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Functional Selectivity and Downstream Signalling Bias of Ghrelin Receptor Ligands

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Acknowledgements

This research was funded in part by Science Foundation Ireland in the form of a Research Centre grant (SFI/12/RC/2273) to the APC Microbiome Institute. We would like to thank Naomi Hoevenaars for her help and assistance with internalisation assay experiments. Graphic work was carried out by Felipe G. Serrano (www.illustrative-science.com).

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Running title: Characterisation of ligand-mediated GHS-R1a receptor signalling bias.

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Number of text pages:

Number of tables:

Number of figures:

Number of references:

Number of words Abstract:

Number of words Introduction:

Number of words Discussion:

Nonstandard abbreviations: GHS-R1a, Growth Hormone secretagogue receptor 1a.

Abstract

The ghrelin receptor (or growth hormone secretagogue receptor; GHS-R1a) is a G-protein coupled receptor with significant potential to modulate appetite, and it is therefore a promising pharmacological target for the treatment of metabolic disorders, including obesity, cachexia and malnutrition. The GHS-R1a receptor has a complex pharmacology and is activated by ligand-mediated G-protein dependent as well as G-protein independent signalling pathways. The GHS-R1a receptor is also characterized by high basal ligand-independent signalling. Here, we characterise the GHS-R1a receptor ligand-directed functional selectivity and bias of ligands on GHS-R1a receptor signalling as well as its high constitutive activity. Particularly, we investigated the pharmacological properties of the well-known GHS-R1a receptor ligands ghrelin, MK-0677 and L-692,585, defined as full GHS-R1a receptor agonists, as well as [D-Lys3]-GHRP and JMV2959 used as antagonists and the inverse agonist [D-Arg(1),D-Phe(5),D-Trp(7,9),Leu(11)]-Substance P. These findings will provide novel insight in the pharmacological characteristics of GHS-R1a receptor ligands and their signalling bias, which will have implications for designing new drugs to target eating disorders and other ghrelin receptor signalling functionalities with higher selectivity and, therefore, limited side effects.

Introduction

The gut hormone, ghrelin, is a 28-amino acid peptide that participates in the modulation of several gastrointestinal functions such as gut motility, adiposity and glucose metabolism, as well as the central regulation of appetite and secretion of growth hormone (GH) (Howard et al., 1996; Inui, 2001; Kojima et al., 1999; Muller et al., 2015; Tschop et al., 2000). Additionally, ghrelin plays an important role in central nervous system participating in learning and memory processes, sleep/awake rhythm and reward seeking behaviour (Berrout and Isokawa, 2017; Mason et al., 2014). Ghrelin acts through the activation of the growth hormone secretagogue receptor (GHS-R1a receptor), also called the ghrelin receptor. The GHS-R1a receptor is a GPCR (G-protein coupled receptor), which classically exerts intracellular effects via Gαq and Gαo/i activation (Cong et al., 2010) (Fig. 1). Briefly, the GHS-R1a-Gαq-dependent signalling activates PLC (phospholipase C) responsible for intracellular Ca^{2+} release, which in turn activates PKCβ (protein kinase C beta) (Lee et al., 2014), CAMKIIα (Ca2+/calmodulin-dependent protein kinase II alpha) and AMPK (AMP-activated protein kinase) (Bayliss et al., 2016; Chen et al., 2011). The GHS-R1a-Gαo/i–dependent signalling activates PI3-K (phosphatidylinositol 3 kinase) to regulate the activation of PKA (protein kinase A) (Cavalier et al., 2015), PKCε (protein kinase epsilon) and AKT (serine/threonine-specific protein kinase) (Mousseaux et al., 2006). Furthermore, G-protein-independent signalling also occurs via the β-Arrestin scaffolding protein to activate ERK1/2 (extracellular signal–regulated kinases 1 and 2) and AKT (Bouzo-Lorenzo et al., 2016; Santos-Zas et al., 2013). Moreover, the GHS-R1a receptor has a high and physiological relevant constitutive activity in absence of its endogenous ligand, ghrelin (Camina et al., 2004; Damian et al., 2012; Els et al., 2010; Holst and Schwartz, 2004; Mear et al., 2013; Petersen et al., 2009). Due to the complexity of the downstream signalling at the GHS-R1a receptor and the multitude of physiological functions it is believed that specific signalling cascades may contribute to distinct functional responses. It has been

suggested that GHS-R1a-Gq-Ca^{2+} dependent signalling is mainly related with GH secretion (Osterstock et al., 2010) and GHS-R1a-β-Arrestin signalling is linked with mitogenic activity and intracellular lipid storage (Santos-Zas et al., 2013). However, the functional outcome of GHS-R1a receptor signalling might also be dependent on the tissue where the receptor is expressed (Gnanapavan et al., 2002).

The GHS-R1a receptor is a promising pharmacological target for the treatment of metabolic disorders, including obesity, cachexia and malnutrition, via the modulation of appetite (Delporte, 2012). Compounds targeting the GHS-R1a receptor have therefore gained significant pharmacological interest (Abegg et al., 2017; Allas and Abribat, 2013; Schellekens et al., 2010; Schellekens et al., 2013a). Nevertheless, the limited success of therapeutics targeting the GHS-R1a receptor in clinical development to date may be due to the complex signalling pharmacology of the GHS-R1a receptor. A better understanding of GHS-R1a receptor signalling, bias and ligand-mediated functionality would contribute to the successful targeted drug discovery and development of ghrelin-mimetics.

Several synthetic compounds have been described in the literature as GHS-R1a receptor ligands. These compounds have been categorised as agonists, antagonists and/or inverse agonists, however recent evidence has demonstrated that the specific GHS-R1a receptor downstream signalling pathways modulated by these ligands display signalling bias with differential functional selectivity (Evron et al., 2014; M'Kadmi et al., 2015; Pastor-Cavada et al., 2016; Sanger, 2014; Toth et al., 2017). The concepts of functional selectivity and ligand bias are becoming increasingly appreciated in present-day of GPCR-drug discovery (Bologna et al., 2017) and specifically in the case of GHS-R1a receptor signalling pathways, which ultimately contribute to the prediction and selection of the most appropriate therapeutic compound for specific GHS-R1a receptor mediated functionalities.

Measurements of receptor activation can be performed at many levels of signal transduction. Here, we investigate several commercially available GHS-R1a receptor ligands and compare them across a number of *in vitro* cellular assays using a human embryonic kidney (HEK293A) cell line stably expressing the human GHS-R1a receptor (Schellekens et al., 2015; Schellekens et al., 2013b; Schellekens et al., 2013c). We evaluate the potential bias of ligands classically described as agonist, such as ghrelin, MK-0677 and L692,585, through GHS-R1a-Gq-dependent signalling and GHS-R1a-β-Arrestin-dependent signalling. Moreover, we assess the capacity of three well-known molecules, described as GHS-R1a antagonists or inverse agonist, [D-lys^{3}]-GHRP-6 (Dlys), JMV2959 and [D-Arg(1), D-Phe(5), D-Trp(7,9), Leu(11)]-substance P (SP-analog), to effectively block specific GHS-R1a receptor signalling cascades. These findings provide a novel insight in the pharmacological characteristics of these well-known GHS-R1a receptor ligands and their bias, which will have important implications in the design of novel, more selective GHS-R1a receptor ligands with predictable functional outcome for pre-clinical and clinical drug development.

Material and Methods.

Compounds

Ghrelin (#SP-GHRL) was purchased from Innovagen (Lund, Sweden). MK-0677 (#5272), L-692,585 (#2261), [D-lys^{3}]-GHRP-6 (Dlys) (#1922,) and [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-Substance P (SP-analog) (#1946) were purchased from Tocris Bioscience (Ellisville, MO). JMV2959 (#345888) was purchased from Calbiochem (San Diego, CA). Stocks were prepared in assay buffer (1x Hank's Balanced Salt Solution - HBSS with 20 mM HEPES).

Cell culture

Human embryonic kidney cells (HEK293A) from Invitrogen (Carlsbad, CA) were stably transfected with a plasmid construct containing the human GHS-R1a receptor gene tagged with

an Enhanced Green Fluorescent Protein (HEK293A-GHSR1a-EGFP), as previously described (Schellekens et al., 2013b; Schellekens et al., 2013c; Torres-Fuentes et al., 2014). Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, #D5796, Sigma-Aldrich, Wicklow, Ireland) supplemented with 10% heat inactivated FBS (#F7524, Sigma-Aldrich), 1% Non-Essential Amino Acids (NEAA) (11140035, Gibco Life Technologies, Gaithersburg, MD) and 500 ng/µl G-418 (#345812, Calbiochem) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Intracellular calcium mobilisation assay

Receptor-mediated changes in intracellular calcium [Ca^{2+}] were monitored with the use of the FLIPR Tetra® High-Throughput Cellular Screening System (Molecular Devices, LLC Sunnyvale, CA). HEK293A cells with the stable expression of the GHSR-1a-EGFP receptor were seeded in black 96-well microtiter plates at a density of 2.5×10^5 cells/ml (2.5×10^4 cells/well) and incubated overnight at standard culture conditions. Growth media was replaced 24 h before each experiment with serum free DMEM containing 1% NEAA. At the day of experiment cells were incubated for 90 min with 80 µl of the Ca5 dye diluted in assay buffer (1xHBSS, containing 20 mM HEPES) according to the manufacturer's protocol (#R8186; Molecular Devices). Fluorescent readings were taken for a total of 120 seconds with excitation wavelength of 485 nm and emission wavelength of 525 nm. For analysis of agonism of GHS-R1a receptor ligands, 40 µl/well were added from compound plates using the liquid handling option of the FLIPR Tetra® High-Throughput Cellular Screening System. For analysis of antagonism or inverse agonism, cells were pre-incubated for 90 min with ligands and Ca5 dye followed by addition of agonist. The relative increase in intracellular calcium [Ca^{2+}] was calculated as the difference between the maximum and baseline fluorescence, and depicted as percentage relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained with 3.3% Fetal bovine serum (FBS). Background fluorescence was

recorded in cells in assay buffer alone and subtracted from RFUs. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA). The concentration-response curves of GHSR-1a ligands were generated using nonlinear regression. The curves were fitted to a 3-parametric logistic equation, allowing for the determination of EC_{50} and E_{max} values.

