Characterisation of the Molecular Mechanisms Regulating Luteinizing Hormone Receptor Trafficking and Signalling:

Role of APPL1 in GPCR Action and Reproduction

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Statement of originality

All experiments included in this thesis were performed by myself, Silvia Sposini, excluding Figure 5.3 (experiments executed by Dr. Aylin Hanyaloglu) and Figure 5.10 (experiments executed by Dr. Frederic Jean-Alphonse).

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Abstract

G protein coupled receptors (GPCRs) are the major family of membrane proteins transducing extracellular stimuli into intracellular signals. The luteinizing hormone receptor (LHR) is a GPCR expressed in gonadal and extragonadal tissues where it plays pivotal roles in reproduction and pregnancy. Endocytic trafficking of GPCRs represents a key mechanism in defining cellular responses by controlling signalling at both temporal and spatial level. After ligand activation, LHR is internalised into very early endosomes (VEEs), small vesicles (~400nm in diameter) close to the plasma membrane and distinct from the classic early endosome. Our laboratory identified the Adaptor Protein containing pleckstrin homology (PH) domain, PTB domain and Leucine zipper motif 1 (APPL1) as a marker of ~50% of VEEs where LHR is internalised to. My aims were to characterise the VEE by identifying the mechanisms dictating LHR post-endocytic sorting from this novel compartment, the role of APPL1 and how this impacts receptor signalling. Imaging LHR recycling in real time at single event resolution, enabled me to identify the kinetics and the machinery involved. While APPL1 was not required for LHR localisation in VEEs, it is essential for receptor recycling and negative regulation of cAMP production; two functions never ascribed before to APPL1 for any membrane cargo. These two functions of APPL1 are regulated in an opposing manner by phosphorylation of Ser410 of APPL1, achieved by activation of the $G\alpha_s$-cAMP-PKA pathway, highlighting the mutual regulation of GPCR trafficking and signalling. I also demonstrated that both $G\alpha_s$-cAMP and $G\alpha_{q/11}$-calcium signalling require LHR internalisation, that endosomal signalling and recycling involves distinct adenylate cyclases and that $G\alpha_s$ activation is restricted to microdomains of the VEE. Finally, LHR recycling and cAMP signalling are also regulated by APPL1 in primary human endometrial stromal cells, where LHR trafficking and signalling could be perturbed in pathological conditions such as PCOS.

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The one who deserves the biggest thank above all is Giulio, for being the best person I could share my life with.

List of abbreviations

%; percent

°C; degree Celsius

a.u.; arbitrary units

2-HE; 2-dideoxyestradiol

AC; adenylate cyclase

AD; Alzheimer's disease

AdipoR; adiponectin receptor

AKAP; A-kinase anchoring protein

ALIX; ALG-interacting protein X

AMPK; adenosine-5'-monophosphate activated protein kinase

APEX; ascorbic acid peroxidise

APP; β-Amyloid precursor protein

APPL1; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1

Arf6; ADP-rybosylation factor 6

ARNO; ARF nucleotide binding site opener

ARRDC; arrestin-domain containing protein

ART; assisted reproductive technology

ATP; adenosine-5'-triphosphate

B1AR; β2-adrenergic receptor

B2AR; β2-adrenergic receptor

BAR; Bin1-Amphiphysin-Rvs167

bp; base pairs

BPP; between PH and PTB

BRET; bioluminescence resonance energy transfer

BSA; bovine serum albumine

Ca^{2+}; calcium cations

$CaCl_2$; calcium cloride

cAMP; cyclic adenosine monophosphate

CCP; clathrin coated pit

CDE; clathrin dependent endocytosis

CFP; cyan fluorescent protein

CIE; clathrin independent endocytosis

CO_2; carbon dioxide

Cryo-EM; Cryo-electron microscopy

CXCR4; chemokine receptor 4

DAG; diacylglycerol

DCC; deleted in colorectal cancer

ddA; 2'-5'-dideoxyestradiol

DMEM; Dulbecco's modified eagles medium

DMSO; dimethyl sulfoxide

DNA; deoxyribonucleic acid

DNase I; deoxyribonuclease I

dNTP; deoxynucleotide triphosphate

DOR; δ-opioid receptor

DS; Down's syndrome

DTT; dithiothreitol

ECL; extracellular loop

EE; early endosome

EEA1; early endosome antigen 1

EGF; epidermal growth factor

EGFR; epidermal growth factor receptor

Em; emission

EPAC; exchange protein directly activated by cAMP

ER; endoplasmic reticulum

ERK; extracellular signal-regulated kinase

ESCRT; endosomal sorting complex required for transport

Ex; excitation

FACS; fluorescence activated cell sorting

FBS; fetal bovine serum

FEME; fast endophilin mediated endocytosis

FRET; fluorescence resonance energy transfer

FSH; follicle stimulating hormone

FSHR; follicle stimulating hormone receptor

g; gram

g; gravity force

GAP; GTPase activating protein

GAPDH; glyceraldehyde 3-phosphate dehydrogenase

GDI; guanine nucleotide dissociation inhibitor

GDP; guanosine-5'-diphosphate

GEF; guanine nucleotide exchange factor

GFP; green fluorescent protein

GIPC; GAIP-interacting protein C-terminus

GLP1R; glucagon-like peptide 1 receptor

GnRH; gonadotrophin releasing hormone

GnRHR; gonadotrophin releasing hormone receptor

GPCR; G protein coupled receptor

GRK; GPCR kinases

GSK3; glycogen synthase kinase 3

GTP; guanosine-5'-triphosphate

h; hour

H_2O; water

hCG; human chorionic gonadotrophin

HCl; hydrochloric acid

HEK 293; human embryonic kidney 293

HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hESC; human endometrial stromal cell

HRP; horseradish peroxidase

Hrs; HGF regulated tyrosine kinase substrate

IBMX; 3-isobutyl-1-methylxanthine

ICL; intracellular loop

IF; immunofluorescence

IP3; inositol-1,4,5-trisphosphate

IP3K; inositol-1,4,5-trisphosphate 3-Kinase

ISO; isoproterenol

IVF; *in vitro* fertilization

JNK; c-Jun N-terminal kinase

kDa; kilodaltons

L; litre

LB; lysogeny broth or Luria Bertani broth

LE; late endosome

LH; luteinizing hormone

LHR; luteinizing hormone receptor

MAPK; mitogen-activated protein kinase

M; molar

mg; milligram

$MgCl_2$; magnesium chloride

min; minute

ml; millilitre

mM; millimolar

MOR; μ-opioid receptor

MPA; medroxyprogesterone

MVB; multi vesicular body

MVE; multi vesicular endosome

NA; numerical aperture

NaCl; sodium chloride

NAM; negative allosteric regulator

Nb; nanobody

nm; nanometre

nM; nanomolar

NP-40; nonylphenyl polyethylene glycol

OCRL; Oculo-Cerebro-Renal syndrome of Lowe

o/n; overnight

PALM; photo-activable localisation microscopy

PAM; positive allosteric regulator

PAR; protease activated receptor

PBS; phosphate buffered saline

PCOS; polycystic ovary syndrome

PCR; polymerase chain reaction

PDE; phosphodiesterase

PDZ; postsynaptic density 95/disc large/zonula occludens-1

PFA; paraformaldehyde

PH; pleckstrin homology

PI3K; phosphatidylinositol-3-phosphate kinase

PI3P; phosphatidylinositol-3-phosphate

PI4P; phosphatidylinositol-4-phosphate

PIP2; phosphatidylinositol-4,5-bisphosphate

PKA; protein kinase A

PKB; protein kinase B (also known as Akt)

