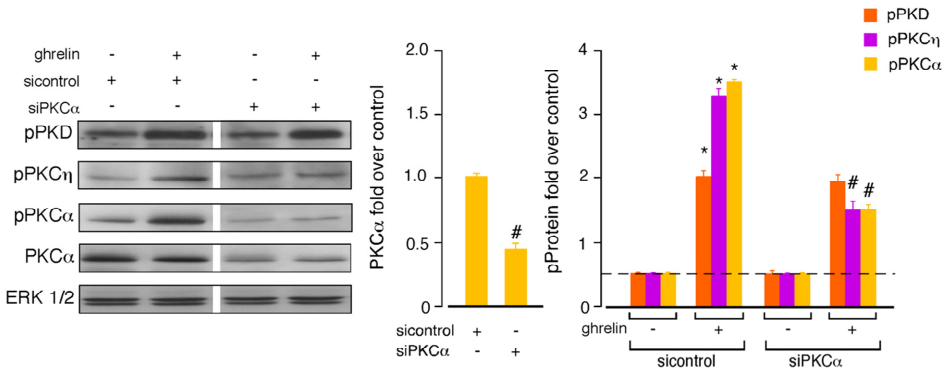

Figure 25. PKCs activation through $G_{q/11}$ route. HEK-GHSR1a cells were transfected with PKCα or control siRNA and treated with ghrelin 100nM 5min. The levels of PKCα and actin were quantified by densitometry, normalized to total ERK1/2, and expressed as fold relative to the siRNA control. The levels of pPKCα(T638/641), pPKCη(T655), and pPKCμ(S916), were quantified by densitometry and normalized to ERK1/2**.** Immunoblots are representative of three independent experiments. The data are expressed as the mean of the fold change relative to the unstimulated cells ± SEM (*, $p < 0.05$).

[171] Wang QJ.PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol Sci.* 2006 Jun;27(6):317-23.

To deeper analyze whose PKCs are activating PKD under ghrelin stimulation (100nM) several PCKs inhibitors were used. As would be expected, Gö6850, which inhibits all PKCs, reduced PKD phosphorylation. On the contrary, Gö6976, inhibitor of cPKCs (α/β and γ), did not attenuate PKD phosphorylation. Finally, treatment with Gö6983, inhibitor of cPKCs (α, β, and γ) and nPKCs (ε, δ, η and θ), decreased PKD activation but in lesser extent than Gö6850, suggesting that PKD is activated by atypical PKCs. Taken together all the results, PKCη activation is dependent on the activation of $G_{q/11}$/PKCα pathway whereas PKD is activated via $G_{i/o}$/PI3K by novel and atypical PKCs.

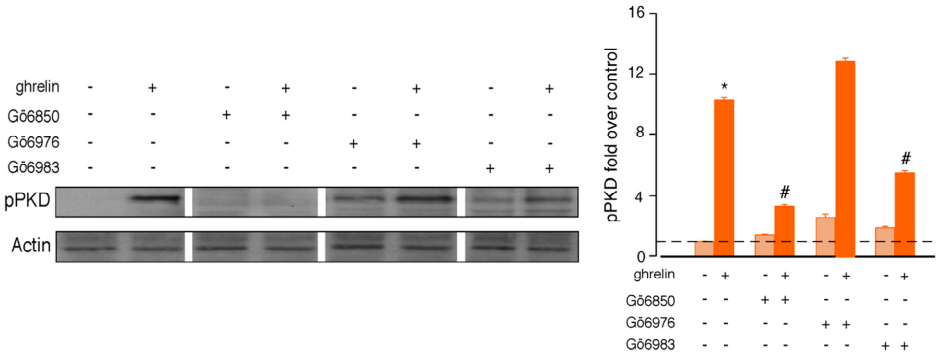

Figure 26. PKD activation mediated by PKCs. HEK-GHSR1a cells were treated with Gö6850, Gö6983 or Gö6976 100nM 30min and 5min with ghrelin (100nM). The levels of PKCα and actin were quantified by densitometry, normalized to total actin, and expressed as fold relative to the siRNA control. The levels of pPKCα(T638/641), pPKCη(T655), and pPKCμ(S916), were quantified by densitometry and normalized to actin**.** Immunoblots are representative of three independent experiments. The data are expressed as the mean of the fold change relative to the unstimulated cells ± SEM (*, $p < 0.05$).

β-arrestin binding to the phosphorylated GHSR1a form is mediated by PKCα/GRK2-dependent pathway

G protein-coupled receptor kinases (GRKs) play an important role in the desensitization of G protein-mediated signalling of GPCRs. GRK phosphorylation of the receptors leads to the subsequent binding of β-arrestins, which partially quenches receptor coupling to G proteins[172]. Each of the GRKs can potentially phosphorylate different S and T residues on a given receptor, and the phosphorylation pattern can be biased by the receptor conformation established by bound ligand. The diversity of the barcoding to flexible β-arrestin explains the multidimensional nature of signalling in the GPCR superfamily. GRKs share certain characteristics but are distinct enzymes with specific regulatory properties. GRK2, 3, 5 and 6 are ubiquitously expressed in mammalian tissues, whereas GRK1, 4 and 7 are confined to specific organs. GRK1 and 7 are expressed in retinal rods and cones, respectively, and GRK4 is present in testis, cerebellum and kidney[92,173]. Transfection of GRK siRNAs into HEK-GHSR1a cells reduced expression of the targeted GRK compared to either non-silencing or control siRNA-transfected cells. Besides, all siRNAs demonstrated marked isoform specificity depleting GRK levels by 60-80% (Figure 27A). The ability of GRKs to support ghrelin-induced phosphorylation of GHSR1a was estimated in cells transfected with siRNAs targeting the GRKs. Stimulation with ghrelin (100nM) for 5min led to prominent phosphorylation of the receptor, which was significantly reduced in cells transfected with GRK2 or GRK6 siRNAs (Figure 27B). After transfection with GRK3, or 5 siRNAs, no effect in receptor phosphorylation was observed upon ghrelin stimulation (100nM) (Figure 27B).

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[172] Ritter SL andHall RA. Fine-tuning of GPCR activity by receptor-interacting proteins.*Nature Rev*.2009Dec;10:819-830

[173] Walther C, Ferguson SS. Arrestins: role in the desensitization, sequestration, and vesicular trafficking of G protein-coupled receptors. *Prog Mol Biol Transl Sci*. 2013;118:93-113.

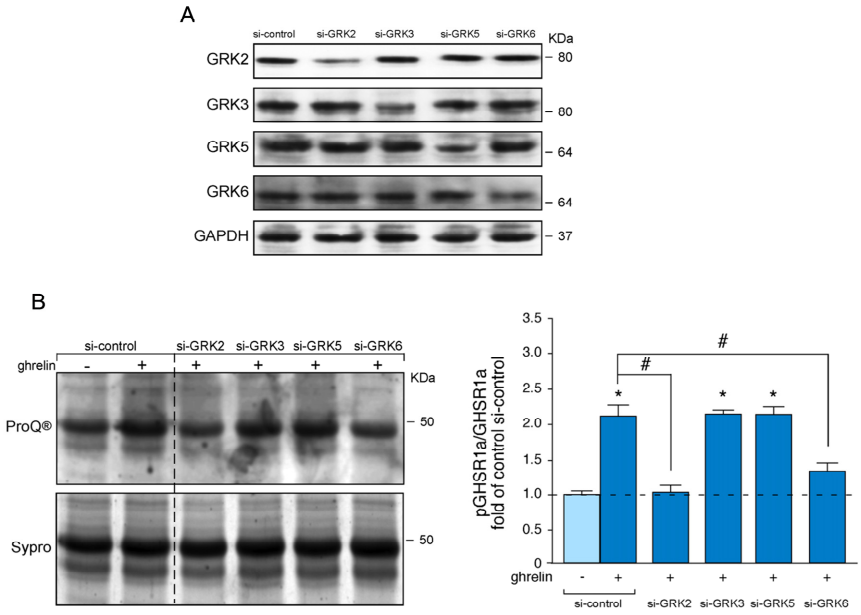

Figure 27. Analysis of GRKs implication on GHSR1a phosphorylation. A, HEK-GHSR1a cells were transfected with GRK2, 3, 5, 6 or control siRNA and treated with ghrelin 100nM 5min. The levels of GRKs and GAPDH were acquired by densitometry as silencing control. Immunoblots are representative of three independent experiments. **B**, *Left panel*, ProQ staining of phosphoproteins was performed in the same HEK293 cells transfected with GRK2, 3, 5, 6 or control siRNA. *Right panel,* levels of phosphorylation were quantified by densitometry, normalized to Sypro signal, and expressed as fold increase relative to the SiRNA control cells. Immunoblot is representative of three independent experiments. The data are expressed as the mean ± SEM (*,$^{\#}$ $p < 0.05$).

Next, the effect of CRISPR suppression of GRK2 expression was further examined on ghrelin-promoted phosphorylation in HEK293 cells expressing GHSR1a-WT or mutants tagged with HA. Phosphorylation of GHSR1a-WT or GHSR1a-DM was significantly reduced in cells transfected with GRK2-CRISPR transfected cells compared to GRK2 control cells. On the contrary, GHSR1a-TM cells were not affected by GRK2 KO-CRISPR transfection (Figure 28). This result suggests the implication of GRK2 in the phosphorylation of the S^{362}, $S^{363}$and T^{366} residues.