β-Arrestin-1 recruitment assay

PathHunter® eXpress GHSR1a U2OS β-Arrestin-1 GPCR Assay (93-0242E3CP5S, Discoverx, Fremont, CA) was used to analyse the effects of GHS-R1a receptor ligands on both basal and agonist-mediated β-Arrestin-1 recruitment. Procedures were performed according to the manufacturer's instructions. Briefly, cryopreserved PathHunter® eXpress GHSR1a U2OS cells were added to the cell plating reagents and plated at a density of 8.3 x 10^4 cells/mL (2.5 x 10^3 cells/well) in 96-well plates. After 48 h incubation at standard culture conditions, cells were treated with GHS-R1a receptor ligands for 60 minutes. Luminescence signal was read with the use of Synergy 2 (Biotek Instruments, Winooski, VT). Data were shown as RLU and analysed using GraphPad Software.

Receptor internalisation assay

Ligand-mediated GHS-R1a receptor translocation was quantified by monitoring the EGFP fluorescent trafficking away from the cellular membrane into vesicles within the cytosol. HEK293-GHS-R1a-EGFP cells were seeded in 96-well microtiter plate at density of 2.5 x 10^5 cells/ml (2.5 x 10^4 cells/well) and incubated for 48 h at standard culture conditions. 24 h before experiment media was replaced with serum free DMEM containing 1% NEAA. Cells were incubated with different concentrations of GHSR-1a receptor ligands for 60 minutes in 37°C. To investigate the effect of antagonists/inverse agonists on ghrelin-mediated internalisation, cells were pre-incubated with different concentrations of those compounds for 60 minutes prior

the addition of increasing concentration of GHS-R1a agonist and further incubation for 60 minutes. After treatment, cells were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 20 min, and washed two times with 1x PBS. Cells were imaged on the GE Healthcare IN Cell Analyzer 1000 (GE Healthcare Life Science, Buckinghamshire UK) and analysed using the IN Cell Analyzer Developer Toolbox V1.6 Software (GE Healthcare) as previously described (Pastor-Cavada et al., 2016). Data were analysed and depicted using GraphPad Prism software.

ERK1/2 signalling assay

HEK293-GHS-R1a-EGFP cells were seeded in 6-well microtiter plate at density of 2.5 x 10^6 cells/well and incubated for 24 h at standard culture conditions. Media was replaced with serum free DMEM media containing 1% NEAA and incubated for another 24 h. Cells were exposed to ligands at different concentrations for 60 minutes. The effect of GHS-R1a receptor antagonists/inverse agonists on ghrelin-induced extracellular signal-regulated kinases (ERK1/2) was investigated by pre-incubating cells for 60 minutes followed by the addition of agonists for 60 minutes incubation. Phosphorylation of ERK1/2 was measured by Western blot. Cells were lysed in RIPA extraction buffer (Thermo Fisher Scientific, Pierce, Sunnyvale, CA) supplied with protease inhibitor cocktail (cOmplete ULTRA, Roche, Indianapolis, IN) and phosphatase inhibitor (PhosSTOP, Roche). Cell lysates were clarified by centrifugation for 15 min at 14000 g at +4 °C. Protein concentration was measured by BCA Protein Assay kit (Thermo Fisher Scientific) and equal protein amount was loaded on the gel (20µg). Proteins were separated on 4–20% polyacrylamide electrophoresis gel (GenScript, Piscataway, NJ), and transferred onto/to a 0.2 µm Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA) in wet mini-transfer system Owl VEP-2 (Thermo Fisher Scientific). After transfer membranes were incubated with primary antibodies in 5% BSA (Sigma-Aldrich) against phospho-ERK1/2 Thr202/Tyr204, (#9101; 1:1000; Cell Signalling Technology Inc, Beverly, MA), and then

stripped using Restore Western blot stripping buffer (#21059; Thermo Scientific) and incubated with anti-ERK1/2 (#4695; 1:1000; Cell Signalling Technology Inc) overnight at +4°C. Additionally, anti-GFP-HRP (#ab6663; 1:1000; Abcam) and anti-β-actin (#A2228; 1:5000; Sigma) were used as loading control. Incubation with secondary antibodies (HRP-conjugated, Sigma) was done in 5% fat-free milk for 1 h at room temperature. Detection was performed using Amersham ECL Prime reagent (GE Healthcare Life Sciences) and the LAS-3000 Imager/Image Reader LAS-3000 2.2 (FujiFilm, Tokyo, Japan). Quantitative image analysis was done in ImageJ software. The density of phosphorylated protein bands was normalized to the corresponding total protein bands.

Statistical analysis

Statistical analysis for calcium mobilisation, β-Arrestin-1 recruitment assays, ERK1/2 and internalisation assay was performed using one-way ANOVA with Dunett's post-hoc test analysed using Prism Software (GraphPad Software, Inc). All data are presented as mean ± SEM. The differences between groups were deemed significant at $p<0.05$.

Results

Potency and efficacy profiles of GHS-R1a receptor ligands

We selected two synthetic agonists of the GHS-R1a receptor, MK-0677 and L692,585, and the endogenous ligand ghrelin, to assess differences in functional selectivity towards diverse signalling pathways, focusing on calcium mobilisation, β-Arrestin-1 recruitment, receptor internalisation and downstream ERK1/2 signalling. Firstly, we compared the effects of the ligands in calcium mobilisation in HEK293A cells that overexpress the GHS-R1a receptor. In agreement with previous results (Evron et al., 2014; Holst et al., 2005) MK-0677 and L692,585 produced a strong agonist effect, (Fig. 2A; Table I), with L692,585 being the most potent agonist (EC_{50} 0.69 nM) compared to ghrelin (EC_{50} 27.57 nM) and with MK-0677 (EC_{50} 2.45

nM). SP-analog and Dlys did not shown a Ca^{+2}-dependent agonist activity, in contrast, JMV2959 displayed a slight agonist activity (20.44 ± 2.4 % of maximal activation) which has been suggested before (M'Kadmi et al., 2015). To investigate the effect of these GHS-R1a receptor ligands in the β-Arrestin dependent signalling, the β-Arrestin-1 recruitment was analysed in a U2OS cell line. Ghrelin, MK-0677 and L692,585 (500 nM) all showed a classic agonist response increasing β-Arrestin-1 recruitment, however this effect was not observed after treatment with 500 nM pyridone, which was previously identified as a novel non-peptide GHS-R1a receptor agonist in our laboratory (Pastor-Cavada et al., 2016) (Fig. 2B). Interestingly, 1 µM SP-analog produced a significant decreases in the β-Arrestin-1 recruitment in basal condition which confirm its ability to attenuate the ligand-independent constitutive activity of the GHS-R1a receptor (Els et al., 2010).

Next, the effects of the ligands on GHS-R1a receptor internalisation into endosome, a characteristic of receptor desensitisation, were examined. Both desensitisation and internalisation process provide crucial physiological feedback mechanism that protect against both acute and overstimulation of receptors (Ritter and Hall, 2009). Clear internalisation of the GHS-R1a receptor was observed after treatment with the endogenous agonist ghrelin (500 nM), as shown before (Torres-Fuentes et al., 2014) (Fig 2C and D). In addition, both MK-0677 and L692,585 agonist resulted in a high increased GHS-R1a receptor internalisation with respect to untreated cells (cells in assay buffer) (Fig. 2C and D). Ghrelin, MK-0677 and L692,585 agonists demonstrated a significant and concentration-dependent increase of GHS-R1a-EGFP receptor internalisation after 1 h of ligand exposure (Fig. 2D, Table II). MK-0677 presented the higher potency (EC_{50} 2.35 nM) compared to ghrelin (EC_{50} 38.87 nM) and with L692,585 (EC_{50} 21,89 nM), however there was no significant difference in the efficacy (E_{max}) between ligands (Table II). In contrast, treatment with the inverse agonist SP-analog did show a dose-dependent decrease in EFGP fluorescence intensity within the cytoplasm (EC_{50} 76.01 nM) (Fig.

2D). Interestingly, both JMV2979 and Dlys treatment also decreased GHS-R1a-EGFP receptor in the cytoplasm with respect to untreated cells (JMV2959 EC_{50} 74.65 nM; Dlys EC_{50} 1.6 μM) (Fig. 2D and Table II). This implies that both compounds have inverse agonist properties on the GHS-R1a receptor, which has not been reported before.

Previously it has been shown that both GHS-R1a receptor-G-protein-dependent and GHS-R1a-β-Arrestin dependent signalling can activate the 44- and 42 kDa extracellular signal-regulated kinase (ERK1/2) in different cell lines (Bouzo-Lorenzo et al., 2016; Lodeiro et al., 2009; Mousseaux et al., 2006). The early phosphorylation peak of ERK1/2 activation is a G-protein-dependent process that happens after few minutes of GHS-R1a receptor endogenous ligand challenge, while the late peak phase of ERK1/2 activation is a β-Arrestin dependent mechanism that occurs after 1 h of ghrelin treatment (Bouzo-Lorenzo et al., 2016; Camina, 2006). We measured the late phase of ERK1/2 activation after 1 h of ghrelin incubation (Fig. 2E) and observed that ghrelin at concentrations of 10 and 100 nM produced a significant increase in ERK1/2 activation (Fig. 2E). Treatment with JMV2979 and Dlys had no effect, while treatment with SP-analog produces a significant decrease in the basal ERK1/2 phosphorylation (Fig. 2F), which confirms the inverse agonist nature of this compound.