PKC; protein kinase C

PLC; phospholipase C

PstIns; phosphoinositide

PTHR; pituitary hormone receptor

PTM; post translational modification

RGS; regulator of G protein signaling

Rluc; *Renilla reniformis* luciferase

RNA; ribonucleic acid

rpm; revolution per minute

S1P1R; sphingosine-1-phosphate 1 receptor

sAC; soluble adenylate cyclase

SDS; sodium dodecyl sulphate

SDS-PAGE; SDS-polyacrilamide gel electrophoresis

SEM; stardard error of the mean

SEP; super ecliptic pHluorin

SIM; structured illumination microscopy

siRNA; small interfering RNA

SNX; sorting nexin

STORM; stochastic optical reconstruction microscopy

TBS; tris buffered saline

TBS-T; TBS-Tween

TfR; trasnferrin receptor

TGN; trans Golgi network

TIR-FM; total internal reflected-fluorescence microscopy

TM; transmembrane

tmAC; transmembrane adenylate cyclase

TrkA; tropomyosin receptor kinase A (also known as nerve growth factor receptor)

TSHR; thyroid stimulating hormone receptor

V; volts

V2R; vasopressin receptor 2

v/v; volume/volume

VEE; very early endosome

Vps27; vesicular protein sorting-associated protein 27

w/v; weight/volume

WASH; Wiskott-Aldrich Syndrome Protein and SCAR Homolog complex

WDFY2; WD repeat and FYVE domain-containing protein 2

WT; wild-type

YFP; yellow fluorescent protein

µ; micro

µg; microgram

µm; micron

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Chapter 1:

Introduction

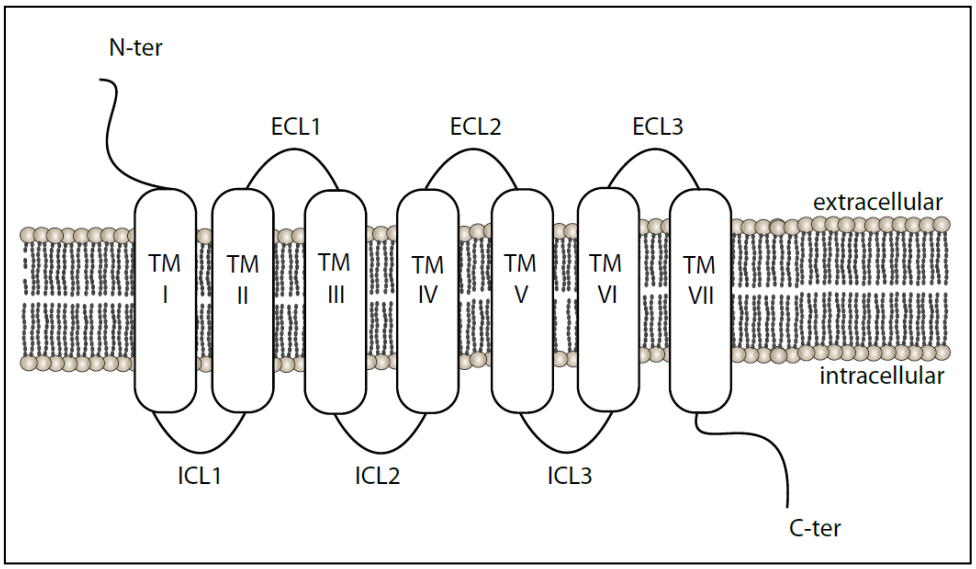
1.1 G protein coupled receptors

G protein coupled receptors (GPCRs) are the largest family of membrane proteins, covering ~3% of the human proteome (Fredriksson and Schioth, 2005). They convert extracellular stimuli into intracellular signals in response to a variety of ligands: ions, light, amino acids, nucleotides, neurotransmitters and hormones. Thanks to their broad responsiveness and to their ubiquity in the human body, GPCRs mediate a vast array of biological processes, thus they are the most exploited drug targets, with 40% of all prescription pharmaceuticals on the market acting on GPCRs (Rask-Andersen et al., 2011).

1.1.1 Structure and classification

Although the GPCR superfamily comprises more than 800 receptors, all of its members share a highly conserved structure; they are composed by seven hydrophobic transmembrane (TM) alpha helices, linked by three intracellular and three extracellular loops, an extracellular amino-terminal domain and an intracellular carboxy-terminal domain. This common signature gives them the name of 7TM receptors (Figure 1.1). GPCR structure can be divided into three modules; the extracellular, the TM and the intracellular domain. The extracellular module, which exhibits the highest variability across GPCRs, is, in most cases, responsible for ligand binding, whilst the intracellular module interacts with cytosolic protein partners including G proteins (Katritch et al., 2013).

A.



B.

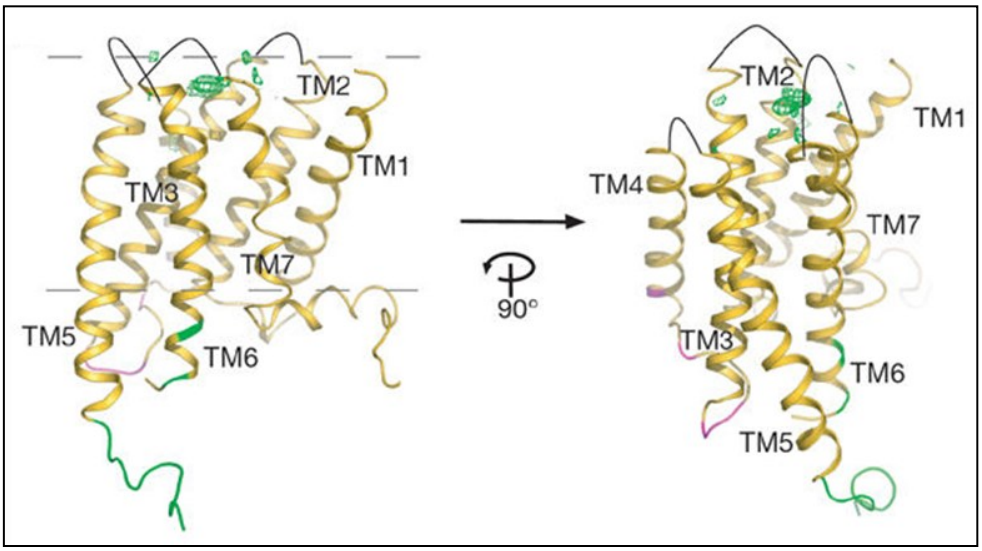


Figure 1.1 Schematic diagram and crystal structure of GPCRs. (A) Cartoon depicting GPCR topology; all GPCRs are composed by seven transmembrane helices (TMs), three extracellular loops (ECLs) and three intracellular ones (ICLs), an extracellular amino-terminal domain (N-ter) and a cytosolic carboxy-terminal tail (C-tail). (B) Structure of human β2-adrenergic receptor crystals depicting the spatial arrangement of the TM bundle. Adapted from (Rasmussen et al., 2007).

The TM module shows the greatest similarity across GPCRs, thus, based on the sequence homology of the TMs, the GPCR superfamily has been divided into 5 families: Rhodopsin,

Adhesion, Secretin, Glutamate and Frizzled (Fredriksson et al., 2003). Of these, Rhodopsin is the largest in vertebrates and the most studied. Rhodopsin is also the first GPCR for which the crystal structure was solved, thanks to its extreme stability in a detergent-solubilised state and its natural abundance in bovine retinas (Palczewski et al., 2000). Due to the difficulties in expressing and purifying GPCRs, it took almost 10 years for the second GPCR crystal structure, the β2-adrenergic receptor (B2AR), to be determined ((Rasmussen et al., 2007) and Figure 1.1 B). As a result of the tremendous advance in the development of methods for the crystallisation of GPCRs, exploiting T4 lysozyme fragments or the camelid single-domain antibodies (nanobodies) to stabilise receptors in a certain conformation, ~100 crystal structures of 38 receptors have now been reported (White, 1998); they include GPCRs from all different families, in both inactive and active state though seldom in conjunction with the cognate G protein or β-arrestin. Additionally, a highly innovative approach has been recently developed to determine GPCR structures: single particle Cryo-electron microscopy (Cryo-EM). Overcoming most of the requirement for crystal structure determination, Cryo-EM has already provided the tools to resolve two GPCR structures at nearly atomic resolution (Zhang et al., 2017b, Liang et al., 2017). GPCR structure determination has enabled a deeper understanding of the mechanistic aspects of how these proteins work, as well as providing new opportunities for drug development.