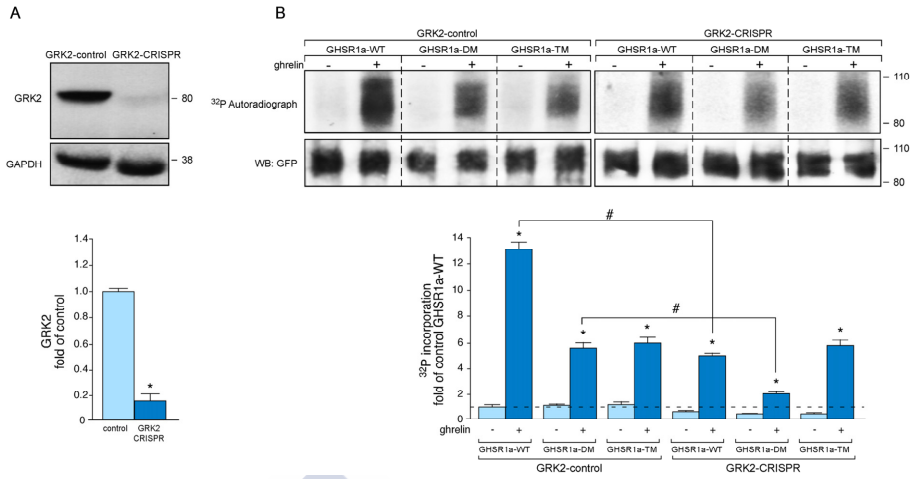

Figure 28. Detailed analysis of GRK2 implication on GHSR1a phosphorylation. A, HEK293 cells stably expressing the GHSR1a tagged with HA in the presence or not of the GRK2 KO CRISPR/Cas9 plasmid were used as control of GRK2 inhibition. Immunoblot is representative of three independent experiments. The data are expressed as the mean ± SEM (*,# $p < 0.05$). **B**, Upper *panel*, ^{32}P labeling of HEK293 cells stably expressing the GHSR1a or its mutants tagged with HA in the presence or not of the GRK2 KO CRISPR/Cas9 plasmid. *Bottom panel,* levels of ^{32}P were quantified by densitometry, normalized to GHSR1a-HA, and expressed as fold increase relative to the control cells expressing the GHSR1a-WT. Immunoblot is representative of three independent experiments. The data are expressed as the mean ± SEM (*,# $p < 0.05$)

To test the role of GRK2 in GHSR1a ghrelin-mediated signaling, the effect of CRISPR-mediated GRK2 depletion (~80%, Figure 29A) was measured on ERK1/2 and Akt activation. In ghrelin-stimulated HEK-GHSR1a control cells, ERK1/2 activation reached maximal levels rapidly and remained stable for up to 10-20min (Figure 29A). By contrast, ghrelin stimulation (100nM) of GRK2 KO-CRISPR-transfected cells caused a rapid and transient ERK activation compared to control cells and the loss of the persistent phase of the signal, previously shown to be β-arrestins-

mediated. Similarly, after depletion of GRK2 the rapid and transient Akt activation was observed, whereas β-arrestin-dependent persistent phase was missed (Figure 29B). Taken together, these results suggest that GRK2 empower β-arrestins-mediated signaling to ERK1/2 and Akt.

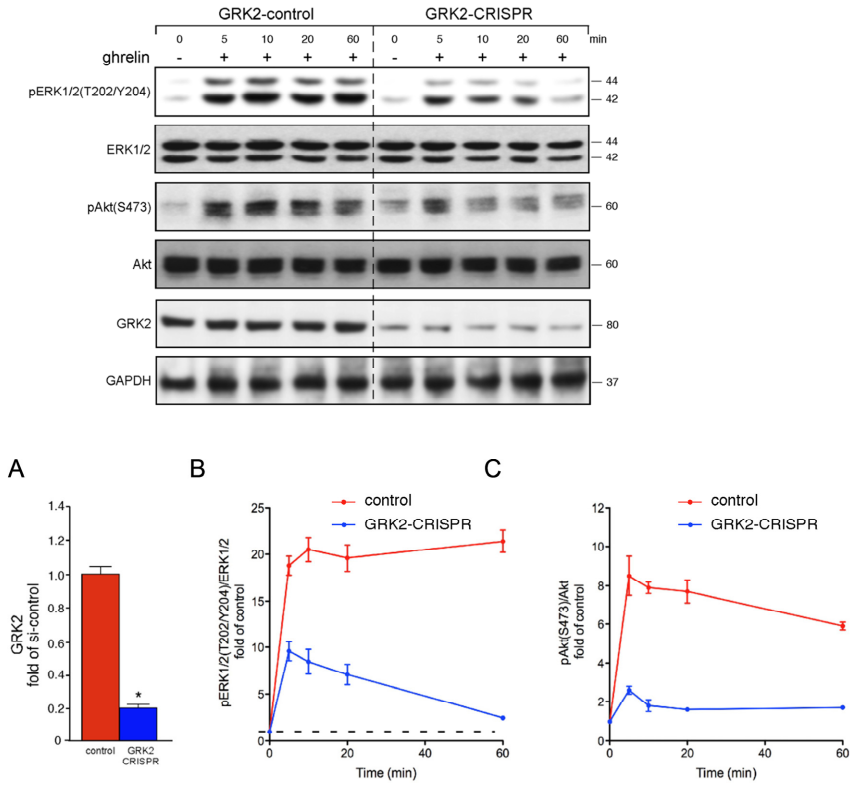

Figure 29. GRK2 effect on ERK1/2 and Akt activation. HEK-GHSR1a cells were transiently transfected with GRK2 KO CRISPR/Cas9 plasmid and stimulated with ghrelin (100nM) for the indicated times. **A**, Effect of GRK2 KO CRISPR depletion. Levels of GRK2 were quantified by densitometry and normalized to GAPDH **B**, The levels of pERK1/2(T202/Y204) were quantified by densitometry and normalized to total ERK1/2. Results are expressed as the fold change relative to the unstimulated cells **C**, The levels of pAkt(S473) were quantified by densitometry and normalized to total Akt. Results are expressed as the fold change relative to the unstimulated cells **A-C**, Immunoblots are representative of three independent experiments. The data are expressed as the mean ± SEM.

Previous studies described that β-arrestins signaling is dependent on the G_q and $G_{i/o}$ protein activation[81,82], which advocates that certain key components of the G protein-dependent routes are required to determine β-arrestins recruitment and signaling. This prompted us to speculate about the implication of GRK2 and/or second messenger kinases like PKCs as the missing link between G proteins and β-arrestins. Firstly, was tested whether $G_{i/o}$ protein inactivation by PTX alters GRK2 signaling from GHSR1a. Upon ghrelin stimulation of control cells, GRK2 phosphorylation at S670 reached maximal levels rapidly and decreased for up to 10min very slowly (Figure 30B). By contrast, in PTX-treated cells, ghrelin action caused a similar kinetic pattern with the persistent phase lost. As would be expected, PKCα activation was not affected by PTX treatment as it is dependent of G_q. After inactivation of $G_{i/o}$, only very rapid and transient ERK1/2 activation was observed (Figure 30C). Moreover, $G_{i/o}$ inactivation caused a similar kinetic pattern of Akt activation, but with elevated levels of S473 phosphorylation, compared to control cells (Figure 30D). These results ruled out the implication of $G_{i/o}$ -dependent signaling and pointed the implication of G_q-dependent signaling on the early GRK2 phosphorylation.

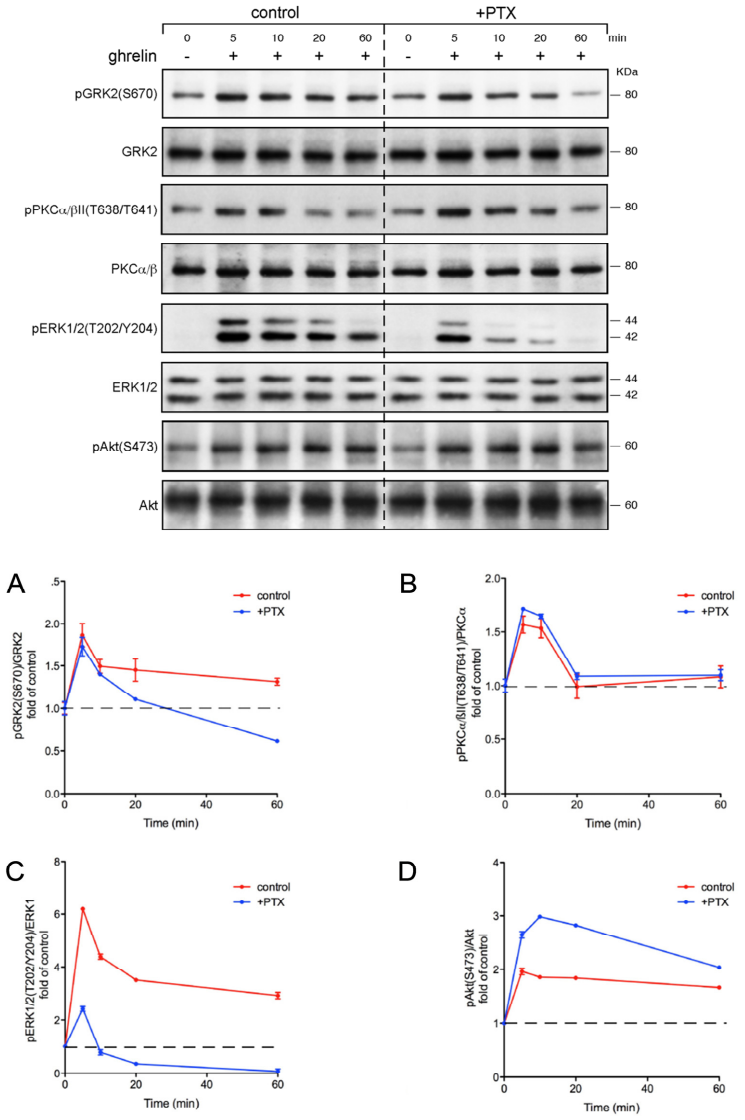

Figure 30. $G_{i/o}$-dependent signaling. HEK-GHSR1a cells were treated with PTX 100ng/ml 12h and stimulated with ghrelin (100nM) the indicated times. **A**, The levels of pPKCα (T638/641), were quantified and normalized to total PKCα**. B,** The levels of pGRK2 (S670) were quantified and normalized to total GRK2. **C**, The levels of pERK1/2(T202/Y204) were quantified and normalized to total ERK1/2. **D**, The levels of pAkt(S473) were quantified and normalized to total Akt. **A-D**, Immunoblots are representative of three independent experiments. The data are expressed as the mean of the fold change relative to the unstimulated cells ± SEM (*, $p < 0.05$).