Antagonist characteristics of [D-lys^3]-GHRP-6 ligand

Next, we evaluated the effect of Dlys as an antagonist of GHS-R1a receptor signalling (Fig. 3). This ligand has been widely used in *in vitro* and *in vivo* studies to antagonise the GHS-R1a receptor (Beck et al., 2004; Torres-Fuentes et al., 2014), however it has not been established whether this compound has biased antagonist properties. Hek-GHS-R1a-EGFP cells were incubated for 1 h with 3 different concentration of Dlys and then they were challenged with increasing concentration of ghrelin, MK-0677 and L692,585 for 1 h (Fig. 3 A; panel 1, 2 and 3). We observed that Dlys 1 μM pre-treatment blocked ghrelin- and L692,585-mediated

internalisation producing a shift in the curve to the right, however no significant blocking effect was observed by Dlys for MK-0677-mediated internalisation (Fig. 3A). However, the Dlys-mediated attenuation of GHS-R1a receptor internalization did not significantly reduce EC50 values nor efficacy (Table II). In addition, we determined the effect of Dlys on ERK1/2 activation after agonist's treatment (Fig. 3B; panel 1, 2 and 3). According with the internalisation results, Dlys pre-treatment (1 μM) significantly decreased ghrelin- and L692,585-dependent ERK1/2 activation, however the ERK1/2 activation after MK-0677 treatment was not reduced (Fig. 3B; panel 2). Interestingly, Dlys did not significantly change calcium mobilisation after agonists treatment (Fig. 3C; panel 1, 2 and 3 and Table I), suggesting that this antagonist might possess a preferential blocking signalling cascade, corresponding to the GHS-R1a-β-Arrestin pathway.

Antagonist characteristics of JMV2959 ligand

Next, we set out to determine the potential antagonist effect of JMV2959 on GHS-R1a receptor-G-protein-dependent versus β-Arrestin-dependent pathway (Fig. 4). JMV2959 is a small molecule that has shown antagonist effect on GHS-R1a receptor signalling (Moulin et al., 2013) and is able to bind to human GHS-R1a receptor in an orthosteric manner (Moulin et al., 2007). Ligand-mediated internalisation was investigated using different concentrations of JMV2959 followed by GHS-R1a receptor challenge with increasing concentration of ghrelin, MK-0677 and L695,585. JMV2959 was able to decrease the ghrelin receptor internalisation producing a rightwards shift of the ghrelin concentration-response curve (Fig. 4A; panel 1). Rightwards shift were also obtained for JMV2959 on MK-0677 and L692,585 mediated GHS-R1a receptor internalisation (Fig. 4A; panel 2 and 3). Interestingly, JMV2959 pre-treatment also produced a downward shift of L692,585 dose-concentration curve (Fig. 4A; panel 3). However, only the highest concentration of JMV2959 (1 μM) was able to significantly reduce EC_{50} or E_{max} (Table II). Next, the effect of JMV2959 on agonist-mediated ERK1/2 activation

was evaluated (Fig 4B; panel 1, 2 and 3). We confirmed that JMV2959 1 μM was able to significantly reduce ERK1/2 phosphorylation mediated by the three agonists tested in a concentration dependent fashion, with the L692,585 agonist dependent response most strongly attenuated by JMV2959 (Fig. 4B, panel 3).

The ability of JMV2959 to act as antagonist in Gq-coupled GHS-R1a receptor signalling was further assessed using the calcium mobilisation assay (Fig. 4C; panel 1, 2 and 3). As noted earlier (Fig. 2A), ghrelin, MK-0677 and L692,585 all significantly increase intracellular calcium influx via GHS-R1a receptor-dependent activation of Gq. JMV2959 was able to significantly reduce GHS-R1a receptor-mediated calcium influx by ghrelin, MK-0677 and L692,585 (Fig. 4C; panel 1, 2 and 3). The antagonist, JMV2959 significantly reduced the potency (EC_{50}) of all three agonist and reduced maximal efficacy for MK-0677 and L692,585 at 1 μM and 0.1 μM (Table I). These data highlight that JMV2959 does not have a functional bias on GHS-R1a receptor signalling and behaves as full antagonist to the endogenous ligand, ghrelin, and the two agonists, MK-0677 and L692,585, attenuating both Gq- and β-Arrestin mediated signalling.

Antagonist and inverse agonist characteristics of [D-Arg(1), D-Phe(5), D-Trp(7,9), Leu(11)]-substance P (SP-analog)

The GHS-R1a receptor inverse agonists SP-analog has shown to decrease the high constitutive activity of the GHS-R1a receptor, leading to an increase of GHS-R1a receptor availability on the membrane (Holst et al., 2003). We analysed ligand-mediated GHS-R1a receptor internalisation with a pre-incubation of increasing concentration of SP-analog (Fig. 5; panel 1, 2 and 3). In the presence of 1 μM SP-analog, GHS-R1a receptor internalisation was significantly enhanced at increasing concentration of ghrelin and MK-0677, as demonstrated by a greater cytoplasmic internalisation compared to ghrelin and MK-0677 exposure alone

(Fig, 5A; panel 1 and 2) and significant increase in E_{max} (Table II) (ghrelin E_{max} = 1.37 ± 0.02; ghrelin + 1 μM SP-analog E_{max} = 1.80 ± 0.05, $p<0.05$; MK-0677 E_{max} = 1.39 ± 0.02; MK-0677 + 1 μM SP-analog E_{max} = 1.86 ± 0.04, $p<0.05$) and a significant increase in EC_{50} for SP-analog effect on MK-0677 (MK-0677 E_{max} = 2.35 nM; MK-0677 + 1 μM SP-analog E_{max} = 35.31 nM, $p<0.001$, Table II). In contract, SP-analog pre-treatment showed a decreased GHS-R1a receptor internalisation at lower concentrations of ghrelin and MK-0677 exposure, which suggest the SP-analog ligand to also act as a GHS-R1a receptor antagonist (Fig. 5A; panel 1 and 2). However, the capacity of varying concentrations of L585,692 to promote the GHS-R1a receptor internalisation was completely blocked by the pre-treatment with SP-analog increasing E_{50} (L692,585 E_{50} = 21.89 nM; L692,585 + 1 μM SP-analog E_{50} = 332.6 nM, $p<0.05$) (Fig. 5A; panel 3 and Table II). In addition, SP-analog pre-treatment (1 μM) was able to block the levels of phosphorylated ERK1/2 phosphorylation induced by low doses of ghrelin, MK-0677 and L692,585 (1 and 10 nM), but not at higher concentrations (100 nM) in cells expressing the GHS-R1a receptor (Fig. 5B; panel 1, 2 and 3). In the calcium mobilisation assay, we observed that increasing concentration of SP-analog resulted in significant higher calcium mobilisation mediated by ghrelin, MK-0677 and L692,585 with corresponding increases in E_{max} (Fig. 5C; panel 1, 2 and 3 and Table I). Therefore, this compound is acting as an inverse agonist of the Gq-dependent signalling.

Discussion

Understanding the basis of interactions between ligands and their target GPCR receptors is central to efforts in pharmacology and development of novel therapeutic ligands. Moreover, in the case of GPCRs that are investigated as targets for the design of small molecule ligands to treat diseases, a detailed level of understanding of receptor signalling is key for the design of appropriate ligands with high selectivity to limit toxicity and off-target side effects.

The GHS-R1a receptor has been investigated as a promising pharmacological target for the modulation of the appetite and food intake (Howick et al., 2017; Pietra et al., 2014; Vodnik et al., 2016). However, the non-peptide GHS-R1a agonist anamorelin, currently in phase 3 trials for the treatment of cancer-anorexia-cachexia, is the only therapeutic targeting the GHS-R1a receptor so far (Garcia et al., 2015; Zhang and Garcia, 2015) .

The limited success of development of molecules targeting the GHS-R1a receptor from bench to bedside may be due to the complex signalling of the GHS-R1a receptor. Therefore, a better understanding of GHS-R1a receptor ligands and their signalling bias and selective functionality is poised to improve the development of ghrelin-mimetics and GHS-R1a receptor antagonist.

Indeed, recent evidence has demonstrated that the GHS-R1a receptor is functionally biased, favouring specific signalling transduction pathways over others depending on the specific ligand present (Evron et al., 2014; Sivertsen et al., 2013). In addition, GHS-R1a receptor agonists have been shown to act both as GHS-R1a agonists as well as allosteric modulators of the receptor (Holst et al., 2005). An example of biased agonism for the GHS-R1a receptor is the ligand JMV3018, which demonstrated to drive partial Gαq-protein coupling and signalling, but was unable to recruit β-Arrestin and also induced Gαi-protein coupling (Mary et al., 2012). Moreover, the hexapeptide KwFwLL was identified to favour the Gαq-protein over the Gα12/13-protein signalling pathway, therefore being labelled a biased inverse agonist (Holst

et al., 2007). Moreover, signalling bias for GHS-R1a receptor ligands towards β-Arrestin activity as compared to G protein-dependent activity was shown in addiction therapy *in vivo* (Toth et al., 2017).

Here, we investigated several GHS-R1a receptor ligands and modulators to establish the specific signalling pathways that they regulate and to shed light on the complex GHS-R1a receptor pharmacology, aimed to expand the current knowledge of the functional selectivity and biased signalling of the GHS-R1a receptor. Additionally, we provide evidence for "probe of dependence" at this receptor, which shows that the characteristics of the antagonist compounds or allosteric modulation, depend on the identity of the orthostheric agonist examined (Christopoulos, 2014; Keov et al., 2011).