1.1.2 Activation

As mentioned above, crystal structure analysis has hugely increased our knowledge on GPCR activation and conformational changes. Receptors exist in multiple inactive and active forms, determined by the interaction with the ligand or other protein partners. The inactive states of the receptor are maintained by inter-helical stabilizing interactions; ligand binding disrupts these intra-molecular constrains leading to a new and energetically favourable conformational state of the receptor. While the structural mechanisms assigned to receptor activation are similar for many GPCRs, the processes causing them are quite different. Not all the receptors share the same inactive conformation and the energy associated with their inactive states greatly affects the receptor shift to the active state (McCudden et al., 2005). Furthermore, receptor activation is also influenced by ligand efficacy and affinity that

depend on the chemical properties of the ligand. Accordingly, ligands can be divided into groups: agonists, which fully activate the receptor; partial agonists induce submaximal activation of coupled G protein even at saturating concentration; inverse agonists inhibit basal activity of the receptor; antagonists, which have higher affinity for the receptor compared to agonists but no efficacy, thus do not activate the receptor and, once bound, inhibit the function of agonists (Kobilka, 2007). After the first step, the sequence of events during receptor activation is shared by all GPCRs and this similarity is much higher at the cytoplasmic side of the membrane bundle where the activated receptor is coupled to the heterotrimeric G protein. Although the pre-association of the receptor with its cognate G protein is still a matter of debate, growing evidence points to the fact that the "pre-coupling" model could explain the high specificity of coupling and the rapidity of the G protein activation (Oldham and Hamm, 2008). Heterotrimeric G proteins are composed by three subunits: Gα (45 kDa), Gβ (35 kDa), Gγ (7 kDa). In humans there are 21 different Gα subunits, 6 Gβ and 12 Gγ. In the inactive state, the Gα subunit is bound to GDP and is associated with Gβγ subunits, with the latter heterodimer increasing the affinity of Gα to GDP. Receptor activation enables it to act as a guanine nucleotide exchange factor (GEF) leading to the Gα-bound GDP to be exchanged for GTP. The GTP-bound Gα dissociates from the Gβγ dimer where each can then go on to modulate the activity of various downstream effectors. The hydrolysis of GTP to GDP is induced by GTPase-activating proteins (GAPs). Gα has weak intrinsic GTPase activity, thus additional molecules called Regulator of G protein signalling (RGS) facilitate this process, triggering heterotrimeric complex re-association and the return of the system to the basal state (Figure 1.2) (Oldham and Hamm, 2008, McCudden et al., 2005).

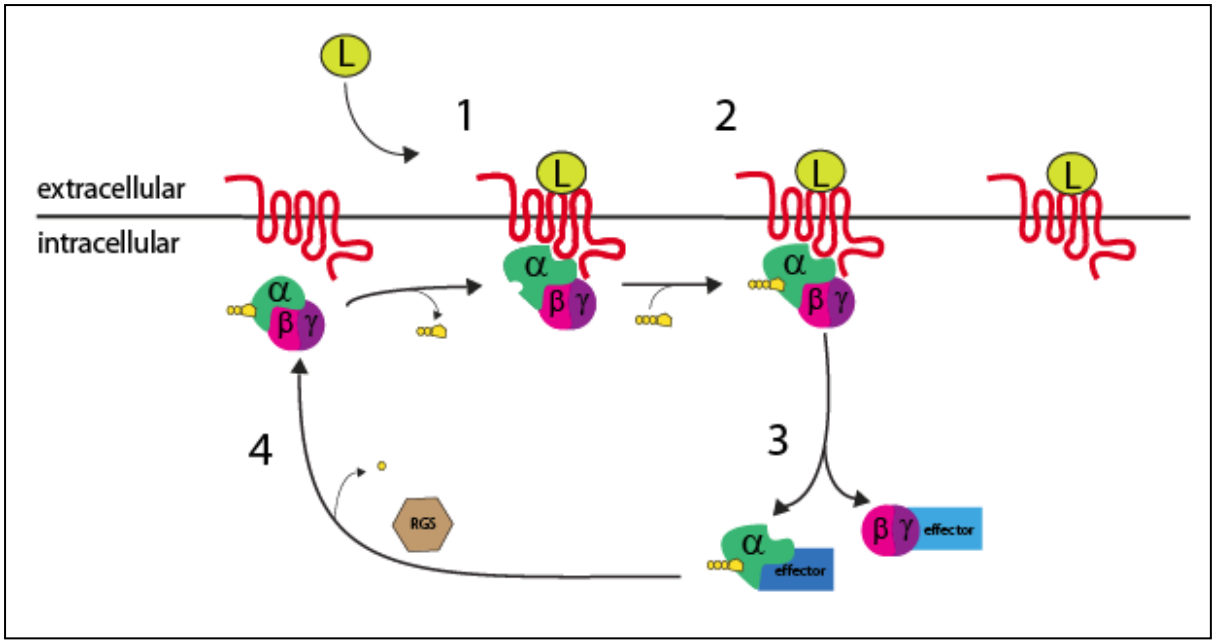


Figure 1.2 Model of heterotrimeric G protein activation. Following ligand binding (1), the activated GPCR switches on the cognate heterotrimeric (α,β,γ) G protein by promoting the exchange of GDP for GTP in the α subunit (2). Both α and β/γ subunits detach from the GPCR and start the signal transduction through cellular effectors (3). Regulators of G protein signalling (RGS) stimulate the GTPase activity of Gα, thus the re-association with β/γ and the return to the basal state (4).

1.1.3 Signalling pathways

Based on the primary sequence similarity of Gα, G proteins have been divided into four classes: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ ((Milligan and Kostenis, 2006) and Figure 1.3). $G\alpha_s$ and $G\alpha_{i/o}$ are both involved in the regulation of cAMP production by acting on membrane anchored enzymes called adenylate cyclases (ACs), with $G\alpha_s$ stimulating and $G\alpha_{i/o}$ inhibiting ACs (Stryer and Bourne, 1986). Active ACs catalyse the conversion of ATP to cAMP, which binds to its effectors such as the two regulatory subunits of PKA, which triggers conformational changes that lead to the release of the two catalytic subunits of the PKA tetrameric holoenzyme; the active enzyme can now phosphorylate its targets on specific Ser/Thr (Beebe, 1994). The $G\alpha_{q/11}$ class acts on another type of enzymes, phospholipases, which catalyse the conversion of phospholipids. $G\alpha_{q/11}$ main target is phospholipase C (PLC)-β, which hydrolyses the membrane phospholipid phosphatidylinositol-4,5-bisphosphate

(PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 triggers Ca^{2+} from intracellular storages and DAG stimulates PKC activity (Strathmann and Simon, 1990). $G\alpha_{12/13}$ is the most recently discovered class of G proteins. They act on the members of the Rho-GEF family, which, upon activation by $G\alpha_{12}$ or $G\alpha_{13}$, traslocate from the cytosol to the plasma membrane and activate the small GTPase RhoA, that targets downstream effectors including cytoskeletal proteins (Strathmann and Simon, 1991).