On the other hand, GRK2 has also been shown to be substrate for PKCs[174,175]. Considering the ghrelin-induced-PKC activation, it is possible to speculate about the role of PKCα/β and/or PKCε as GRK2 phosphorylation mediators. However, the PKCε dependence of $G_{i/o}$-protein activation ruled out this PKC and pointed directly to PKCα/β as upstream target for GRK2 phosphorylation. Indeed, PKCα depletion by siRNA transfection (~70%; Figure 31A) caused a decreased in ghrelin-induced GRK2 S670 phosphorylation (Figure 31B). Furthermore, after inhibition of PKCα, only very rapid and transient ERK1/2 and Akt activation was observed under ghrelin stimulation (100nM) (Figure 31C and 31D).

[174] Chuang TT, LeVine H, De Blasi A. Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. J Biol Chem 1995; 270: 18660–18665

[175] Krasel C, Dammeier S, Winstel R, Brockmann J, Mischak H, Lohse MJ. Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. J Biol Chem. 2001 Jan 19;276(3):1911-5

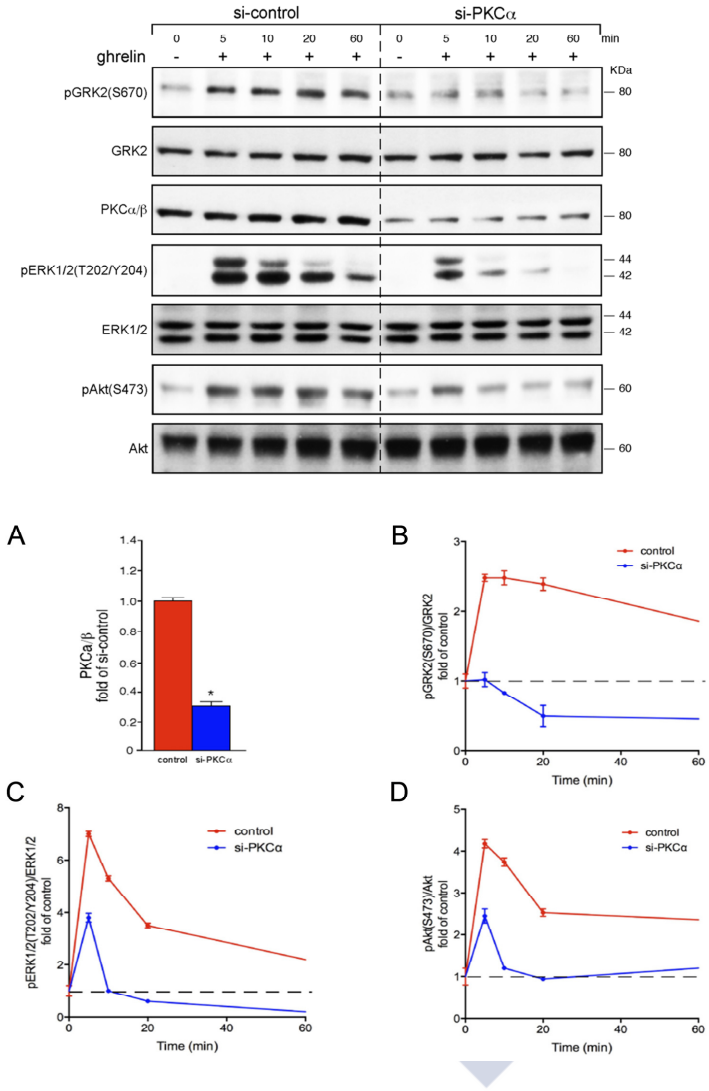

Figure 31. PKCα mediated GRK2 phosphorylation and associated ERK1/2 and Akt activity. **A**, HEK-GHSR1a cells were transfected with PKCα SiRNA and stimulated with ghrelin (100nM) the indicated times. The levels of PKCα were quantified by densitometry, normalized to total actin, and expressed as fold relative to the siRNA control. **B**, The levels of pGRK2(S670), were quantified and normalized to total GRK2. **C**, The levels of pERK1/2(T202/Y204), were quantified and normalized to total ERK1/2. **D**, The levels of pAkt (S473) were quantified by densitometry and normalized to total Akt. **A-D,** Immunoblots are representative of three independent experiments. The data are expressed as the mean of the fold change relative to the unstimulated cells ± SEM (*, $p < 0.05$).

CHAPTER 3

Determination of active conformations of GHSR1a

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Effect of ghrelin truncations in GHSR1a signaling

To determine the key structural elements of ghrelin which ensure its bioactivity, three different ghrelin truncations was used to evaluate the GHSR1a signaling: COOH-GSS(n-octanoyl)FL-NH_2 (1-5 ghrelin), COOH-GSS-(n-octanoyl)-FLSPEHQRVQQ-NH_2 (1-14 ghrelin) and, COOH-GSS-(n-octanoyl)-FLSPEHQRVQQRKES-NH_2 (1-18 ghrelin) (Figure 1A). First, the capacity to phosphorylate GHSR1a was tested by ^{32}P incorporation under ghrelin or truncates stimulation (100nM, 5min) in HEK-GHSR1a cells. The radiactive labeling of the immunoprecipitate receptor showed that 1-5 ghrelin induced phosphorylation level comparable to that of ghrelin, while 1-14 and 1-18 ghrelin showed significant decrease on its ability to phosphorylate GHSR1a (~60% and ~37% reduction of ghrelin signal, respectively; Figure 32B).

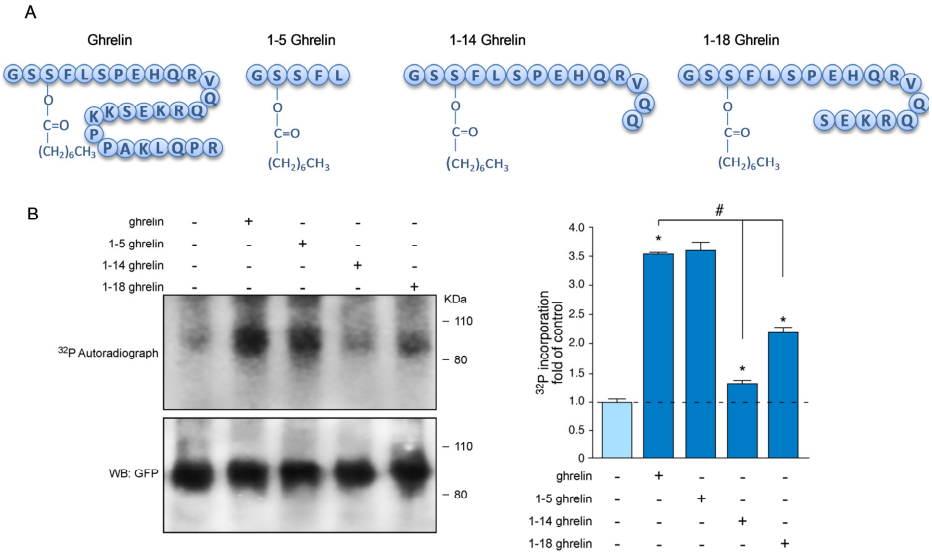

Figure 32. Ghrelin truncates phosphorylation. **A**, Illustration of the ghrelin truncations. **B**, *Left panel*, HEK-GHSR1a cells were stimulated 5min with ghrelin or the corresponding truncate (100nM) and ^{32}P labeled. *Right panel,* levels of ^{32}P were quantified by densitometry, normalized to GHSR1a-EGFP, and expressed as fold increase relative to the control cells unstimulated. Immunoblot is representative of three independent experiments. The data are expressed as the mean ± SEM (*,$^{\#}$ $p < 0.05$).