Antagonists are defined by their capacity to bind to their target receptors, while lacking the ability to modulate receptor signalling. Although JMV2959 has been identified as GHS-R1a receptor agonist, we observed that JMV2959 produces a slight increase in calcium intracellular mobilisation in line with previous findings (M'Kadmi et al., 2015). However, increasing concentration of JMV2959 demonstrated an increase in GHS-R1a receptor localization on the membrane, suggesting a reduced GHS-R1a receptor constitutive activity. This data reinforces the complex pharmacology of this antagonist. JMV2959 was also shown to inhibit agonist-induced intracellular calcium mobilisation, receptor internalisation and ERK1/2 phosphorylation, in a dose-dependent manner, as expected from a full GHS-R1a receptor agonist.

The peptide-based [D-lys^3]-GHRP-6 ligand, has been used as a GHS-R1a receptor antagonist and is described in several *in vivo* and *in vitro* studies (Lien et al., 2016; Liu et al., 2016; Traebert et al., 2002). Here we observed that Dlys did not affect the ghrelin, MK-0677 or L692,585-mediated calcium mobilisation, but was able to attenuate the ghrelin and L692,585-

dependent receptor internalisation and ERK1/2 activation, but unexpectedly, it did not block MK-0677 receptor internalisation. This effect shows a probe of dependence effect and specificity in its inhibitor capacity, demonstrating a clear antagonist biased behaviour towards GHS-R1a-β-Arrestin dependent signalling. Interestingly, under basal conditions, Dlys did show a reduction of receptor internalisation indicating that they may have potential to reduce GHS-R1a receptor constitutive activity, similar to the GHS-R1a receptor inverse agonists, of [D-Arg(1), D-Phe(5), D-Trp(7,9), Leu(11)]-substance P (SP-analog). Indeed, SP-analog showed a reduction of the β-Arrestin-1 recruitment, a significant reduction in ERK1/2 phosphorylation and in GHS-R1a receptor internalisation compared to basal conditions in a ligand-independent manner, displaying a strong effect as a GHS-R1a receptor inverse agonist.

In addition, SP-analog showed a significant potentiation of GHS-R1a receptor agonist-mediated calcium signalling, hypothesized to be due to an increased membrane expression of the GHS-R1a receptor as previously reported (Liu et al., 2007). In fact, it has been shown previously that SP-analog potentiates the MK-0677 and ghrelin-dependent calcium activation (Holst et al., 2003). However, it is surprising that SP-analog behaves as competitive antagonist of the L695,585 agonist in the receptor internalisation assay, suggesting an overlap with the binding site for L692,585 ligand. In addition, SP-analog produces a relative low potency antagonist effect for ghrelin- and MK-0677-induced ERK activation of GHS-R1a receptor (<100 nM) which has been observed previously (Bennett et al., 2009). For that reason, the ratio between GHS-R1a receptor agonist/antagonist concentrations needs to be taken in account when *in vivo* studies are designed.

Additionally, the endogenous GHS-R1a receptor agonist ghrelin was compared to non-peptide GHS-R1a receptor agonists, MK-0677 and L692,585. All receptor agonist were found to increase intracellular calcium mobilisation, β-Arrestin-1 recruitment, receptor internalisation and ERK1/2 activity. Particularly, L692,585 appears to be a more potent agonist that the

endogenous ligand ghrelin, increasing the potency in G-protein dependent assays. At the same time, MK-0677 displayed a more potent activity than ghrelin and L692,585 in G-protein independent signalling measured by the internalization assay. In agreement with our results, it has been shown previously that MK-0677 is a bioavailable compound that activates growth hormone secretion *in vitro*, and has increased potency and efficacy compared with ghrelin in [35S]GTPγS scintillation proximity assay (Bennett et al., 2009). In the case of L692,585, it has also been reported that this ligand presents higher efficacy and potency than ghrelin in the [35S]GTPγS scintillation proximity assay (Bennett et al., 2009), however in our results we observed an increased potency but not a higher efficacy. Interestingly, these two agonists dock to the GHS-R1a receptor in the same site that ghrelin (Holst et al., 2009), beings orthosteric agonists, for that reason they present similar signalling pathway characteristics than ghrelin agonist. In addition, we tested the ability of pyridone, a novel non-peptide GHS-R1a receptor agonist (Pastor-Cavada et al., 2016) to activate the β-Arrestin recruitment. This compound was unable to activate this signalling pathway, suggesting that pyridone has a GHS-R1a-G-protein dependent pathway bias.

In summary, we have demonstrated that ligands reported as GHS-R1a receptor antagonist display functional selectivity and biased signalling properties and also modulate GHS-R1a receptor constitutive activity. These data thus challenge the conventional characterization of GHS-R1a receptor ligands into agonist, antagonist or inverse agonist. Further studies of GHS-R1a receptor are required to examine the role of the functional selectivity in mediating the effects of GHS-R1a receptor ligands *in vivo*. It is possible that the therapeutic effects of a particular selective GHS-R1a receptor antagonist is mediated via specific GHS-R1a-coupled second messenger signalling pathway, and its side effects via another. Specific molecular signalling pathways of this complex receptor may translate in the very divergent functionalities and behaviours *in vivo*. The findings in this study provide novel implications for designing

future drugs targeting the GHS-R1a receptor with higher selectivity and therefore, limited side effects.

Figure Legends

Figure 1. The Ghrelin signalling pathway. Ghrelin signalling begins with the interaction between ghrelin ligand and the GHS-R1a (growth hormone secretagogue type 1a) receptor, which exerts it effects via Gαq-, Gαi/o- or β-Arrestin- dependent mechanism. The Gαq activation leads to activation of phospholipase C (PLC), which leads to increase of intracellular Ca^{+2} levels. Ca^{2+} then activates several proteins including protein kinase C beta (PKCβ), calcium calmodulin dependent protein kinase II (CamKII) and AMP-activated protein kinase (AMPK). The activation of Gαi/o produces the activation of phosphatidylinositol 3 kinase (PI3K), which in turns produces the activation of protein kinase epsilon (PKCe), protein kinase A (PKA) and serine/threonine-specific protein kinase (AKT). In the β-Arrestin dependent pathway, the binding of ghrelin ligand to GHS-R1a receptor activates the extracellular signal–regulated kinase (ERK) and AKT that ultimately leads to receptor internalisation.

Figure 2. Characteristic of GHS-R1a receptor ligands on Gq- and β-Arrestin- depend signalling. (A) Concentration-response curves of five GHS-R1a receptor ligands in calcium mobilisation assay in Hek-GHS-R1a-EGFP cell line shown as % of FBS response (n=3-6, in duplicate). **(B)** Effect of GHS-R1a receptor ligands (500 nM, 1 h) on β-Arrestin-1 recruitment. RLU=Relative Luminescence Units (n=3, in triplicate). **(C)** Representative images of GHS-R1a receptor internalisation following of 500 nM ligands incubation for 1 h. Receptor internalisation is detected by EGFP fusion protein at C-terminus of GHS-R1a receptor using IN Cell analyser 1000. The cytoplasmic intensity of EGFP was evaluated as receptor internalisation. **(D)** Concentration-response curves of five ligands of GHS-R1a receptor internalisation. Results are normalized to buffer B (n=3-5, in triplicate). **(E, F)** Representative blots and analysis of phospho-ERK1 relative to Total ERK1/2 level are shown after 1, 10 and 100 nM of ghrelin treatment for 1 h or 1 μM of Dlys, JMV2959 or SP-analog treatment for 1 h (n=4) GFP and actin are shown as loading control. Data are the mean ± S.E.M * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ANOVA, Dunnett's post hoc test.

Figure 3. Dlys antagonist characteristics on GHS-R1a receptor-mediated signalling. (A) GHS-R1a receptor internalisation assay. HEK-GHS-R1a-EGFP cells were pre-treated for 1 h with 0.01, 0.1 or 1 μM Dlys and then challenged with increasing concentration of ghrelin, MK-0677 or L692,585 for 1 h. The intracellular intensity of EGFP was measured. Data were normalized to Buffer B (n=3-4, in triplicate). **(B)** Representative blots and quantitative analysis of p-ERK1/2 relative to total ERK1/2. HEK-GHS-R1a-EGFP cells were treated with 1 μM Dlys for 1 h and then treated with 1, 10, 100 nM of ghrelin, MK-0677 or L692,585. Data were normalized to Buffer B (n=3). **(C)** Calcium mobilisation assay. HEK-GHS-R1a-EGFP were treated along with 0.01, 0.1 or 1μM Dlys and then treated with increasing concentration of ghrelin, MK-0677 or L692,585 (n=4-5, in duplicate). Data showed as % of FBS response. Data are the mean ±S.E.M * $p<0.05$, ** $p<0.01$, ANOVA, Dunnett's post hoc test.

Figure 4. JMV2959 antagonist characteristics on GHS-R1a receptor-mediated signalling. (A) GHS-R1a receptor internalisation assay. HEK-GHS-R1a-EGFP cells were pre-treated for 1h with 0.01, 0.1 or 1 μM JMV2959 and then challenged with increasing concentration of ghrelin, MK-0677 or L692,585 for 1 h. The intracellular intensity of EGFP was measured. Data

were normalized to Buffer B (n=3-4, in triplicate). **(B)** Representative blots and quantitative analysis of p-ERK1/2 relative to total ERK1/2. HEK-GHS-R1a-EGFP cells were treated with 1 µM JMV2959 for 1h and then treated with 1, 10, 100 nM of ghrelin, MK-0677 or L692,585. Data were normalized to Buffer B (n=3). **(C)** Calcium mobilisation assay. HEK-GHS-R1a-EGFP were treated along with 0.01, 0.1 or 1 µM JMV2959 and then treated with increasing concentration of ghrelin, MK-0677 or L692,585 (n=4, in duplicate). Data showed as % of FBS response. Data are the mean ±S.E.M * $p<0.05$, ** $p<0.01$, ANOVA, Dunnett's post hoc test.