The Gβγ heterodimer has been recognised to perform other functions apart from acting as a guanine nucleotide dissociation inhibitor (GDI) for Gα. It has been shown to couple at least to four effectors: PLC, K^+ and Ca^{2+} channels, PI3K and ACs ((Smrcka, 2008) and Figure 1.3). Interestingly, it has been recently shown that the Gβγ of one receptor could mediate the signalling of another GPCR, thus facilitating inter-GPCR crosstalk without physical interaction of the two receptors (Jean-Alphonse et al., 2016).

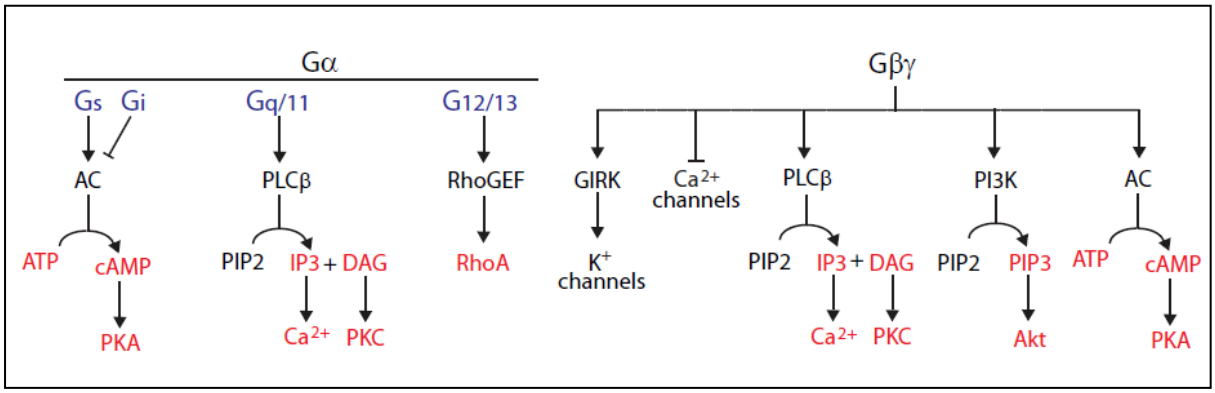


Figure 1.3 Schematic representation of different the G protein subunits and the signalling pathways they activate. There are 4 main Gα classes (blue): stimulatory Gα ($G\alpha_s$) activates AC which converts ATP into cAMP; inhibitory Gα ($G\alpha_i$), inhibit cAMP production; $G\alpha_{q/11}$ activates PLC-β that cleaves PIP2 into DAG and IP3 which activate PKC and Ca^{2+} release from intracellular storages, respectively; $G\alpha_{11/12}$ activates members of the Rho family of GTPases. Second messengers are in red.

All of the G protein subunits described above also contribute to the activation of another signalling pathway, the mitogen-activated protein kinase (MAPK) cascade, whose

components are among the major cellular signalling effectors (Seger and Krebs, 1995). There are four types of MAPKs and all of them are Ser/Thr kinases; these are: ERK, c-Jun N-terminal kinase (JNK); ERK5; p38 (Li et al., 2016). The ERK pathway can be activated by both agonist and antagonist molecules and in both G protein-dependent and -independent manner, in the latter case involving another class of GPCR interacting proteins, β-arrestins (Lefkowitz and Shenoy, 2005). In brief the ERK cascade is composed by the following steps: upon receptor activation, Ras recruits and activates the Ser/Thr kinase Raf; Raf promotes MEK1/2 kinase activation and ERK 1/2 phosphorylation, leading to ERK 1/2-mediated phosphorylation of either cytosolic or nuclear proteins (Chung and Kondo, 2011).

1.1.4 Diversification of GPCR activity

As presented above, the main mechanism for GPCRs to translate messages from the extracellular environment is via coupling to distinct heterotrimeric G proteins. However, given that an individual cell can express more than 100 GPCRs (Vassilatis et al., 2003, Hakak et al., 2003), an outstanding question for many years was to understand how such a limited number of G protein pathways can mediate diverse responses in different tissues. GPCR signalling can be diversified in various ways and at different levels; receptors can aggregate with each other or with other types of receptors forming homo- or hetero-dimers, as well as higher order oligomers; splice variants of receptors can be generated, characterised by altered responsiveness to ligands or coupling to G protein; post-translational modifications modulate receptor interactions with other proteins; membrane trafficking is exploited by cells to target receptors to defined locations, thus distinct signalling fates. Each of these mechanisms will be discussed in a dedicate subparagraph.

1.1.4.1 Dimerisation

The classic model of GPCRs functioning as a single unit at the plasma membrane has been largely revised after the discovery that many GPCRs, if not all, work in conjuction with each other through physical interactions mostly taking place at the TM core (Hiller et al., 2013). The first visual evidence of dimeric GPCRs was made possible in 2003, when rhodopsin

dimers were observed in native disc membranes (Fotiadis et al., 2003). Since then, a number of crystal structures, as well as functional complementation studies *in vivo*, confirmed the dimeric arrangement of GPCRs (Manglik et al., 2012, Wu et al., 2010, Rivero-Muller et al., 2010), as well as strengthening the findings obtained by molecular, biochemical and biophysical studies showing the existence of multi-unit GPCR complexes (Hebert et al., 1996, Hernanz-Falcon et al., 2004, Kroeger et al., 2001). Resonance energy transfer approaches such as bioluminescence- and fluorescence resonance energy transfer (BRET and FRET, respectively) have been widely used techniques in this field, allowing direct assessment of GPCR dimerisation in live cells in real time (McVey et al., 2001, Angers et al., 2000, Herrick-Davis et al., 2006). These approaches revealed that dimers could form constitutively or in response to ligand, and even when constitutive dimers were observed, ligand stimulation induced rearrangements in the dimeric unit. GPCR oligomeric units are diverse; dimers can be formed by two units of the same (homo-) or different (hetero-dimers) receptors, as well as more than two units can form higher order trimers and tetramers, and even oligomers with more than three different GPCRs (Ferre et al., 2009, Ferre et al., 2014). A vast array of functions has been proposed for both homo- and hetero-dimers/oligomers. Homo-dimerisation could play a central role in folding, maturation and cell surface delivery of GPCRs (Bulenger et al., 2005). Dimers have been detected not only at the plasma membrane, but also in the ER (Herrick-Davis et al., 2006). This suggests that dimerisation could be involved in the biosynthetic pathway, as receptor-receptor interaction could mask ER retention sequences or function as chaperones promoting mutual folding (Ellgaard and Helenius, 2003). Furthermore, homo-dimerisation provides effective signal amplification allowing receptors to bind more than one ligand or G protein molecule at the same time (Pascal and Milligan, 2005, Jonas et al., 2015, Capra et al., 2017). On the other hand, hetero-dimerisation is able to induce changes in the pharmacology of a co-expressed pair of GPCRs, where the heteromer comprises a new functional unit with distinct properties from its monomeric counterparts (Ferre et al., 2009). For example, stimulation of dopamine receptor 1 and 2 hetero-dimer results in PLC-mediated Ca^{2+} release, although D1 and D2 receptors usually trigger stimulation and inhibition of AC, respectively (Lee et al., 2004). Besides alteration of pharmacological properties, hetero-dimerisation may also influence receptor trafficking. For example, the δ-opioid receptor (DOR) undergoes robust agonist-induced internalisation, whilst the κ-opioid receptor does not; when the two receptors are co-

expressed, the same ligand is unable to induce internalisation of DOR (Jordan and Devi, 1999). It is important to note that hetero-dimerisation is obligatory for GABA B receptor type 1 and 2 correct functioning and membrane localisation; when expressed individually, GABA B type 1 is retained intracellularly, whilst type 2 is addressed to the cell surface but remains non-functional (Pin et al., 2004, Kaupmann et al., 1998). In addition to heteromer-specific properties (e.g. signalling, trafficking and/or ligand binding), it is important that other two criteria are fulfilled to define the existence of GPCR heteromers in native tissues: the monomeric units should co-localise and interact, and the heteromer disruption should cause loss of heteromer-specific functions (Gomes et al., 2016). These directives will greatly help the characterisation and screening for GPCR heteromers that could be targeted for therapeutic purposes.