Following, the effect of ghrelin truncates on receptor endocytosis was examined. Internalization of GHSR1a tagged with EGFP was analyzed 20 and 60min after agonist treatment. Consistent with previous results, the fluorescence associated with the GHSR1a in the resting cells, was predominantly localised to the plasma membrane while exposure to ghrelin for 20 and 60min caused the redistribution of this fluorescence into a population of intracellular vesicles spread throughout the cytoplasm (Figure 33A). In the case of 1-5 ghrelin, at 20min intracellular vesicles were shown disseminated all over the cytoplasm although part of the fluorescence associated with the GHSR1a remained in the plasma membrane of the cells suggesting a reduction in GHSR1a-EGFP internalization. At longer times of stimulation (60min) with 1-5 ghrelin, the extent of receptor internalization increased although the fluorescence associated to the plasma membrane persisted (Figure 33B). Stimulation with 1-14 ghrelin exhibited a diffuse distribution of fluorescence in the cytoplasm but close to plasma membrane at 20min. The diffuse distribution was maintained at longer times of stimulation (60min) but with reduction in the number of intracellular vesicles compared to ghrelin (Figure 33C). Internalization of GHSR1a induced by 1-18 ghrelin also showed a cytoplasmic diffuse distribution of the GHSR1a-associated fluorescence at 20min. However, the extent of intracellular vesicles increased at 60min, indicating slow rate of GHSR1a internalization (Figure 33D).

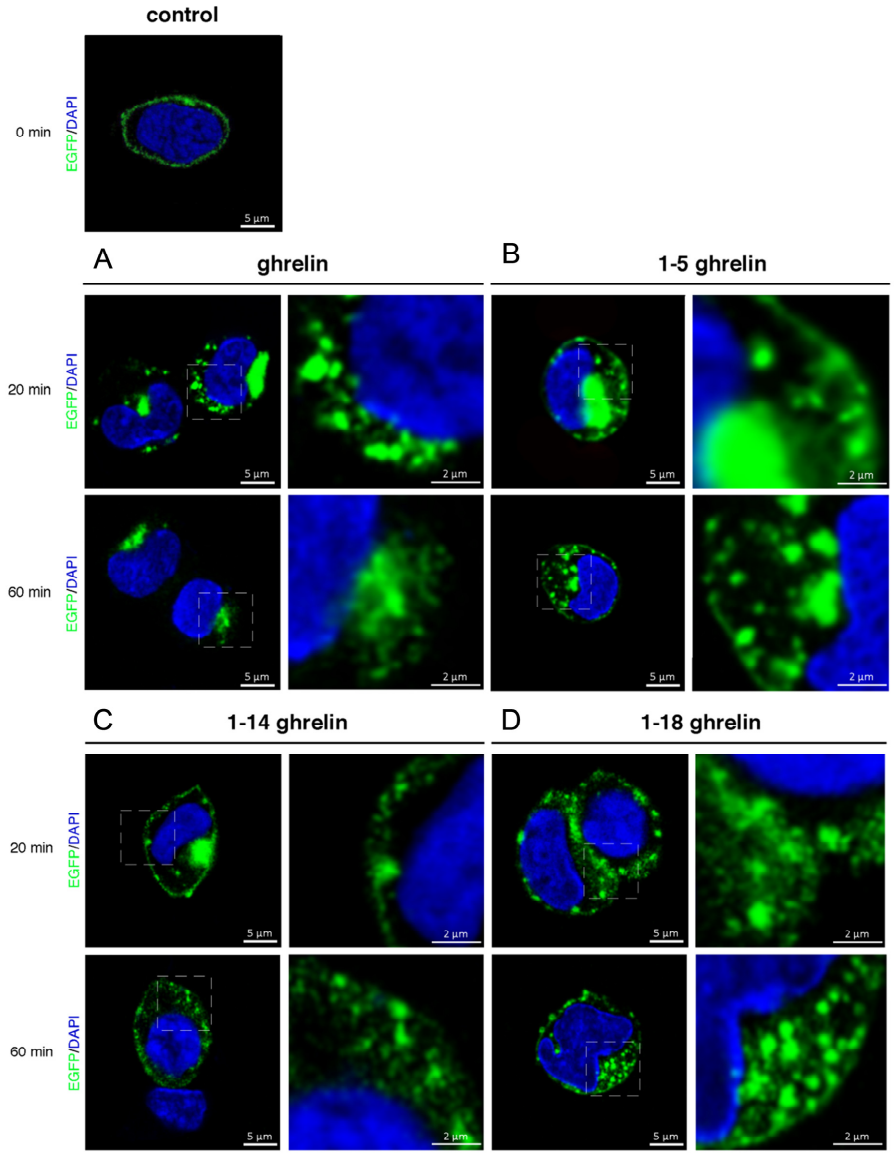

Figure 33. Endocytosis time course of the EGFP-tagged GHSR1a mediated by ghrelin truncates. HEK-GHSR1a cells after stimulation with ghrelin or the corresponding truncate (100nM) for different times at 37°C were analyzed by confocal microscopy. Confocal images are representative of three independent experiments.

Going a step forward, the effect of ghrelin truncates on ERK1/2 and Akt signaling from the activated GHSR1a was tested (Figure 34). 1-5 ghrelin stimulation (100 nM) caused a similar kinetic pattern of ERK1/2 activation than stimulation with ghrelin (100nM), but with elevated levels of activation of ERK1/2. A slow rate of Akt activation compared to control ghrelin was observed maintaining the transient and persistent phases (Figure 34). Stimulation with 1-14 ghrelin (100nM) showed to modify the rapid and transient ERK1/2 activation. This effect was more pronounced in Akt in which the transient Akt activation was lost. By contrast, 1-18 ghrelin (100nM) caused a similar kinetic pattern of ERK1/2 and Akt activation, but with decreased levels compared to ghrelin (Figure 34).

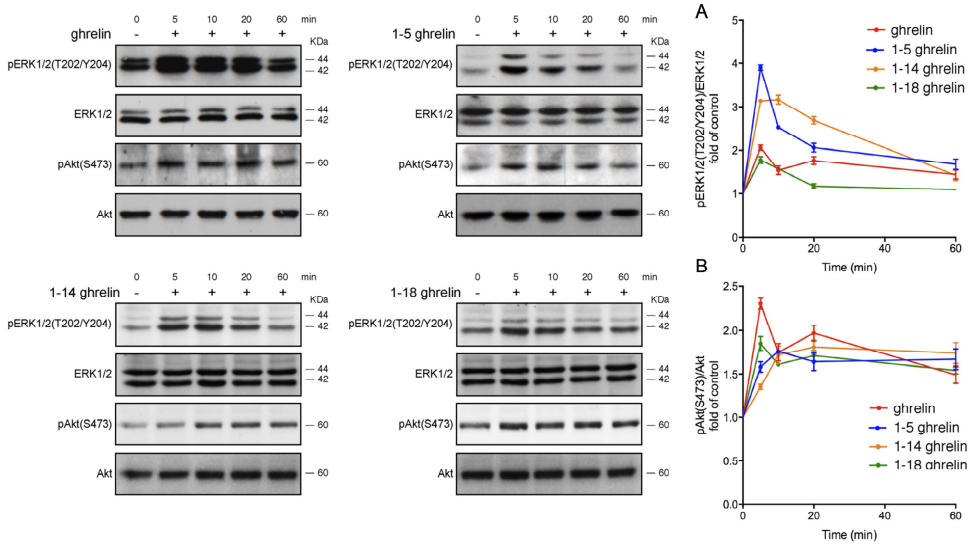

Figure 34. Ghrelin truncates action on ERK1/2 and Akt activation. The HEK-GHSR1a cells were stimulated with ghrelin or the corresponding truncate (100 nM) for the indicated times. **A**, The levels of pERK1/2(T202/Y204) were quantified by densitometry, normalized to total ERK1/2, and expressed as the fold change relative to the unstimulated cells. **B**, The levels of pAkt(S473) were quantified by densitometry, normalized to total Akt, and expressed as the fold change relative to the un-stimulated cells. **A and B**, Immunoblots are representative of three independent experiments. The data are expressed as the mean ± SEM.

To further examine the action of ghrelin truncates on GHSR1a-associated signaling, the mitogenic and adipogenic outcome was measured by BrdU incorporation and Oil red staining respectively. Figure 35A shows that the proliferative effect after 1-5 ghrelin stimulation (100nM) was similar to that observed in ghrelin-stimulated HEK-GHSR1a cells. By contrast, the mitogenic capacity associated to GHSR1a was significantly reduced when cells where stimulated with 1-14 or 1-18 ghrelin (100nM). However, similar effect on lipid droplet accumulation during adipogenesis was shown for all the ghrelin truncates tested in comparison to that observed for ghrelin in 3T3-L1 cells.

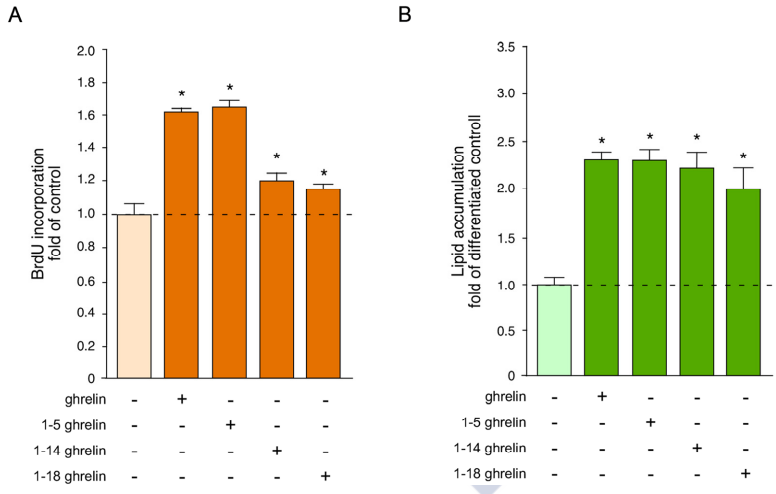

Figure 35. Functional consequences of ghrelin truncates-mediated activation of ERK1/2 and Akt. A, Mitogenic effect of ghrelin or the corresponding truncate (100nM) on HEK-GHSR1a (n=6). Results were expressed as a-fold of the BrdU incorporation relative to control cells. The data are expressed as the mean ± SEM (*,# $p < 0.05$). **B**, Effect of ghrelin or the corresponding truncate (100nM) on terminal adipogenesis in 3T3-L1 cells. The cells were stained with Oil red O and the lipid droplet accumulation were analyzed using the spectrophotometric absorbance at 520 nm. The results are expressed as the fold change of lipid accumulation relative to the differentiation control.