Figure 5. SP-analog antagonist/inverse agonist characteristics on GHS-R1a receptor-mediated signalling. (A) GHS-R1a receptor internalisation assay. HEK-GHS-R1a-EGFP cells were pre-treated for 1h with 0.01, 0.1 or 1 µM SP-analog and then challenged with increasing concentration of ghrelin, MK-0677 or L692,585 for 1 h. The intracellular intensity of EGFP was measured. Data were normalized to Buffer B (n=3-4, in triplicate). **(B)** Representative blots and quantitative analysis of p-ERK1/2 relative to ERK1/2. HEK-GHS-R1a-EGFP cells were treated with 1 µM SP-analog for 1 h and then treated with 1, 10, 100 nM of ghrelin, MK-0677 or L692,585. Data were normalized to Buffer B (n=3). **(C)** Calcium mobilisation assay. HEK-GHS-R1a-EGFP were treated along with 0.01, 0.1 or 1 µM SP-analog and then treated with increasing concentration of ghrelin, MK-0677 or L692,585 (n=4, in duplicate). Data showed as % of FBS response. Data are the mean ±S.E.M * $p<0.05$, ** $p<0.01$, ANOVA, Dunnett's post hoc test.

References

Abegg K, Bernasconi L, Hutter M, Whiting L, Pietra C, Giuliano C, Lutz TA and Riediger T (2017) Ghrelin receptor inverse agonists as a novel therapeutic approach against obesity-related metabolic disease. *Diabetes Obes Metab* **19**(12): 1740-1750.

Allas S and Abribat T (2013) Clinical perspectives for ghrelin-derived therapeutic products. *Endocr Dev* **25**: 157-166.

Bayliss JA, Lemus MB, Stark R, Santos VV, Thompson A, Rees DJ, Galic S, Elsworth JD, Kemp BE, Davies JS and Andrews ZB (2016) Ghrelin-AMPK Signaling Mediates the Neuroprotective Effects of Calorie Restriction in Parkinson's Disease. *J Neurosci* **36**(10): 3049-3063.

Beck B, Richy S and Stricker-Krongrad A (2004) Feeding response to ghrelin agonist and antagonist in lean and obese Zucker rats. *Life Sci* **76**(4): 473-478.

Bennett KA, Langmead CJ, Wise A and Milligan G (2009) Growth 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* **76**(4): 802-811.

Berrout L and Isokawa M (2017) Ghrelin upregulates the phosphorylation of the GluN2B subunit of the NMDA receptor by activating GHSR1a and Fyn in the rat hippocampus. *Brain Res*.

Bologna Z, Teoh JP, Bayoumi AS, Tang Y and Kim IM (2017) Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. *Biomol Ther (Seoul)* **25**(1): 12-25.

Bouzo-Lorenzo M, Santo-Zas I, Lodeiro M, Nogueiras R, Casanueva FF, Castro M, Pazos Y, Tobin AB, Butcher AJ and Camina JP (2016) Distinct phosphorylation sites on the ghrelin receptor, GHSR1a, establish a code that determines the functions of ss-arrestins. *Sci Rep* **6**: 22495.

Camina JP (2006) Cell biology of the ghrelin receptor. *J Neuroendocrinol* **18**(1): 65-76.

Camina JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG and Casanueva FF (2004) Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. *Endocrinology* **145**(2): 930-940.

Cavalier M, Crouzin N, Ben Sedrine A, de Jesus Ferreira MC, Guiramand J, Cohen-Solal C, Fehrentz JA, Martinez J, Barbanel G and Vignes M (2015) Involvement of PKA and ERK pathways in ghrelin-induced long-lasting potentiation of excitatory synaptic transmission in the CA1 area of rat hippocampus. *Eur J Neurosci* **42**(8): 2568-2576.

Chen JH, Huang SM, Chen CC, Tsai CF, Yeh WL, Chou SJ, Hsieh WT and Lu DY (2011) Ghrelin induces cell migration through GHS-R, CaMKII, AMPK, and NF-kappaB signaling pathway in glioma cells. *J Cell Biochem* **112**(10): 2931-2941.

Christopoulos A (2014) Advances in G protein-coupled receptor allostery: from function to structure. *Mol Pharmacol* **86**(5): 463-478.

Cong WN, Golden E, Pantaleo N, White CM, Maudsley S and Martin B (2010) Ghrelin receptor signaling: a promising therapeutic target for metabolic syndrome and cognitive dysfunction. *CNS Neurol Disord Drug Targets* **9**(5): 557-563.

Damian M, Marie J, Leyris JP, Fehrentz JA, Verdie P, Martinez J, Baneres JL and Mary S (2012) High constitutive activity is an intrinsic feature of ghrelin receptor protein: a study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. *The Journal of biological chemistry* **287**(6): 3630-3641.

Delporte C (2012) Recent advances in potential clinical application of ghrelin in obesity. *J Obes* **2012**: 535624.

Els S, Beck-Sickinger AG and Chollet C (2010) Ghrelin receptor: high constitutive activity and methods for developing inverse agonists. *Methods Enzymol* **485**: 103-121.

Evron T, Peterson SM, Urs NM, Bai Y, Rochelle LK, Caron MG and Barak LS (2014) G Protein and beta-arrestin signaling bias at the ghrelin receptor. *The Journal of biological chemistry* **289**(48): 33442-33455.

Garcia JM, Boccia RV, Graham CD, Yan Y, Duus EM, Allen S and Friend J (2015) Anamorelin for patients with cancer cachexia: an integrated analysis of two phase 2, randomised, placebo-controlled, double-blind trials. *Lancet Oncol* **16**(1): 108-116.

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

Holst B, Brandt E, Bach A, Heding A and Schwartz TW (2005) Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. *Molecular endocrinology* **19**(9): 2400-2411.

Holst B, Cygankiewicz A, Jensen TH, Ankersen M and Schwartz TW (2003) High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol* **17**(11): 2201-2210.

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

Holst B, Mokrosinski J, Lang M, Brandt E, Nygaard R, Frimurer TM, Beck-Sickinger AG and Schwartz TW (2007) Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism. *The Journal of biological chemistry* **282**(21): 15799-15811.

Holst B and Schwartz TW (2004) Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends in pharmacological sciences* **25**(3): 113-117.

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG and Van der Ploeg LH (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**(5277): 974-977.

Howick K, Griffin BT, Cryan JF and Schellekens H (2017) From Belly to Brain: Targeting the Ghrelin Receptor in Appetite and Food Intake Regulation. *Int J Mol Sci* **18**(2).

Inui A (2001) Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nature reviews Neuroscience* **2**(8): 551-560.

Keov P, Sexton PM and Christopoulos A (2011) Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. *Neuropharmacology* **60**(1): 24-35.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H and Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**(6762): 656-660.

Lee JH, Patel K, Tae HJ, Lustig A, Kim JW, Mattson MP and Taub DD (2014) Ghrelin augments murine T-cell proliferation by activation of the phosphatidylinositol-3-kinase, extracellular signal-regulated kinase and protein kinase C signaling pathways. *FEBS Lett* **588**(24): 4708-4719.

Lien GS, Lin CH, Yang YL, Wu MS and Chen BC (2016) Ghrelin induces colon cancer cell proliferation through the GHS-R, Ras, PI3K, Akt, and mTOR signaling pathways. *Eur J Pharmacol* **776**: 124-131.

Liu C, Huang J, Li H, Yang Z, Zeng Y, Liu J, Hao Y and Li R (2016) Ghrelin accelerates wound healing through GHS-R1a-mediated MAPK-NF-kappaB/GR signaling pathways in combined radiation and burn injury in rats. *Sci Rep* **6**: 27499.

Liu G, Fortin JP, Beinborn M and Kopin AS (2007) Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. *The Journal of pharmacology and experimental therapeutics* **322**(3): 1036-1043.

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

M'Kadmi C, Leyris JP, Onfroy L, Gales C, Sauliere A, Gagne D, Damian M, Mary S, Maingot M, Denoyelle S, Verdie P, Fehrentz JA, Martinez J, Baneres JL and Marie J (2015) Agonism, Antagonism, and Inverse Agonism Bias at the Ghrelin Receptor Signaling. *J Biol Chem* **290**(45): 27021-27039.

Mary S, Damian M, Louet M, Floquet N, Fehrentz JA, Marie J, Martinez J and Baneres JL (2012) Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. *Proceedings of the National Academy of Sciences of the United States of America* **109**(21): 8304-8309.

Mason BL, Wang Q and Zigman JM (2014) The central nervous system sites mediating the orexigenic actions of ghrelin. *Annu Rev Physiol* **76**: 519-533.

Mear Y, Enjalbert A and Thirion S (2013) GHS-R1a constitutive activity and its physiological relevance. *Front Neurosci* **7**: 87.

Moulin A, Brunel L, Boeglin D, Demange L, Ryan J, M'Kadmi C, Denoyelle S, Martinez J and Fehrentz JA (2013) The 1,2,4-triazole as a scaffold for the design of ghrelin receptor ligands: development of JMV 2959, a potent antagonist. *Amino Acids* **44**(2): 301-314.

Moulin A, Demange L, Berge G, Gagne D, Ryan J, Mousseaux D, Heitz A, Perrissoud D, Locatelli V, Torsello A, Galleyrand JC, Fehrentz JA and Martinez J (2007) Toward potent ghrelin receptor ligands based on trisubstituted 1,2,4-triazole structure. 2. Synthesis and pharmacological in vitro and in vivo evaluations. *Journal of medicinal chemistry* **50**(23): 5790-5806.

Mousseaux D, Le Gallic L, Ryan J, Oiry C, Gagne D, Fehrentz JA, Galleyrand JC and Martinez J (2006) Regulation of ERK1/2 activity by ghrelin-activated growth hormone secretagogue receptor 1A involves a PLC/PKCvarepsilon pathway. *Br J Pharmacol* **148**(3): 350-365.