1.1.4.2 Post-translational modifications

Post translational modifications (PTMs) are covalent changes of proteins at specific residues within a consensus sequence. Altering the chemical properties of amino acids, PTMs function as regulatory mechanisms to reshape the interaction with other proteins, the location and the activity of the modified protein. The most common PTMs observed in GPCRs are phosphorylation, palmitoylation, glycosylation and ubiquitination.

Proteins are glycosylated during their biosynthesis through the addition of a carbohydrate often on an asparagine residue within the NxS⁄T sequence (N-glycosylation), but other glycosydic linkages exist (O- or C-glycosylation). GPCRs are N-glycosylated on their extracellular portions, either the N-terminus or ECLs. Besides ensuring proper protein folding, glycosylation plays different roles in different GPCRs; correct destination within the cell, receptor dimerisation, ligand recognition and signalling have been observed (Boer et al., 2000, Michineau et al., 2006, Tansky et al., 2007, Gellynck et al., 2012).

Palmitoylation of GPCRs was first demonstrated for rhodopsin (O'Brien and Zatz, 1984), but it is now recognized that at least 80% of GPCRs are subjected to this modification (Probst et al., 1992). The addition of a C16 fatty acid chain on a cysteine within the C-tail determines, in some receptors, the formation of a fourth ICL, which tunes receptor activity by

modulating G protein coupling (Altenbach et al., 1999). Palmitoylation is an extremely flexible modification that is dynamically regulated by the cell, for example by ligand exposure (Sadeghi et al., 1997). Additionally, it has been proposed to positively or negatively regulate phosphorylation of the receptor C-tail, by either favouring or sterically impairing kinase activity; this could also reflect in altered receptor trafficking (Munshi et al., 2001, Hawtin et al., 2001). Furthermore, it could serve as a location signal, targeting receptors to lipid rafts, thus dictating their trafficking and signalling.

Unlike glycosylation and palmitoylation, which have a receptor-specific role, phosphorylation and ubiquitination mediate the same downstream events in all GPCRs. Ubiquitination is the linkage, by the ATP-dependent activity of three enzymes, of an evolutionary conserved 76 amino acid peptide to a lysine in the C-tail of the target GPCR. Although in yeast ubiquitin serves as an internalisation signal, internalisation of mammalian GPCRs is largely ubiquitin-independent (Hicke and Riezman, 1996, Marchese and Benovic, 2001, Wolfe et al., 2007). GPCRs acquire ubiquitin moieties at the plasma membrane after ligand binding, sometimes with the requirement of adaptor proteins like β-arrestin (Shenoy et al., 2001). If ubiquitin remains attached to the receptor after its internalisation, this can act as signal to target the receptor to the lysosomes for degradation; alternatively, de-ubiquitinases remove the ubiquitin moieties and the receptor is recycled back to the plasma membrane (Marchese and Trejo, 2013). Both processes are mediated by a number of ubiquitin interacting proteins, the combination of which is receptor-specific.

The phosphorylation state of a certain protein is determined by the action of two enzymes with opposing functions, kinases and phosphatases. The phospho-group is transferred by kinases from ATP to a serine, threonine or tyrosine residue of the target protein. The majority of kinases acting on GPCRs are Ser/Thr kinases, namely PKA, PKC and GRKs, that phosphorylate residues within the C-tail or ICL3 important for receptor desensitisation (Pitcher et al., 1998). GRKs preferentially phosphorylate ligand-bound receptors resulting in β-arrestin recruitment and G protein uncoupling, whilst protein kinase A and C (PKA and PKC), second-messenger effectors, function in a negative feedback loop phosphorylating already active GPCRs to quench their activity and prevent further signalling (Krupnick and Benovic, 1998). Interestingly, different ligands provoke specific “phosphorylation barcodes”

at receptors, causing bias in either signalling, trafficking or both of the receptor (Butcher et al., 2011).

Finally, it is important to note that not only GPCRs are subjected to PTMs but also their adaptor proteins, signalling effectors and trafficking machinery, thus amplifying the complexity of GPCR regulation.

1.1.4.3 Splice variants

Splicing is a cellular mechanism acting at RNA levels where a pre-mRNA molecule, made of both exons and introns, is depleted of introns by the enzymatic activity of the spliceosome, that also fuses the remaining exons forming a mature mRNA molecule (Jurica and Moore, 2003). Alternative splicing highly increases the number of mature mRNAs obtained from a single pre-mRNA; these will be variable in size and nucleotide composition compared to their classically spliced RNA, as exon skipping or intron retention will lead to a shorter or longer product, respectively (Black, 2003). Although 50% of GPCR genes are intronless, thus generating single products of their genes, GPCRs containing introns do undergo alternative splicing that produce truncated forms of the receptor, lacking one or more transmembrane domains and/or the N- or C-termini (Markovic, 2013). Most of these are not targeted to the plasma membrane as they do not meet the biosynthetic pathway quality requirements, hence are retained in the ER or degraded (Tanoue et al., 2002). Those truncation mutants stable enough to reach the plasma membrane are either ligand binding- or signalling-deficient, whether they lack the N- or C-terminus, respectively, and in both cases they fail to activate downstream signalling responses. Nevertheless, they can modulate GPCR activity via dimerisation (Wise, 2012). As detailed in paragraph 1.1.4.1, GPCRs tend to from oligomers, as early as during their biosynthetic pathway; in this way, even truncated receptors retained in the ER can affect the cell surface expression of their WT counterparts, retaining them intracellularly by a dominant-negative effect, or vice versa, with the WT receptor positively affecting the plasma membrane delivery of its truncated version (Markovic and Challiss, 2009). This has a great physiological impact when the WT receptor is either constitutively active or inactive. When both WT and truncated receptors, the latter often lacking TM7 or TM6-7, are inserted into the plasma membrane, their dimerisation

generates a signalling unit with altered or totally new pharmacological properties, similarly to what has been reported for full-length heterodimers (Milligan, 2004). Thanks to the properties described above, GPCR truncation mutants could be exploited in GPCR-targeting therapies, to rescue or quench the activity of an inactive or overactive receptor, respectively (Conn et al., 2007).

1.1.4.4 Membrane trafficking

Another mechanism that cells exploit to control GPCR signalling output is membrane trafficking (Jean-Alphonse and Hanyaloglu, 2011). After ligand binding, GPCRs are uncoupled from their G proteins and internalised to lipid vesicles called endosomes. From there, GPCRs can either be recycled back to the plasma membrane for another round of ligand stimulation, or degraded if delivered to the lysosomes (Figure 1.4). The first step contributes to rapid desensitisation and ensures cells are not excessively stimulated; resensitisation is achieved through receptor recycling and prevents prolonged hormone resistance; degradation determines permanent signal termination (Hanyaloglu and von Zastrow, 2008). This classic view has now been recently revised thanks to the ground-breaking findings that internalised GPCRs are not quiescent but can prolong or perform new signalling activities (Sposini and Hanyaloglu, 2017, Irannejad et al., 2014). The highly complex multi-step process of GPCR sorting through membrane trafficking involves endosomes and other intracellular compartments, that serve as both sorting and signalling stations, and is exquisitely orchestrated by a number of adaptor proteins whose multiple roles have just started to be uncovered.