Finally, was also tested the consequences of stimulation with ghrelin truncates on GHSR1a-induced GH secretion in GC-GHSR1a cells by ELISA. Stimulation with 1-14 or 1-18 ghrelin (100nM) enhanced the GH secretion to similar levels than those of ghrelin, while the activation with 1-5 ghrelin (100nM) failed to induce GH secretion (Figure 36).

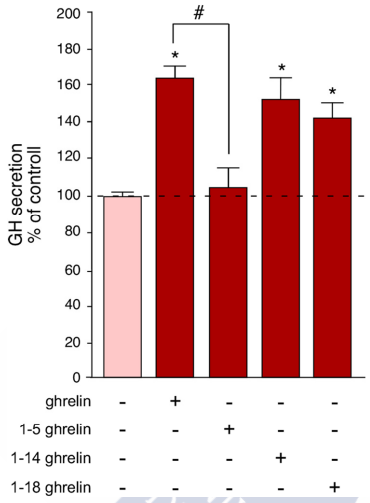

Figure 36. GH secretion mediated by ghrelin truncates. Analysis of the effect of ghrelin or the corresponding truncate (100nM) on the GH release in GC-GHSR1a cells. Data are from three independent experiments and were performed in quadruplicate. The data are expressed as mean of a-fold over unstimulated control ± SEM (*, $p < 0.05$).

DISCUSSION

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CHAPTER 1. GHSR1a phosphorylation.

The interplay of the classic G protein-mediated signaling and β-arrestin-dependent pathways largely determines the cellular consequences of the GHSR1a activation. One of the most important post-translational modifications mediated by agonist stimulation is the phosphorylation of the GPCRs. The covalent modifications produced on the receptor mediates its desensitization as well as provides a mechanism by which receptors can engage with β-arrestins and trigger specific downstream signaling pathways[83,84]. However, until now the phosphorylation status of the GHSR1a remained to be elucidated.

The map of the specific phosphorylation sites of the GHSR1a obtained by mass spectrometry-based proteomic approach allowed the elucidation of the functional capabilities imparted by these specific phosphorylation events on the β-arrestin signaling. While the phosphor-acceptors S^{362}, $S^{363}$and T^{366} residues were primarily responsible for β-arrestin 1 and 2 binding, receptor internalization and β-arrestin-induced ERK1/2 and Akt activation, the T^{350} and S^{349} sites stabilized the interaction between the GHSR1a and β-arrestins, and as a whole contributed to desensitization. These results are consistent with a model in which the phosphor-acceptor residues of the GHSR1a C-tail are able to lead to distinct patterns of phosphorylation and consequently induce different β-arrestins interactions and determine the ultimate cellular consequences of β-arrestins-induced pathways[91,92]. The obtained data support the hypothesis of receptor phosphorylation barcode, whereby the distinct pattern of phosphorylation triggers defined downstream signaling and are further consistent with the concept that the C-terminal phosphorylation pattern establishes the subtle differences in β-arrestins conformation that expose distinct sets of binding partner sites determining the downstream signaling events associated to β-arrestins functions.

The failure to bind β-arrestins and internalize exhibited by the GHSR1a when the S^{362}, S^{363} and T^{366} residues were deleted (GHSR1a-TM and GHSR1a-Total), corroborates the classic model for GPCR signaling which predicts that receptor phosphorylation at multiple intracellular S and T positions creates binding sites for β-arrestins. By contrast, deletion of T^{350} and S^{349} of the GHSR1a C terminus (GHSR1a-DM) demonstrated β-arrestins recruitment and receptor internalization, ruling out any implication for switching-in the β-arrestins binding. These results suggest that the most terminal phosphor-acceptor sites S^{362}, S^{363} and T^{366} of the GHSR1a confer crucial high-affinity binding for β-arrestins recruitment and also facilitate receptor internalization coordinating protein-protein interactions. This finding supports the notion of a bulk negative charge on the C-tail responsible for driving receptor/β-arrestins interactions. Despite the phosphorylation of these three sites (S^{362}, S^{363} and T^{366}) can promote β-arrestins interaction with the GHSR1a, it is the phosphorylation distal to T^{350} and S^{349} residues that is required to determine the ultimate outcome of receptor internalization and β-arrestin signaling. Loss of the phosphorylation sites T^{350} and S^{349} modified the internalization partner from a typical population of intracellular vesicles to redistribution into a diffuse cytoplasmic granular pattern. Besides, a deeper study using BRET to closely analyze real-time interplay of β-arrestins with the GHSR1a-DM, revealed that β-arrestin 1 and 2 are both recruited to this mutant receptor but with a lower apparent affinity, corroborating that while S^{362}, S^{363} and T^{366} phosphorylation can promote β-arrestins recruitment with the GHSR1a, distal phosphorylation of T^{350} and S^{349} residues is required to stabilize the receptor/β-arrestins interactions. Finally, the fact that GHSR1a-DM had not shown the capacity of translocate ERK1/2 to the nucleus and induce cell proliferation, hallmarks of β-arrestins-mediated downstream signaling of the GHSR1a, further support the observation that β-arrestins action is dependent on the receptor's phosphorylation pattern or 'barcode'[81]. Although there are studies indicating that differences in β-arrestins signaling are dependent upon the stability of

the complex formed[176,177,178], it is possible to speculate that differences in stability contribute or affect to the binding domains on β-arrestins that are exposed, resulting in association of certain putative binding partners and exclusion of others[91,179,180]. Recent studies using a β-arrestins biosensor to detect gross changes in conformation suggest phosphorylation of the different sets of sites engenders the distinct functionality of β-arrestins by inducing different conformations of the receptor-bound β-arrestins[91,179]. These findings are consistent with a model where the patterning of receptor phosphorylation sites establishes a code that determines the conformation of the bound β-arrestins and, subsequently, its functional capabilities. Thus, differences in β-arrestins binding in response to recruitment to GHSR1a *versus* GHSR1a-DM might underlie the differences in signaling we observed.

Upon stimulation a GPCR is activated and β-arrestins are mobilized from its dimeric or tetrameric storage in its basal conformation. The proposed model for the interaction between both involves a biphasic mechanism which starts with the connection of the phosphorylated C-tail of the receptor and the N-terminal domain of β-arrestins. In the next step, the

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insertion of the finger loop of β-arrestins within the receptor core brings additional binding sites into action consequently inducing additional conformational changes to yield a high affinity receptor/ β-arrestins complex[73,181,182]. Following this complex formation, the phosphor-acceptor residues further act in agreement with other structural elements of the intracellular core of the receptor. Indeed, recent studies have shown that P^{148} and L^{149} amino acids located in the second intracellular loop of GHSR1a generate receptors with a strong bias to G protein and β-arrestins, respectively, supporting a role for conformation-dependent signaling bias in the wild-type receptor[93]. Thus, the nature of the active conformation of β-arrestins and the signaling outcome is determined by the complex ensemble of the GHSR1a phosphorylation sites within the C-tail in combination with structural elements within intracellular loops that confer the functional selectivity.

An interesting aspect is the functional selectivity associated to the differential GHSR1a-stimulated G protein- and β-arrestin-mediated signaling to control particular cellular response. The β-arrestin knockdown results revealed that the GH-releasing activity results from G protein signaling with no implication of β-arrestin signaling because this action occurs upon activation of β-arrestin-impaired GHSR1a. With the mutation of phosphor-acceptor sites, the proliferative effect of ghrelin was impaired indicating the implication of β-arrestins-mediated ERK1/2 pathway. Furthermore, the β-arrestins-scaffolded complex positively determines Akt activity and adipocyte differentiation. The implication of these scaffolding proteins during ghrelin-

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induced adipogenesis in 3T3-L1 cells was previously demonstrated. β-arrestins determined the adipocyte differentiation and the expression levels of master regulators of early, the CCAAT/enhancer-binding protein β (C/EBPβ) and the CCAAT/enhancer-binding protein δ (C/EBPδ), and terminal, the peroxisome proliferator-activated receptor (PPARγ) and the CCAAT/enhancer-binding protein α (C/EBPα), adipogenesis[137]. Initially, these findings imply the existence of independent G protein- and β-arrestins-mediated pathways. However, this is not the case at all, especially if we take into consideration the fact that β-arrestin signaling is dependent on the $G_{i/0}$ protein activation[81,82]. This fact suggests that certain key components of the $G_{i/0}$ protein-dependent signaling pathways are required to determine β-arrestins recruitment and signaling. This prompted us to speculate about the implication of GRKs or second messenger kinases, i.e. PKCs, on the receptor phosphorylation and consequent β-arrestins binding as the missing link between $G_{i/0}$ protein and β-arrestins. These data highlight the concept that the functions of β-arrestins may be pre-specified by the particulars of the GRK/PKC-receptor interaction. This is consistent with previous work on multiple different receptors showing a requirement for GRKs-mediated receptor to activate specific transducers as well as to affect transducer functionality in a selective manner[91,183]. Thus, the patterning of receptor phosphorylation sites, barcode, engenders subtle differences in β-arrestins/receptor interactions that lead to divergent β-arrestins-dependent signaling events. Ultimately, the fact that GHSR1a can direct functionality via different pathways unveils a tremendous potential for new approaches in developing therapeutics at this receptor particularly taking into account the physiological and pathophysiological effects in both neural and peripheral tissue.