Muller 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, Dieguez 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, Laferrere 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 and Tschop MH (2015) Ghrelin. *Mol Metab* **4**(6): 437-460.

Osterstock G, Escobar P, Mitutsova V, Gouty-Colomer LA, Fontanaud P, Molino F, Fehrentz JA, Carmignac D, Martinez J, Guerineau NC, Robinson IC, Mollard P and Mery PF (2010) Ghrelin stimulation of growth hormone-releasing hormone neurons is direct in the arcuate nucleus. *PLoS One* **5**(2): e9159.

Pastor-Cavada E, Pardo LM, Kandil D, Torres-Fuentes C, Clarke SL, Shaban H, McGlacken GP and Schellekens H (2016) A Novel Non-Peptidic Agonist of the Ghrelin Receptor with Orexigenic Activity In vivo. *Sci Rep* **6**: 36456.

Petersen PS, Woldbye DP, Madsen AN, Egerod KL, Jin C, Lang M, Rasmussen M, Beck-Sickinger AG and Holst B (2009) In vivo characterization of high Basal signaling from the ghrelin receptor. *Endocrinology* **150**(11): 4920-4930.

Pietra C, Takeda Y, Tazawa-Ogata N, Minami M, Yuanfeng X, Duus EM and Northrup R (2014) Anamorelin HCl (ONO-7643), a novel ghrelin receptor agonist, for the treatment of cancer anorexia-cachexia syndrome: preclinical profile. *J Cachexia Sarcopenia Muscle* **5**(4): 329-337.

Ritter SL and Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10**(12): 819-830.

Sanger GJ (2014) Ghrelin and motilin receptor agonists: time to introduce bias into drug design. *Neurogastroenterol Motil* **26**(2): 149-155.

Santos-Zas I, Lodeiro M, Gurriaran-Rodriguez U, Bouzo-Lorenzo M, Mosteiro CS, Casanueva FF, Casabiell X, Pazos Y and Camina JP (2013) beta-Arrestin signal complex plays a critical role in adipose differentiation. *Int J Biochem Cell Biol* **45**(7): 1281-1292.

Schellekens H, De Francesco PN, Kandil D, Theeuwes WF, McCarthy T, van Oeffelen WE, Perello M, Giblin L, Dinan TG and Cryan JF (2015) Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction. *ACS chemical neuroscience* **6**(7): 1186-1197.

Schellekens H, Dinan TG and Cryan JF (2010) Lean mean fat reducing "ghrelin" machine: hypothalamic ghrelin and ghrelin receptors as therapeutic targets in obesity. *Neuropharmacology* **58**(1): 2-16.

Schellekens H, Dinan TG and Cryan JF (2013a) Ghrelin at the interface of obesity and reward. *Vitam Horm* **91**: 285-323.

Schellekens H, McNamara O, Dinan TG, McCarthy JV, McGlacken GP and Cryan JF (2013b) Semagacestat, a gamma-secretase inhibitor, activates the growth hormone secretagogue (GHS-R1a) receptor. *The Journal of pharmacy and pharmacology* **65**(4): 528-538.

Schellekens H, van Oeffelen WE, Dinan TG and Cryan JF (2013c) Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *The Journal of biological chemistry* **288**(1): 181-191.

Sivertsen B, Holliday N, Madsen AN and Holst B (2013) Functionally biased signalling properties of 7TM receptors - opportunities for drug development for the ghrelin receptor. *British journal of pharmacology* **170**(7): 1349-1362.

Torres-Fuentes C, Theeuwes WF, McMullen MK, McMullen AK, Dinan TG, Cryan JF and Schellekens H (2014) Devil's Claw to suppress appetite--ghrelin receptor modulation potential of a Harpagophytum procumbens root extract. *PLoS One* **9**(7): e103118.

Toth K, Slosky LM, Pack TF, Urs NM, Boone P, Mao L, Abraham D, Caron MG and Barak LS (2017) Ghrelin receptor antagonism of hyperlocomotion in cocaine-sensitized mice requires betaarrestin-2. *Synapse*.

Traebert M, Riediger T, Whitebread S, Scharrer E and Schmid HA (2002) Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. *J Neuroendocrinol* **14**(7): 580-586.

Tschop M, Smiley DL and Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* **407**(6806): 908-913.

Vodnik M, Strukelj B and Lunder M (2016) Ghrelin Receptor Ligands Reaching Clinical Trials: From Peptides to Peptidomimetics; from Agonists to Antagonists. *Horm Metab Res* **48**(1): 1-15.

Zhang H and Garcia JM (2015) Anamorelin hydrochloride for the treatment of cancer-anorexia-cachexia in NSCLC. *Expert Opin Pharmacother* **16**(8): 1245-1253.

Figure 1

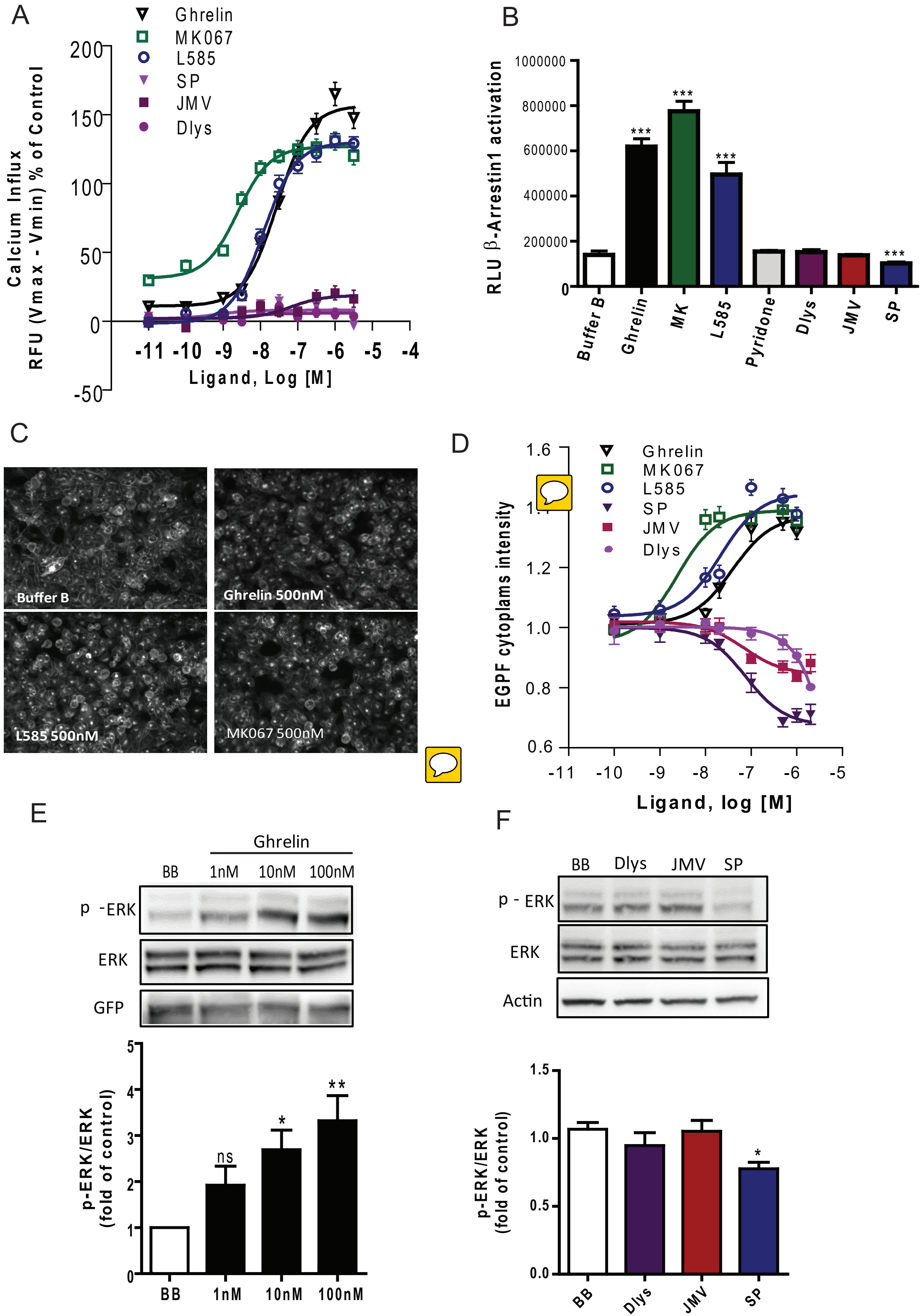
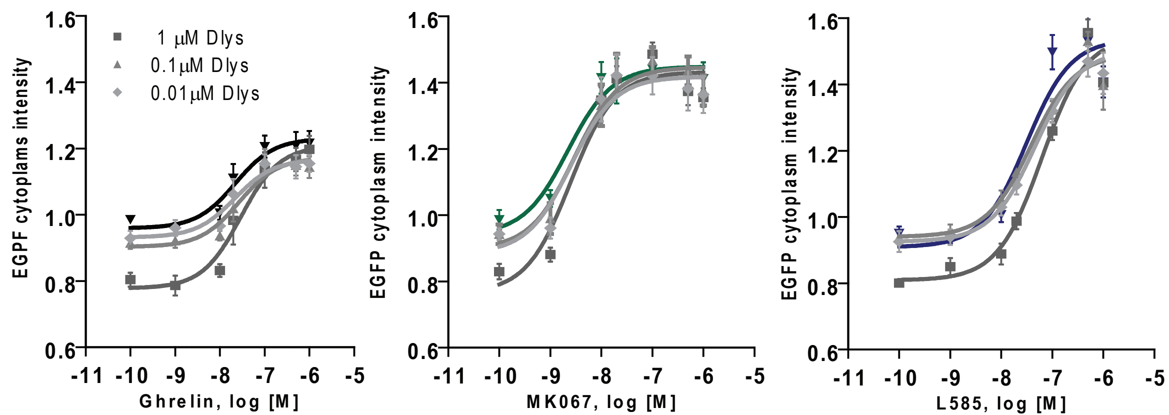
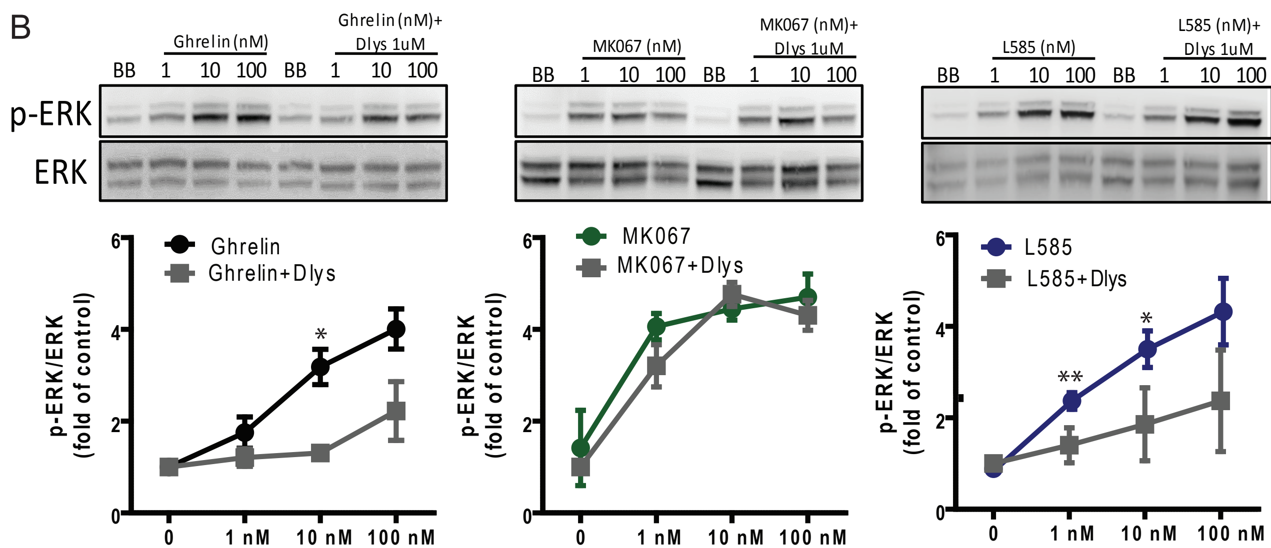