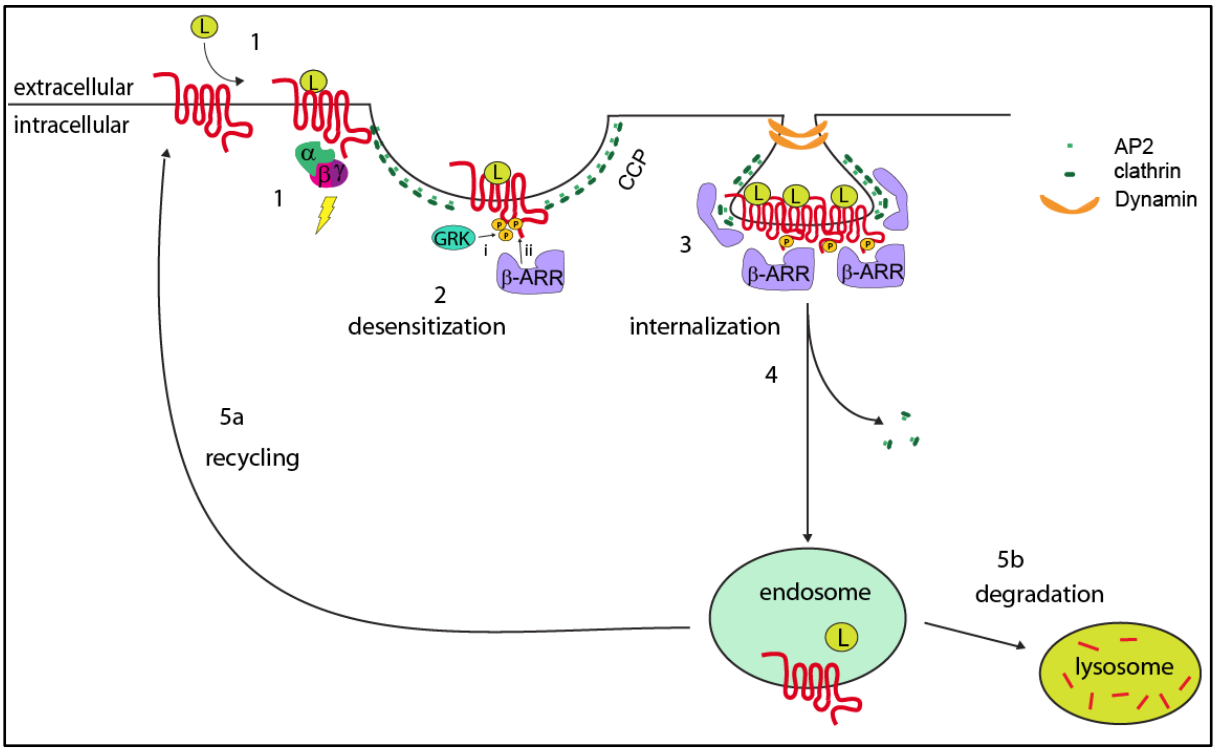


Figure 1.4 Steps of GPCR activation, internalisation and intracellular sorting. The ligand activated receptor starts signalling from the plasma membrane through the coupled G protein (1). After G protein dissociation, the receptor is phosphorylated on multiple intracellular sites by GPCR kinases (GRKs) phosphorylation (2i) and subsequent β-arrestin binding (2ii). Concurrently receptors are clustered into forming-vesicles marked by clathrin and adaptor protein 2 (AP2), the clathrin coated pits (CCPs) (3). Dynamin-mediated CCP scission from the plasma membrane and subsequent uncoating originate endosomes which act as sorting stations for GPCRs, which can be targeted back to the plasma membrane (5a) or to lysosomes (5b).

1.2 Endosomal trafficking and intracellular signalling

1.2.1 Desensitisation and internalisation

The archetypal model for internalisation of many GPCRs occurs primarily via CCPs and involves a mechanism whereby GRKs and β-arrestins play a central role ((Moore et al., 2007, Barki-Harrington and Rockman, 2008) and Figure 1.4). Four isoforms of arrestin have been identified: arrestin-1 and -4 are restricted to the visual system and accordingly named 'visual arrestins' (Wilden et al., 1986, Craft et al., 1994), whereas the other two isoforms,

arrestin-2 and -3, also called β-arrestin-1 and -2, are ubiquitously distributed (Attramadal et al., 1992, Lohse et al., 1990). In this model, the ligand-activated receptor is the target of GRK-mediated phosphorylation and subsequent arrestin binding. GRKs are a family of seven Ser/Thr kinases with tissue-specific distribution. As more than one GRK type can be expressed in a given cell, as well as more than one Ser/Thr can be phosphorylated by GRKs, different receptors, or even the same receptor activated by different ligands, could show distinct phosphorylation patterns, the so called "phosphorylation barcodes" (Nobles et al., 2011, Butcher et al., 2011). This generates diverse modes of β-arrestin binding to the receptor, either through the core (ICLs), the C-tail or both (Shukla et al., 2014). How β-arrestin binds the receptor influences its conformation and determines its functions. β-arrestin canonical roles are uncoupling receptor and G protein, leading to signal desensitisation at the G protein level, and receptor clustering into CCPs, through the binding of both GPCR and the CCP proteins clathrin and its adaptor AP2. Additionally, only when engaging with both GPCR core and tail, β-arrestin can mediate G protein-independent signalling by scaffolding various components of signalling pathways such as the MAPK. Considering that only 2 isoforms of arrestins are exploited by GPCRs in comparison to the tens of G protein types, one must really appreciate the capacity of β-arrestins to sense, through their N-terminal domain, the different phosphorylation forms of the receptor. This determines conformational changes in the β-arrestins themselves resulting in "conformational signatures" recognized by different downstream effectors as another way to diversify GPCR activity (Lee et al., 2016, Yang et al., 2015).

Once GPCRs are concentrated in CCPs, the scission of clathrin-coated vesicles from the plasma membrane is achieved by the large GTPase dynamin, resulting in GPCR internalisation to endosomes. Although β-arrestins appear to be fundamental in GPCR endocytosis, β-arrestin-independent GPCR internalisation has been reported (Smith et al., 2016, van Koppen and Jakobs, 2004). Thus, GPCRs greatly differ in how they utilise this GRK/arrestin/CCP model for their desensitisation and internalisation. Although GRK and β-arrestin induce rapid desensitisation, there are several studies indicating that these common internalisation and desensitisation mechanisms have expanding roles in spatio-temporal control of GPCR signalling. For example, there is known heterogeneity in CCP function, where different GPCRs can be organised within distinct subsets of CCPs to dictate

subsequent post-endocytic fate between recycling or lysosomal pathways (Mundell et al., 2006, Lakadamyali et al., 2006). Such divergent sorting of GPCRs at the plasma membrane was mechanistically dependent on GRK/arrestin (for CCPs directing receptors to the recycling pathway) and receptor phosphorylation by second messenger kinases (for lysosomal sorting). CCPs may also function as signalling microdomains (West and Hanyaloglu, 2015). This is supported by the ability of GPCRs to regulate their own residency time in CCPs. For the B2AR, interaction with scaffold proteins, namely postsynaptic density 95/disc large/zonula occludens-1 (PDZ) proteins, tethers receptors to cortical actin and extend the occupancy time of a receptor in a CCP by delaying the recruitment of dynamin (Puthenveedu and von Zastrow, 2006). However, the μ-opioid receptor (MOR), which does not have a PDZ ligand within its intracellular domain, modulates its residency time by delaying the scission activity rather than recruitment of dynamin and involves a mechanism whereby receptor ubiquitination dictates CCP residency time (Henry et al., 2012, Roman-Vendrell et al., 2012). Interestingly, a recent study on the β1-adrenergic receptor (B1AR) has demonstrated that arrestin signalosomes at the CCP may not require alteration of the residency time of a GPCR. In fact, although B1AR internalises more slowly and poorly clusters compared to GPCRs such as the B2AR, it robustly recruits β-arrestin to CCPs without detectable receptor localised to CCPs and this CCP-bound β-arrestin was shown to activate a prolonged MAPK signal profile (Eichel et al., 2016). Although clathrin-associated adaptors such as β-arrestin and AP2 represent core CCP machinery utilised by many kinds of receptors, it is likely there are additional players that can direct receptors into distinct pits via differential phosphorylation of their intracellular domains.