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CHAPTER 2. PKCs and GRKs: role in GHSR1a signaling.

Originally, PKC were considered to be the mediators of GPCR phosphorylation and desensitization[184]. Despite they seemed to be relegated to a secondary action after the discovery of the role of GRKs and β-arrestins in GPCR desensitization[185,186], these kinases have shown to be crucial in GPCRs regulation as second messenger transducers during years[187]. PKC family enzymes regulate the function of other proteins by phosphorylation of the hydroxyl groups of S and T residues. Based on their second messenger requirements, PKC family is divided into three subfamilies: conventional (α, β, and γ isoforms), novel (δ, ε, η, and θ isoforms), and atypical (ζ and ι / λ isoforms). These PKC isotypes are unique, not only with respect to primary structure, but also on the basis of expression patterns, subcellular localization, activation *in vitro* and responsiveness to extracellular signals [170,188,189]. Upon ghrelin stimulation was observed that GHSR1a was able to activate PKC α/β and η through $G_{q/11}$ route while PKC ε, δ, θ, μ and ζ were activated via $G_{i/o}$/PI3K/PKD1 pathway[81]. More interesting, was the fact that PKCμ exhibited the capacity to be a direct substrate of novel and atypical

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PKCs via $G_{i/o}$/PI3K, demonstrating that PKCμ firstly catalogued as a PKC it is actually a PKD which acts as a binding partner of PKC and might confer specificity to PKC signaling through their direct interaction and consequently mediate many receptor transduction pathways[190,191].

In the particular case of GHSR1a, β-arrestin signaling had shown to be dependent on the G-protein activation[81,82,98]. This fact suggests that certain key components of the G protein-dependent signaling pathways are required to determine β-arrestin recruitment and signaling. Indeed, previous works demonstrated that ghrelin leads to Akt activation through an early $G_{i/o}$-protein-dependent cascade and a late pathway mediated by β-arrestins[82,98]. The starting point is the $G_{i/o}$-protein dependent PI3K activation promoting the membrane recruitment of Akt, which is phosphorylated at Y by c-Src with the subsequent phosphorylation at A-loop (T308) and HM (S473) by PDK1 and mTORC2, respectively. Once the receptor is activated, a second signaling pathway is mediated by β-arrestins 1 and 2, involving the recruitment of at least β-arrestins, c-Src and Akt. This β-arrestin-scaffolded complex leads to full activation of Akt. In agreement with these results, assays performed in 3T3-L1 preadipocyte cells indicate that β-arrestins and c-Src are implicated in the activation of Akt in response to ghrelin through the GHSR1a[82]. This result supports the implication of G protein-dependent proteins, such as Src activation via $G_{i/o}$-protein, prompting β-arrestins-mediated signaling events, i.e. full activation of Akt. Additionally, the impact of GRKs or second messenger kinases like PKCs on the receptor phosphorylation and the consequent β-arrestin recruitment might be proposed as the missing link between G proteins and β-arrestins. Our data highlight the possibility that the functions of β-arrestins may be pre-specified by GRK/PKC-receptor interaction. This is consistent with previous work on

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other receptors that exhibited GRKs requirement to activate specific transducers as well as to affect transducer functionality in a selective manner[91],[192]. How GRKs are activated by GPCRs is not currently clear; however, several studies suggested a link between PKC and GRKs activation[174,175,193]. From the data presented so far, GRK2 and GRK6 are critical regulators of GHSR1a phosphorylation. With a focus on GRK2 activation, the lack of effect of inhibition of $G_{i/o}$ by PTX treatment ruled out the implication of $G_{i/o}$-protein dependent activation, and subsequently the involvement of PKCs which are activated via $G_{i/o}$-protein (PKC ε, δ, θ, μ and ζ). Considering the kinetic pattern of PKCs, PKCα/β may have a role in the GRK2 activation. Indeed, siRNA silencing of PKCα decreased GRK2 phosphorylation demonstrating the link between them; moreover, PKCα had exhibited before the capacity to activate GRK2 leading to kinase action175,[193]. Intriguingly, using mutational and conformational studies was described that PKCα activates GRK2 by phosphorylation of S29[175,194]; however the unavailability of a specific S29 antibody when this work was performed lead us to evaluate the phosphorylation of GRK2 at S670. Despite S670 is located in the C-terminal domain instead of being in the N-terminal domain as S29, both residues are involve in a $G_{\beta\gamma}$-binding site. While S29 favor GRK2 interaction with $G_{\beta\gamma}$ subunit with the consequent translocation to the plasma membrane and activation, S670 had exhibited the opposite effect uncoupling GRK2 and $G_{\beta\gamma}$ subunit[195]. This GRK2 desensitization by S670

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phosphorylation was also described as a negative feedback mechanism mediated by ERK1/2[196]. However, assays developed with siRNA directed against PKCα inhibited significantly GRK2 phosphorylation at S670 and, curiously, β-arrestin-activated EKR1/2 and Akt signaling. These results disagree the inhibitory role of S670 phosphorylation by PKCα given that an incresed β-arrestin-dependent ERK1/2 and Akt activity should be observed under siRNA-mediated knockdown of PKCα. Despite additional studies are required to clarify this point, the missing link between G proteins and β-arrestins seems to be related to $G_{q/11}$- and $G_{i/o}$-protein-dependent pathways through GRKs and Scr signaling.

Going a step forward, we also demonstrated the ability of GRK2 and 6 to phosphorylate the ghrelin receptor. In particular, GRK2 appears to phosphorylate specifically the S^{362}, $S^{363}$and T^{366} residues of the C-tail of the GHSR1a following ghrelin activation. Furthermore, GRK2 had proved to be crucial for β-arrestins-mediated signaling to ERK1/2 and Akt at the same time that PKCα. Besides, the model by which, GRKs induce functionality in a selective manner was demonstrated in other receptors. β2-adrenergic receptor, thyrotropin-releasing hormone receptor, angiotensin II receptor signaling or V2 vasopressin receptor had shown that receptor phosphorylation mediated by GRK2 and GRK3 leaded to receptor internalization, while receptor phosphorylation induced by GRK5 and GRK6 was necessary for β-arrestin-dependent ERK1/2 signaling[91,183]. These findings further support the idea of a GRK-induced phosphorylation barcode at the C-tail of the GHSR1a that regulates the nature of β-arrestin intracellular functions[73]. The fact that only GRK6 had also exhibited capacity to reduce GHSR1a phosphorylation in response to ghrelin and the previous results which pointed at the S^{349}, S^{362}, S^{363}, T^{350} and T^{366} as the key phosphor-

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acceptor sites of the GHSR1a, lead us to suggest that if GRK2 is responsible for the phosphorylation of the distal S^{362}, S^{363} and T^{366} sites, GRK6 could be mediating the phosphorylation of the proximal S^{349} and T^{350} residues and subsequently it might be leading the stabilization of the interactions between GHSR1a and β-arrestins. Unlike GRK2 and GRK3 which are translocated to the plasma membrane where they form a complex with the free βγ subunits of heterotrimeric G protein, making its activation dependent on G protein α/βγ subunit dissociation, GRK5 and GRK6 are constitutively bound to the plasma membrane and thus, can interact with and phosphorylate activated receptors independently of heterotrimeric G protein[197]. However, to corroborate that GRK6 is responsible of the phosphorylation of the S^{349} and T^{350} residues, acting or not through $G_{i/0}$ protein-dependent pathway, and the signaling consequences of this, deeper studies are necessary. It is also essential the development of antibodies against the phosphorylated forms of GRK6 to follow its activation.

Finally, it should be notice that many receptor subtypes like GHSR1a are found in more than one tissue type[198]. Receptors which are expressed in different cell types controlling specific cellular responses would be regulated in a cell type-specific manner. GPCR phosphorylation is a flexible regulatory process that seems to have acceptable diversity and dynamic range to accommodate the variety of physiological events triggered by each GPCR subtype. Thus, the sites that are phosphorylated in a GPCR might encode the signaling properties on the receptor. Phosphorylation mediates regulatory features such as β-arrestins recruitment and internalization and consequently, cell-type specific phosphorylation may lead to particular

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signaling functions and a specialized physiological role of the receptor[199,200]. PKCs and GRKs have shown variation in cellular and tissue distribution as well as in abundance[201,202], which made them the perfect candidates to mediate this tissue-dependent phosphorylation code. Thus, the unique phosphorylation signature on the receptor might be the result of the specific employment of PKCs and GRKs. This could be achieved either by differential expression, activation or scaffolding of the kinases. Subsequently, the specific array of intracellular second messengers available in each tissue is able to determine the activation of one mechanism over another or to induce multiple mechanisms simultaneously. For all of this, the final physiological function mediated by ghrelin through GHSR1a is dependent on the tissue-related PKC and GRK expression. We correlated the specific pattern of receptor phosphorylation by GRK2 with the functions of β-arrestins and propose that the distinct phosphorylation patterns established by different GRKs determine a code that imparts the conformations to the recruited β-arrestin, thus regulating its functional activities.

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CHAPTER 3. Determination of active conformations of GHSR1a.