Figure 2





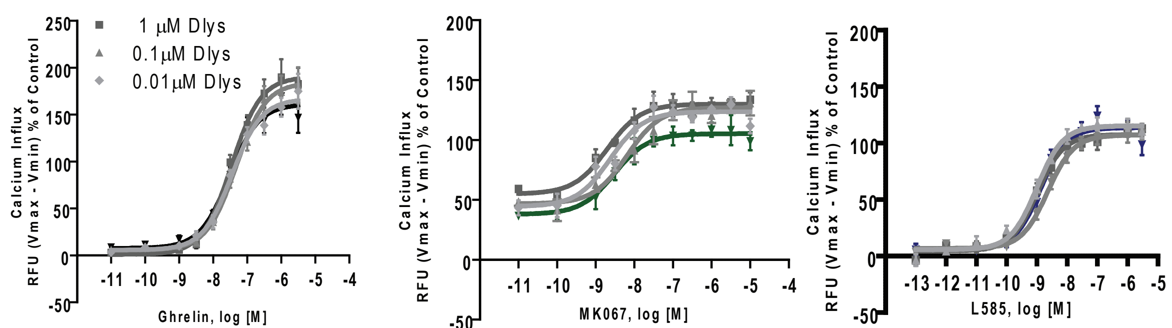


Figure 3

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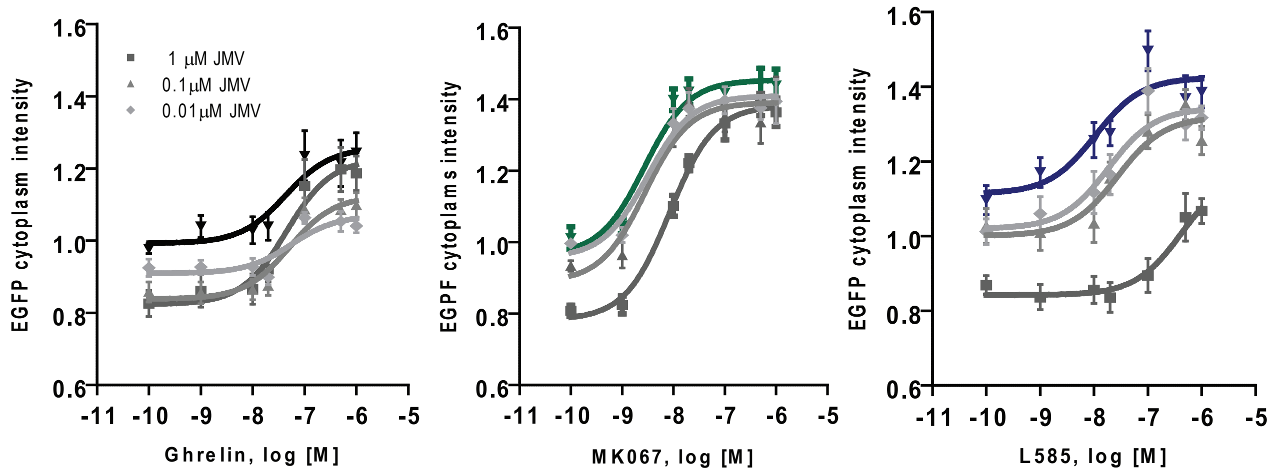

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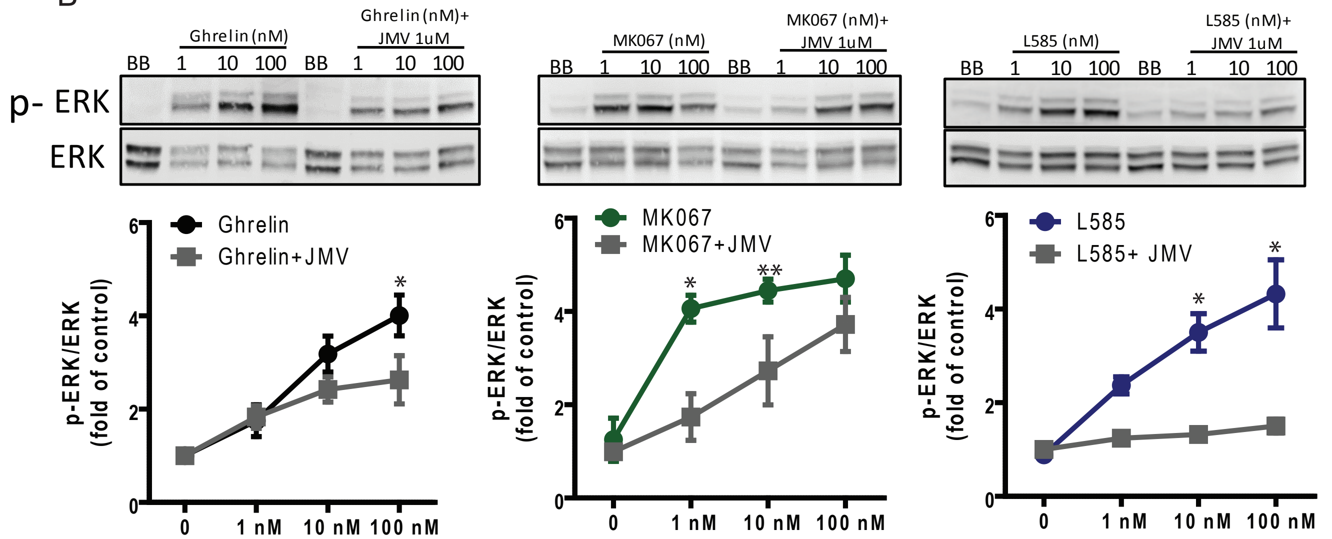