Recently, clathrin independent endocytosis (CIE) has been demonstrated for a number of GPCRs, which internalise via a mechanism called fast endophilin mediated endocytosis (FEME), as it is faster than CDE and utilises endophilin instead of clathrin/AP2, yet still relys on dynamin for pit scission. FEME generates tubulo-vesicular coat-less structures containing the ligand-activated cargo from specific plasma membrane sites and in response to peculiar stimuli (Boucrot et al., 2015). The role for such targeted receptor uptake is still unknown but could represent another level of GPCR regulation (Watanabe and Boucrot, 2017).

1.2.2 Endosomal sorting to the recycling pathway

Regardless of the modality, internalisation takes GPCRs to endosomal compartments where their intracellular fate is decreed. GPCRs targeted to a recycling pathway back to the plasma membrane, resulting in resensitisation or recovery of signalling, require a sequence-directed mechanism involving specific cis-acting sorting sequences in their C-tails. These could be either tyrosine- and dileucine-based motifs or PDZ binding motifs (also known as PDZ ligands as there are recognized by the PDZ domain of interacting proteins). Canonical Tyr- and diLeu-based motifs are recognized by the clathrin adaptors AP-1, -2 and -3, which regulate GPCR trafficking at the TGN and clathrin coated endosomes, CCPs and endosomes, respectively (Marchese et al., 2008). PDZ ligands are mostly present at the distal C-tail of GPCRs, though internal ones have been reported. According to their sequence, PDZ ligands are grouped into three classes, I, II and III, with class I being the most common among GPCRs (Sheng and Sala, 2001). This form of regulated recycling was first identified for the archetypal GPCR B2AR, where it was shown that this form of recycling differs to cargo that recycle in the absence of sorting sequences, which occur in a "default" manner via bulk membrane flow (Cao et al., 1999, Hirakawa et al., 2003). Removal or mutation of the PDZ-ligand not only inhibited recycling, but re-routed the receptor to the degradative pathway. Furthermore, addition of the PDZ ligand to a receptor sorted primarily to a degradative pathway, DOR, altered the post-endocytic trafficking fate to the recycling pathway, demonstrating this recycling sequence was both necessary and sufficient in directing receptors back to the plasma membrane (Cao et al., 1999, Gage et al., 2001). Initial studies suggested the PDZ protein for B2AR was Na^+/H^+ exchange regulatory factor 1. However, subsequent studies identified that this receptor can interact with a subset of PDZ proteins (He et al., 2006), in part distinct from another GPCR with a type 1 PDZ ligand, the B1AR, indicating that unique GPCR/PDZ interactions may define distinct functions. These PDZ proteins identified were primarily plasma membrane, or juxta-membrane localised, with the exception of sorting nexin (SNX) 27, which is EE-localised (Lauffer et al., 2010). Since the identification of a recycling sequence in the B2AR, numerous GPCRs have been shown to require recycling sequences in their C-tails. Interestingly, these recycling sequences are not all PDZ type 1 ligands, with some corresponding to unknown interacting partners, thus this

high diversity in recycling sequences across GPCRs suggests that they bind specific cytoplasmic proteins (Marchese et al., 2008, Hanyaloglu and von Zastrow, 2008).

That recycling of membrane cargo could occur via two distinct modes, regulated and default, combined with the knowledge that multiple distinct GPCR recycling sequences exist, led to the pursuit of whether there are distinct mechanisms of recycling for these different cargos and/or common machinery. A collection of studies indicates that both common and receptor specific mechanisms exist, although these have also highlighted that regulated GPCR recycling was not a one-step mechanism occurring via a protein-interaction with the GPCR recycling sequence, but a complex, multi-step system, where the receptors own signalling plays an integral part. Examples of core endocytic machinery that process receptors to the regulated recycling pathway include the EE-localised adaptor protein HGF regulated tyrosine kinase substrate (Hrs), also termed vesicular protein sorting-associated protein 27 (Vps27). Hrs was first characterised for its role in the first steps of lysosomal sorting but is also essential for regulated recycling of the B2AR, MOR and calcitonin-receptor-like receptor (Hanyaloglu et al., 2005, Hasdemir et al., 2007). Given its role in regulating lysosomal sorting of cargo, loss of Hrs results in EE retention of receptors, suggesting this adaptor protein acts as a sorting point between these divergent endocytic fates. Hrs-dependent recycling is mediated via its association with as yet unknown additional proteins, as Hrs is considered to be an EE scaffolding protein and likely does not associate directly with receptors (Hanyaloglu et al., 2005, Hasdemir et al., 2007, Huang et al., 2009). Additional core sorting proteins could also be the arrestins. For the N-formyl peptide receptor, receptor recycling, but not internalisation is highly dependent on β-arrestins (Abdullah et al., 2016). A core endosomal complex that has gained recent attention is the retromer complex. This macromolecular complex was known for its role in endosome-trans Golgi network (TGN) transport, but identification of its association with the Wiskott-Aldrich Syndrome Protein and SCAR Homolog complex (WASH) has identified additional roles in receptor sorting to the plasma membrane recycling pathway and organisation of receptors into specific endosomal tubules that mediate PDZ-dependent recycling (Puthenveedu et al., 2010). Interestingly, the PDZ protein SNX27 links the receptor and the retromer, via the WASH complex (Temkin et al., 2011, McGarvey et al., 2016). This not only highlights the complex nature of regulated recycling, but there are mechanisms for a cell to regulate

receptors between the two modes of recycling - regulated and default - via physical organisation into biochemically and kinetically defined tubules. That a GPCR could potentially 'switch' between these two modes of recycling was first demonstrated via a study that identified specific receptor C-tail sequences mediating Hrs/Vps27 dependent recycling. Mutation on an acidic-dileucine like sequence located upstream of the B2AR PDZ ligand did not affect ability of the receptor to recycle but switched the recycling to a PDZ-independent default pathway (Hanyaloglu and von Zastrow, 2007). Subsequent studies have also identified additional molecular requirements critical for switching between bulk/default and sequence-dependent recycling modes. For the B2AR, PKA phosphorylation of C-tail, induced by activation of the receptors $G\alpha_s$-cAMP pathway, inhibits entry into the sequence-dependent recycling tubule (Vistein and Puthenveedu, 2013) and more recently demonstrated for the B1AR (Nooh and Bahouth, 2017). The downstream biological significance of this sequence-directed recycling pathway is underscored by demonstration of its direct involvement in regulating cardiac myocyte contractility, where regulated recycling was essential for G protein switching of the B2AR from $G\alpha_s$ to $G\alpha_i$ (Xiang and Kobilka, 2003), while for the MOR there may be a role for this trafficking pathway in opiate tolerance (Roman-Vendrell et al., 2012).