Since the discovery of ghrelin in 1999 as the endogenous ligand for the GHSR1a, several studies were performed trying to determine the minimum structural requirements to induce GHSR1a biological activity. Ghrelin is a GH releasing peptide which consists of 28 amino acid residues with octanoyl modification at S3. This modification has largely demonstrated to be essential for ghrelin activity as GHSR1a recognition of ghrelin is dependent of a large hydrophobic group in this specific position[7,203,204]. The importance of this post-translational modification suggests the amino acid sequence surrounding the S3 might encode a motif directing the octanoyl modification, as the first 10 N-terminal amino acids are identical among the different mammalian species. Despite some studies reported that the the 1-5 truncated form of ghrelin was able to induce intracellular Ca^{+2} mobilization and GH release *in vivo*[7,203] other studies indicated that this truncate form lacked the ability of secrete GH *in vivo*[205]. To elucidate structural features of this peptide necessary for efficient activation of GHSR1a, several short peptides derived from ghrelin were tested in receptor phosphorylation, endocytosis, early signaling events, proliferation, adipogenesis and GH release capacity. The N-terminal part of ghrelin with S(n-octanoyl) in position 3 was preserved, but the chain was gradually shortened by the omission of blocks of several amino acids from the C-terminal end of ghrelin.

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Interestingly, a peptide encompassing only the first 5 residues of ghrelin, GSS(n-octanoyl)FL-NH2 (1-5 ghrelin), activated the GHSR1a phosphorylation as efficiently as ghrelin although the internalization grade was somewhat less potent than ghrelin given that the GHSR1a remained partially in the membrane instead completely internalize. The differences observed in endocytosis could be due to decreased binding affinity to GHSR1a[205] determining a slow rate of GHSR1a internalization.On the other hand, similar activation pattern of ERK1/2 and Akt was observed after stimulation with 1-5 ghrelin, being more potent in the G protein-dependent ERK1/2 signal and less strong in Akt activation compared to ghrelin. Nonetheless, the mitogenic and adipogenic effect was comparable to ghrelin. These results prompted us to speculate to a complete activation of β-arrestin-dependent signaling since proliferative and mitogenic action associated to GHSR1a are mediated by β-arrestins which are related to receptor phosphorylation. However, 1-5 ghrelin failed to activate GH release in GC cells. Considering that GH secretion activity associated to GHSR1a is independent on β-arrestin signaling, 1-5 ghrelin shows characteristics of a partial agonist for G protein-dependent signaling pathways, activating certain G protein-dependent mechanisms. Curiously, 1-5 ghrelin had showed to induce intracellular Ca^{2+} mobilization7, a second messenger involved in GH release. This fact supports multiple active conformations of GHSR1a upon agonist binding and consequently, the efficacy of GHSR1a is recognized as pluridimensional.

The peptide comprising the first 14 residues of ghrelin, COOH-GSS-(n-octanoyl)-FLSPEHQRVQQ-NH_2 (1-14 ghrelin), showed a clear decrease in the GHSR1a phosphsorylation with different pattern and dynamic of endocytosis characterized by smaller vesicles distributed close to the membrane. The activation pattern of ERK1/2 was more potent than ghrelin signal, leading to an early peak phase of ERK1/2 activation that was sustained. According to ERK1/2 pattern, the mitogenic effect was not

observed after 1-14 ghrelin stimulation. By contrast, the early peak phase of Akt activation was absent showing the sustained plateau phase corresponding to β-arrestin signaling. In fact, adipogenic action was comparable to ghrelin. There were no differences in the activation of GH release. Thus, 1-14 ghrelin exhibitss characteristics of partial agonist for G protein and β-arrestin signaling activating G-proteins involved in ERK1/2 and GH release but with lower effect on G-proteins implicated in Akt signalling, i.e. $G_{i/o}$-protein. Differences in the pattern and dynamic of endocytosis may be related to the phosphorylation rate, although this pattern was adequate to induced Akt-associated adipogenic effect by β-arrestin signaling. Curiously, these results reveal the existence of specificity associated to scaffold protein complex. This would be the case for β-arrestin scaffolded ERK1/2 and Akt complexes, which appears to be regulated or determined by different mechanisms of assembly.

Regarding the peptide encompassing only the first 18 residues of ghrelin, COOH-GSS-(n-octanoyl)-FLSPEHQRVQQRKES-NH_2 (1-18 ghrelin), GHSR1a phosphsorylation showed to be higher than in the case of 1-14 ghrelin leading to a dynamic of endocytosis closer to the ghrelin-induced internalization. In this particular case, the activation pattern of ERK1/2 showed the early peak phase associated to G-protein signaling. According to this pattern of ERK1/2 activation, the mitogenic effect was not observed. By contrast, the early peak and sustained phases of Akt activation corresponding to G-protein and β-arrestin signaling were activated. In fact, adipogenic action was similar to that obtained for ghrelin. GH release showed comparable levels to ghrelin. In this particular case, the absent of β-arrestin-dependent ERK1/2 activation explains the lack of effect on mitogénesis similar to 1-14 ghrelin. A complete activation of Akt, i.e. G-protein and β-arrestin signaling, correlates with the adipogenic effect, an effect not observed for 1-14 ghrelin. These results support the postulated specificity associated to scaffold protein complex. Although the receptor

endocytosis activated by 1-18 ghrelin was closer to that activated by ghrelin, this was not adequate to activate β-arrestin-dependent ERK1/2 signaling and, consequently, mitogenic functionality.

In conclusion, these evidences strongly support the idea of multiple active conformations in GHSR1a stabilized as a function of chemical structure of ligands or their pharmacological profiles. Another interesting idea is based on the architecture of ligand binding pockets in the GHSR1a and its association with the multiple active conformations and signaling. However, this hypothesis remains to be tested with rigorous experimentation, for example, by using receptor systems for which well-defined biased ligands and biased receptor mutants are available. Detailed mapping of conformational differences between receptors occupied with agonists remains to be carried out. Such information will be critical both for understanding the mechanism of agonisms at GHSR1a and for aiding in the design of pharmacological tools to selectively manipulate one or the other signaling pathway.

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CONCLUSIONS

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CHAPTER 1. GHSR1a phosphorylation.

1. Mass spectrometry-based proteomic approach allow the mapping of five phosphorylation sites on the C-tail of the GHSR1a that can be divided into 2 different areas. Region 1 comprises T^{350} and S^{349}, while region 2 emcompass S^{362}, S^{363} and T^{366}. Both regions appear to contribute equally to the overall phosphorylation of the receptor.

2. The region 2 was primarily responsible for β-arrestin 1 and 2 binding, receptor internalization, β-arrestin-mediated ERK1/2 and Akt activation. By contrast, region 1 appeared to play a subtler role of stabilizing the interaction between the receptor and β-arrestins.

3. Our data are consistent with a model in which different phosphorylation pattern (barcode) on the GHSR1a can induce distinct interactions with β-arrestins that determine the ultimate cellular consequences of β-arrestins signaling.

CHAPTER 2. PKCs and GRKs: role in GHSR1a signalling.

1. Ghrelin-mediated GHSR1a phosphorylation is dependent on the activation of GRK2 and GRK6. In particular, GRK2 phosphorylates region 2, S^{362}, S^{363} and T^{366}, at the C-tail of GHSR1a.
2. In the system ghrelin/GHSR1a GRK2 is activated by $G_{q/11}$-protein dependent PKCα pathway.
3. GHSR1a phosphorylation mediated by GRK2 is proposed as a key event to activate β-arrestin-signaling, and consequently as a crucial regulator of its functional activities.

CHAPTER 3. Determination of active conformations of GHSR1a.

1. Data obtained from structural features of ghrelin supports the idea of multiple active conformations of GHSR1a which are stabilized as a function of the chemical structure of ligands. Such information will be critical for understanding the mechanism of agonisms at GHSR1a and for aiding in the design of pharmacological tools to selectively manipulate one or the other signaling pathway.

RESUMO

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A ghrelina é una hormona peptídica de 28 aminoácidos, secretada en maior parte polo estómago e en menor proporción polo intestino, o páncreas, o ril, a placenta, o hipotálamo e a hipófise. Tratase do primeiro péptido natural acilado, debido o grupo n-octanoil que posúe na S3, e que ademais é esencial para a súa bioactividade, xa que lle permite traspasar a barreira hematoencefálica e unirse o seu receptor específico, o receptor de secretagogos da hormona do crecemento (GHSR1a). O GHSR1a expresase en diversas áreas do cerebro que inclúen a hipófise, o hipocampo, o núcleo arcuato hipotalámico, a substantia nigra pars compacta, a área tegmental ventral e os núcleos do rafé. A activación do GHSR1a na hipófise estimula a secreción da hormona do crecemento (GH). Ademais, a unión da ghrelina ao seu receptor nos neuropeptidos Y do hipotálamo induce apetito e estimula a inxesta. Ditos comportamentos de alimentación e recompensas asociados a ghrelina son controlados pola área tegmental ventral e o hipocampo. A nivel periférico o GHSR1a expresase nos illotes pancreáticos, a glándula suprarrenal, a tiroide, o miocardio, e o tecido adiposo. Ata o momento foron descritas numerosas accións periféricas da ghrelina que incluen: a regulación do metabolismo da glucosa, a lipoxénese, a supresión da termoxénese na graxa parda, e a mellora das funcións cardiovasculares como a vasodilatación ou a contractilidade.