C

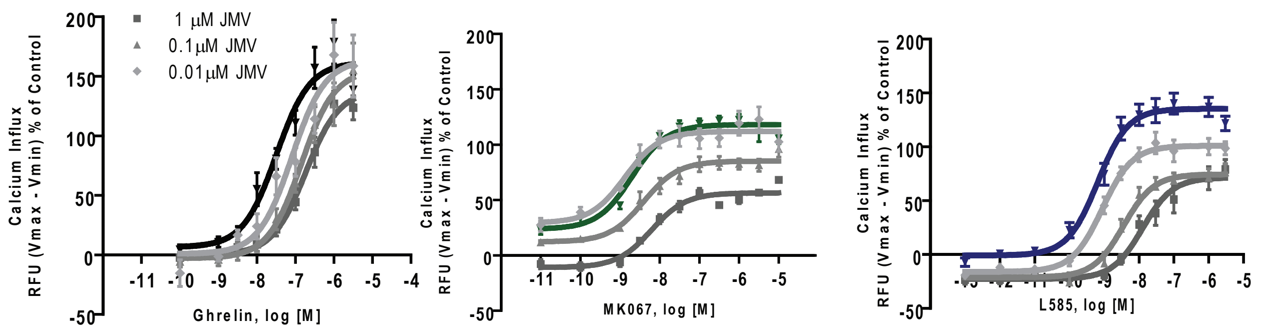

Figure 4

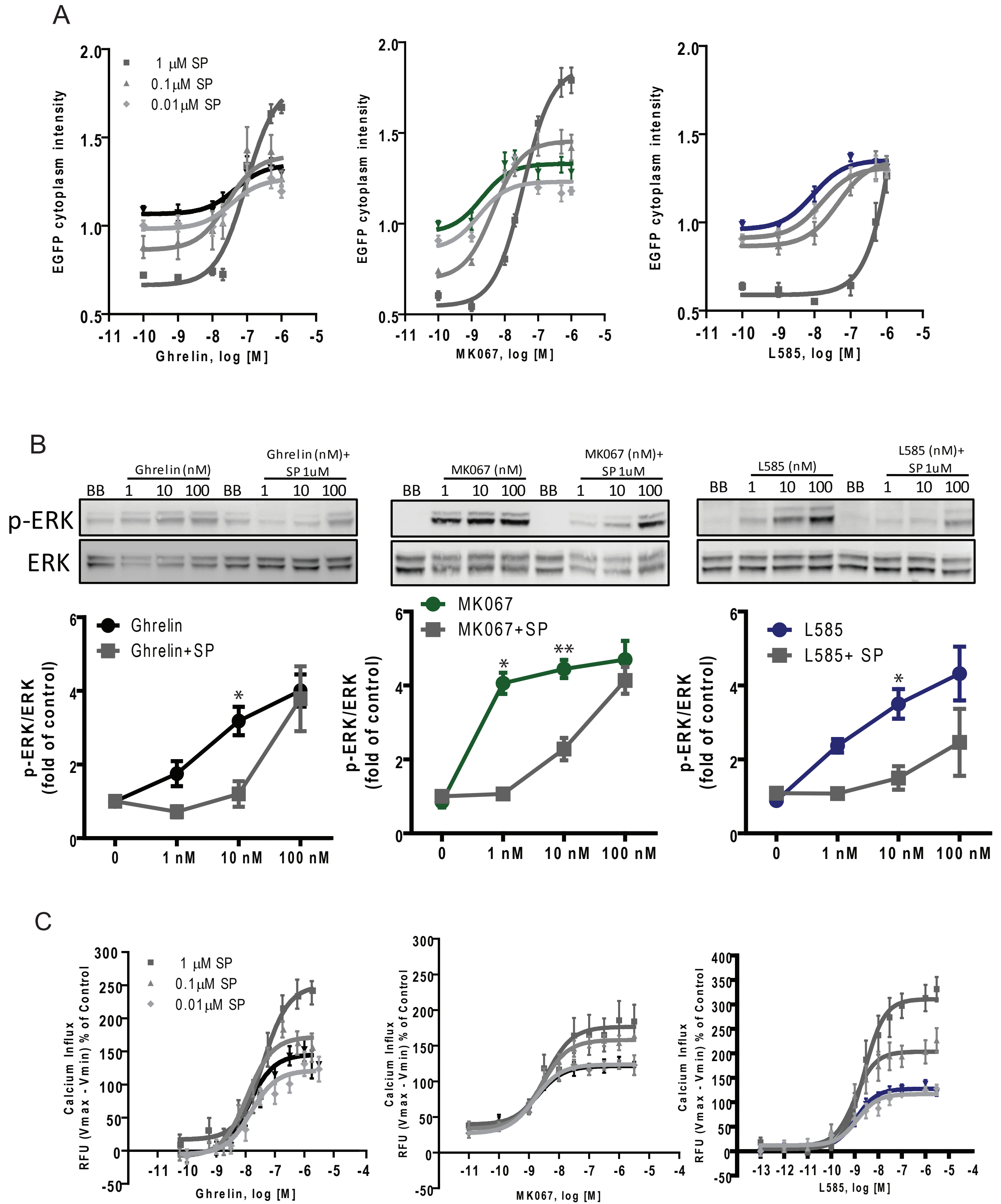

Table I. Potency and efficacy of Ghrelin, MK067 and L585 in the presence on increasing concentrations of Dlys, JMV and SP using GHSR-1a-EGFP internalisation assay. Emax is displayed as normalization with the control (Buffer B). Data are presented as mean S.E.M

Condition	pEC_{50}		E_{MAX}	
Ghrelin	7.65 ± 0.21		1.37 ± 0.02	
+ 0.01 µM Dlys	7.66 ± 0.22		1.17 ± 0.02	
+ 0.1 µM Dlys	7.57 ±0.19		1.17 ± 0.02	
+ 1 µM Dlys	7.53 ±0.18		1.21 ± 0.03	
+ 0.01 µM JMV	7.25 ±0.30		1.07 ± 0.02	*
+ 0.1 µM JMV	7.29 ± 0.22		1.13 ± 0.03	
+ 1 µM ±M JMV	7.35 ± 0.25		1.23 ± 0.04	
+ 0.01 µM SP	7.45 ± 0.39		1.27 ± 0.05	
+ 0.1 µM SP	7.57 ± 0.32		1.40 ± 0.07	
+ 1 µM SP	7.02 ± 0.09		1.80 ± 0.05	*
MK067	8.63 ± 0.16		1.39 ± 0.02	
+ 0.01 µM Dlys	8.59 ± 0.24		1.42 ± 0.03	
+ 0.1 µM Dlys	8.53 ± 0.21		1.45 ± 0.03	
+ 1 µM Dlys	8.60 ± 0.14		1.43 ± 0.02	
+ 0.01 µM JMV	8.51 ± 0.19		1.41 ± 0.02	
+ 0.1 µM JMV	8.55 ± 0.20		1.39 ± 0.02	
+ 1 µM ±M JMV	8.07 ± 0.09	***	1.38 ± 0.02	
+ 0.01 µM SP	8.78 ± 0.27		1.23 ± 0.02	
+ 0.1 µM SP	8.35 ± 0.15		1.46 ± 0.03	
+ 1 µM SP	7.45 ±0.07	***	1.86 ± 0.04	*
L585	7.66 ± 0.13		1.45 ± 0.02	
+ 0.01 µM Dlys	7.21 ± 0.09		1.50 ± 0.04	
+ 0.1 µM Dlys	7.42 ± 0.10		1.49 ± 0.03	
+ 1 µM Dlys	7.21 ± 0.09		1.47 ± 0.04	
+ 0.01 µM JMV	7.66 ± 0.29		1.31 ± 0.04	
+ 0.1 µM JMV	7.59 ± 0.27		1.31 ± 0.04	
+ 1 µM ±M JMV	6.37 ± 0.50	*	1.18 ± 0.14	**
+ 0.01 µM SP	7.81 ± 0.23		1.30 ± 0.03	
+ 0.1 µM SP	7.35 ± 0.26		1.35 ± 0.05	
+ 1 µM SP	5.48 ± 0.82	*	1.31 ± 0.03	

**p* < 0.05, ***p*<0.01, ****p*<0.001 *one-way ANOVA with Dunnett's post hoc test*

Table II. Potency and efficacy of Ghrelin, MK067 and L585 in the presence on increasing concentrations of Dlys, JMV and SP using Calcium mobilisation assay. Emax is displayed as normalization with the control (Buffer B). Data are presented as mean S.E.M

Condition	pEC$_{50}$		E_{MAX}	
Ghrelin	7.56 ± 0.07		156.5 ± 3.7	
+ 0.01 µM Dlys	7.45 ± 0.08		151.8 ± 5.53	
+ 0.1 µM Dlys	7.33 ± 0.10		168.2 ± 7.29	
+ 1 µM Dlys	7.47 ± 0.09		174.3 ± 5.53	
+ 0.01 µM JMV	7.40 ± 0.20		136.5 ± 10.2	
+ 0.1 µM JMV	7.06 ± 0.16		131.7 ± 8.45	
+ 1 µM JMV	6.82 ± 0.11	**	123.5 ± 6.30	
+ 0.01 µM SP	7.75 ± 0.19		117.7 ± 7.61	
+ 0.1 µM SP	7.76 ± 0.14		165.0 ± 7.39	
+ 1 µM SP	7.29 ± 0.15		227.6 ± 11.9	**
MK067	8.61 ± 0.09		120.9 ± 2.46	
+ 0.01 µM Dlys	8.63 ± 0.14		123.6 ± 2.89	
+ 0.1 µM Dlys	8.19 ± 0.16		127.1 ± 3.66	
+ 1 µM Dlys	8.70 ± 0.15		130.0 ± 2.88	
+ 0.01 µM JMV	8.88 ± 0.16		112.0 ± 3.35	
+ 0.1 µM JMV	8.40 ± 0.12		85.17 ± 2.51	
+ 1 µM JMV	8.20 ± 0.09	*	56.5 ± 1.84	**
+ 0.01 µM SP	8.97 ± 0.22		103.2 ± 3.85	
+ 0.1 µM SP	8.58 ± 0.10		156.8 ± 3.48	
+ 1 µM SP	8.68 ± 0.31		177.6 ± 9.24	*
L585	7.93 ± 0.06		129.9 ± 2.64	
+ 0.01 µM Dlys	8.00 ± 0.08		117.5 ± 2.80	
+ 0.1 µM Dlys	7.64 ± 0.07		117.3 ± 2.44	
+ 1 µM Dlys	7.99 ± 0.09		117.5 ± 2.80	
+ 0.01 µM JMV	7.81 ± 0.09		101.0 ± 3.14	
+ 0.1 µM JMV	7.19 ± 0.11	*	77.51 ± 3.81	**
+ 1 µM JMV	6.64 ± 0.12	*	71.55 ± 4.90	***
+ 0.01 µM SP	7.90 ± 0.16		116.7 ± 5.40	
+ 0.1 µM SP	7.99 ± 0.17		203.5 ± 10.11	
+ 1 µM SP	7.61 ± 0.12		311.0 ± 13.47	***

**p< 0.05, **p<0.01, ***p<0.001 one way ANOVA with Dunnett's post hoc test*