A acción da ghrelina na activación do GHSR1a determina as vías de sinalización intracelular alcanzadas e polo tanto as funcións fisiolóxicas asociadas. As cascadas de sinalización descritas de xeito tradicional para o GHSR1a estaban mediadas polas proteínas G, principalmente $G_{q/11}$ e $G_{i/o}$. Sen embargo, más recentemente demostrouse que as β-arrestinas actúan como soporte de gran variedade de proteínas de sinalización en cascadas independentes das proteínas G. Inicialmente describiuse a implicación das β-arrestinas na desensibilización do GHSR1a través da regulación da endocitose. Sen embargo, na actualidade está ben establecido que as β-arrestinas están tamén implicadas na transdución de sinal da proteína

quinasa activada por mitóxenos (ERK1/2) e Akt. A activación de ERK1/2 a través do GHSR1a prodúcese por ambas vías, a mediada por proteínas G e a dependente das β-arrestinas. Na cascada mediada por Gi/o están implicadas: a proteína fosfatidilinositol 3-quinasa (PI3K), a proteína quinasa C ε, e a proteína non-receptor con actividade tirosina quinasa, cSrc. Nunha segunda vía de activación de ERK1/2 dependente de $G_{q/11}$ vense envoltas a proteína quinasa C α/β e cSrc. Por último, existe unha terceira ruta que implica o recrutamento do GHSR1a con cSrc, Raf 1, e ERK1/2 nun complexo no que as β-arrestinas actúan como soporte. Por outro lado, o GHSR1a tamén é capaz de activar Akt a través da interacción de distintos mecanismos de sinalización que implican unha activación tempera dependente de $G_{i/o}$ e una tardía mediada polas β-arrestinas. Na primeira fase, activase a proteína PI3K a través de $G_{i/o}$ e AKt translocase a membrana onde é fosforilada por cSrc en Tyrosina provocando que a piruvato deshidroxenasa kinasa1 (PDK1) fosforile a sua T308 e a diana de rapamicina en células de mamífero2 (mTORC2) a S473 situadas no loop A e no motivo hidrófobo de Akt respectivamente. Tras a activación do receptor, iniciase unha segunda vía de sinalización mediada polas β-arrestinas 1 e 2, que implica o recrutamento de cSrc e Akt. O complexo que se forma coas β-arrestinas como soporte conduce a activación completa de Akt a través de PDK1 e mTORC2, que no están asociados fisicamente o complexo. En ambas vías, cSrc fosforila a Y536 C-terminal da tyrosina fosfatasa SHP1 que exerce un efecto inhibidor sobre PI3K e Akt, o que converte a cSrc nun interruptor para controlar tanto la ruta dependente de $G_{i/o}$ como a mediada polas β-arrestinas. Por outro lado, o noso grupo tamén demostrou que as β-arrestinas median as funcións adipoxénicas do sistema ghrelina/GHSR1a mediante a vinculación do receptor activado con distintos conxuntos de proteínas accesorias e efectoras, controlando deste xeito a especificidade, a eficiencia e maila capacidade das sinais. A nivel molecular, a inhibición das β-arrestinas durante a adipoxénese inducida por ghrelina diminuiu os niveis de C/EBPα, C/EBPδ, C/EBPβ, así como os niveis de PPARγ, provocando una redución significativa na acumulación de lípidos e o

deterioro da diferenciación terminal de adipocitos. Este efecto, combinado coa inhibición significativa da activación de Akt inducida por ghrelina, corrobora que as β-arrestinas son clave na transdución de sinal do sistema ghrelina/GHSR1a. Queda cada vez mais patente que as β-arrestinas, orixinalmente descubertas como meras proteínas adaptadoras necesarias para la endocitose do GHSR1a, teñen un papel moito más amplo no mantemento da homeostase celular.

A pesares de que a importancia do sistema ghrelina/GHSR1a é amplamente recoñecida, aínda non está claro cómo este receptor acoplado a proteínas G (GPCR) controla as súas diversas funcionalidades. No caso de outros GPCRS demostrouse que a fosforilación do receptor establece un código de barras que determina a interacción coas β-arrestinas e polo tanto, a actividade do mesmo. Tamén se viu que este código de barras fosforilación pode ser específico do tecido no que se estea a expresar o receptor, probablemente, porque cada tecido presenta un nivel distinto de expresión de proteínas quinasas, como as proteína quinasa de GPCR (GRKs) ou as proteínas quinasa C (PKC).

Por todo o exposto anteriormente, o noso traballo comezou realizando un estudo sobre o patrón de fosforilación do receptor GHSR1a como elemento determinante da súa actividade. Para levalo a termo, empregamos diferentes aproximacións co fin de elucidar os sitios precisos de fosforilación do GHSR1a tras a súa estimulación con ghrelina. Mediante a xeración de fosfopéptidos trípticos e a análise destes péptidos por cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS) determinamos que a ghrelina induce a fosforilacion do GHSR1a principalmente nos residuos S^{349}, S^{362}, S^{363} T^{350} and T^{366} que están situados todos eles na cola carboxilo terminal do receptor. A continuación, mediante mutaxénese dirixida dos sitios de fosforilación atopados xeráronse diversos mutantes do receptor marcados coa proteína verde fluorescente (EGFP) que sendo expresados nun sistema in vitro permitíronnos determinar a

implicación destes aminoácidos na sinalización derivada da activación do GHSR1a. Mentres os residuos mais terminais do receptor (S^{362}, S^{363} and T^{366}) demostraron promover a interacción das β-arrestinas co GHSR1a, a internalización do receptor así como a proliferación e a adipoxénese mediada por as β-arrestinas; os fosfo aceptores da zona más distal da cola (T^{350} and S^{349}), mostráronse necesarios para estabilizar a interacción das β-arrestinas co GHSR1a e polo tanto, como os responsables finais da sinalización asociada as β-arrestinas. Sen embargo, a sinalización e funcionalidades asociadas o GHSR1a vía $G_{q/11}$ non se viron afectadas polo patrón de fosforilación do mesmo, o que nos leva a pensar que o receptor podería empregar ambas rutas dun xeito independente o que lle permitiría modular a súa actividade e funcionalidade. Finalmente, os resultados derivados desta parte do estudio demostran que os mecanismos de activación da ghrelina asociados a un patrón de fosforilación do receptor GHSR1a son determinantes das acciones biolóxicas de esta hormona.

Una vez elucidado o patrón de fosforilación do GHSR1a e a súa importancia na sinalización e funcionalidades asociadas o mesmo, procedemos a estudar os elementos implicados na indución de dita cascada intracelular asociada a fosforilación do receptor. Como xa mencionamos anteriormente, a especificidade do código de barras de fosforilación pode estar determinada pola expresión de quinasas como as GRK ou as PKC. O estudo do sistema ghrelina/GHSR1a en células HEK293 determinou que a ghrelina é capaz de inducir a activación de PKCs a través de $G_{i/o}$ e $G_{q/11}$. Mais concretamente, a ruta $G_{i/o}$/PI3K/PKD1 regula a activación de PKCδ, PKCθ, PKCμ e PKCζ mentres PKCη e activada a través da vía mediada por Gq/11 e PKCα. Dito estudo confirmou ademais que a proteína quinasa D actúa como substrato de PKCs noveles (δ, ε, η y θ) e atípicas (ζ y ι / λ) a través da ruta dependente de $G_{i/o}$. Así mesmo, con este traballo conseguiuse establecer que a fosforilacion do GHSR1a é debida o papel das GRK2 e 6. De forma mais especifica, viuse que GRK2 é a encargada da fosforilacion dos residuos mais

terminais da cola do GHSR1a S^{362}, S^{363} and T^{366} e polo tanto da unión do receptor coas β-arrestinas e a súa internalización. Finalmente, nesta parte do traballo logrouse conectar a vía das proteínas G coa fosforilación do receptor. Esta conexión viría dada polo feito de que GRK2 é por lavia mediada por $G_{q/11}$ e PKCα. O complexo sistema descrito determinaría a funcionalidade do receptor asociada o tecido diana de expresión.

Por último, neste traballo incluímos un estudo con diferentes truncados da ghrelina co fin de determinar os elementos estruturais determinantes da súa bioactividade. Créese que ademais da diferenza de expresión de GRKs y PKCs, o uso de distintos ligandos podería levar a prevalenza dunha vía de sinalización sobre outra ou a un distinto grao de activación da mesma, o cal nos permitiría seleccionar unha funcionalidade determinada do mesmo receptor. Do mesmo xeito, é importante a determinación da estrutura dos ligandos dun receptor xa que unha parte concreta dos mesmos podería activar unha conformación determinada do receptor e polo tanto inducir de forma especifica unha vía de sinalización. Con este estudo vimos que os cinco primeiros aminoácidos N-terminais da ghrelina son capaces de estimular a fosforilación e a sinalización mediada polo GHSR1a así como a súa actividade proliferativa e adipoxénica en niveis similares a os producidos pola ghrelina pero non son capaces de inducir secreción de GH. Por outro lado, os truncados que inclúen os 14 e os 18 primeiros aminoacidos da ghrelina non foron quen de inducir un nivel de fosforilación comparable o da mesma. Ademais amosaron unha perda da sinalización mediada por β-arrestinas que conduciu a indución dunha menor actividade mitoxénica. Sen embargo, estes truncados si tiveron un efecto similar o da ghrelina na acumulación de lípidos e na secreción de GH. Todo isto, lévanos a pensar que a estrutura química dos ligandos exerce un papel clave na determinación da conformación do GHSR1a e polo tanto no seu perfil farmacolóxico.

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