Considering that sequence-directed recycling enables flexible programming of receptor post-endocytic fate and that these GPCRs primarily internalise to a common endocytic compartment, the EE, it comes natural to envisage a role of these different recycling sequences and adaptor partners in possibly directing receptors to different endosomal compartments other than the EE. Evidence supporting this hypothesis come from studies on the luteinizing hormone receptor (LHR) showing that its internalisation into very early endosomes (VEEs), physically and biochemically distinct from the EE, requires the interaction between its PDZ sequence and the PDZ protein $G\alpha_i$-interacting protein C terminus (GIPC); loss of this interaction inhibits receptor recycling due to the receptor re-routing to EEs (Jean-Alphonse et al., 2014). Similarly, mutation of the PDZ ligand of the P2Y purinergic receptor re-routes the receptor from a Rab5-positive compartment to the TGN, impairing its ability to recycle but not its degradation rates (Cunningham et al., 2013). These studies, in conjunction with the finding that multiple PDZ proteins can interact with the same receptor, may suggest that 'recycling sequences' may be a too limited description for

their role, and these sites may encode multiple functions at distinct points throughout the endocytic life cycle of the receptor.

1.2.3 Endosomal sorting to the degradation pathway

Sorting of internalised GPCRs to a degradative fate results in permanent signal termination or transient signal profile. For GPCRs who are rapidly targeted to this pathway, disruption of receptor sorting would result in overactive, or persistent signalling, which for certain GPCRs such as the chemokine receptor 4 (CXCR4) is associated with invasive breast cancer (Marchese et al., 2008). Furthermore, lysosomal sorting is a key part of receptor downregulation for recycling receptors that are chronically activated and is thought to play a role in drug tachyphylaxis or tolerance (Tappe-Theodor et al., 2007, Hanyaloglu and von Zastrow, 2008). The pharmacological and clinical significance has thus driven studies to identify the molecular mechanisms targeting GPCRs to this pathway. Following internalisation to EEs, GPCRs sorted from this compartment for degradation are trafficked to Rab7 late endosomes leading to involution of receptors to form multivesicular bodies (MVBs), where subsequent fusion with lysosomes results in receptor degradation. The canonical pathway for sorting of many types of membrane cargo, not just GPCRs, is via cargo ubiquitination at lysine residues and endosomal sorting complex required for transport (ESCRT)-dependent degradation. ESCRTs are a series of 4 distinct protein complexes (ESCRT-0, I, II, III) with the first 3 containing proteins with ubiquitin binding domains, which sequentially capture and retain ubiquitinated cargo from the EE-localised ESCRT-0, to involution of cargo in MVBs. Disassembly of ESCRT complexes from the maturing MVB occurs via the AAA-ATPase Vps4 in order to facilitate additional rounds of MVB sorting (Rusten et al., 2011, Henne et al., 2013). Ubiquitination and ESCRT-dependent degradation has been well-described for certain GPCRs such as the CXCR4, protease-activated receptor 2 (PAR2) and B2AR, where distinct ubiquitin E3 ligases mediate receptor ubiquitination during early endocytosis and often necessitates receptor internalisation, without being required for internalisation, with arrestin providing the platform for ligase recruitment (Kennedy and Marchese, 2015). Although several (>30 GPCRs) have been reported to be ubiquitinated, GPCRs either do not require ubiquitination or certain

components of the ESCRT machinery for their lysosomal degradation, such as the DOR (Henry et al., 2011, Hislop et al., 2011, Dores and Trejo, 2015). As ubiquitin is not the sorting signal for such receptors, they require association with additional machinery for lysosomal targeting that directly interact with GPCR C-tails, such as GPCR associated sorting protein-1 (GASP-1) and the autophagy protein Beclin-2, prior to ESCRT targeting (He et al., 2013). However, subsequent studies have indicated that DOR ubiquitination, via the E3 ligase AIP4, may play a role in later steps of lysosomal sorting, specifically for efficient receptor involution or transfer to intraluminal vesicles of MVBs to enable proteolysis of the receptor endodomain (Henry et al., 2011, Hislop et al., 2009). In a similar manner, chronic activation of the MOR, which reprograms its sorting from a sequence-dependent recycling to degradative fate, also employs receptor ubiquitination (specifically in the first intracellular loop that also defines CCP residency time (Henry et al., 2012)) not for lysosomal sorting, but promotion of receptor involution into MVBs (Hislop et al., 2011). GPCR lysosomal sorting does not always require ubiquitination but association with arrestins (Mosser et al., 2008). Diversity in GPCR lysosomal sorting mechanisms is further demonstrated by GPCRs such as the protease activated receptor 1 (PAR1) that requires neither receptor ubiquitination, ESCRT0-0, -I or GASP-1 for its degradation, but does require the MVB ESCRT III complex and Vps4. This receptor is directed to lysosomes via the ubiquitous adaptor protein ALG-interacting protein X (ALIX). ALIX is ubiquitinated by E3 ubiquitin ligases that form a complex at the endomembrane with the arrestin-domain containing proteins (ARRDCs) ARRDC1 and ARRDC3 (Dores et al., 2012a, Dores et al., 2015, Dores et al., 2012b).

As for sorting of GPCRs to the regulated recycling pathway, there is evidence that lysosomal sorting can be regulated by GPCR signalling. For example, degradation of the dopamine D2 and D3 receptors is mediated via phosphorylation of the receptor at specific residues by the second messenger kinase PKC (Zhang et al., 2016, Cho et al., 2013). Interestingly, the $G\alpha_s$ subunit of heterotrimeric G proteins has been demonstrated to have a direct role in post-endocytic trafficking of GPCRs, independent of its GTPase activity (Rosciglione et al., 2014). Heterotrimeric G proteins have been shown to localise to multiple cellular compartments in addition to the plasma membrane, including the endomembranes. Depletion of $G\alpha_s$ was found to inhibit the degradation of distinct GPCRs (CXCR4, DOR, and the angiotensin 1A receptor) known to sort to this pathway via both ubiquitin-dependent and -independent

pathways, due to an inhibition of receptor involution in MVBs. Despite its identified role at a late stage in lysosomal sorting, $G\alpha_s$ associated with early stage lysosomal sorting proteins GASP-1, dysbindin and Hrs (ESCRT) at the EE. Given CXCR4 does not employ a GASP-1/dysbindin pathway for its degradation may suggest $G\alpha_s$ interacts with additional proteins (Beas et al., 2012), but also highlights an adaptor role for this signalling protein in the lysosomal sorting of receptors via multiple mechanisms (Rosciglione et al., 2014). Overall the complex nature, and diverse mechanisms, of lysosomal sorting could also suggest that it provides a platform for flexible reprogramming GPCR fate. Indeed, disruption of the latter stages of lysosomal sorting results in MVB/endosomal retention, while inhibition at earlier steps, upstream of ESCRT-0, can re-route receptors to a recycling pathway, as shown for PAR1 and CXCR4 where inhibition of its ubiquitination via the tyrosine kinase Her-2/ErB2 results in increased surface expression of these receptors in breast cancer and promotion of tumour progression (Marchese et al., 2008). In non-cancer cells, reprogramming GPCR post-endocytic fate could be advantageous in tightly regulating cellular sensitivity such as in development (Mukai et al., 2010, Cadigan, 2010).

1.2.4 Endosomal signalling

As described above, our view of GPCR signalling has significantly evolved. Endocytic trafficking regulates cell surface responses by shaping the signalling profile, however, studies over the past 6 years have also demonstrated that it also contributes to diversify GPCR activity by providing distinct signalling platforms.

1.2.4.1 Signalling from the EE

The first example of GPCR signalling from endosomes was for those receptors that exhibit a sustained association, and co-internalisation, with β-arrestins, mainly class B receptors. These arrestins were shown to sequester MAPK signalling molecules in the cytoplasm by acting as scaffolds that group the components of the signalling module at specific locations in the cell and preventing the active kinases from dephosphorylation by phosphatases (McDonald et al., 2000, DeFea et al., 2000, Terrillon and Bouvier, 2004). Different receptors

require distinct combination of G protein- and/or β-arrestin-mediated signalling to achieve MAPK activation. For example, the MAPK response activated by the angiotensin receptor AT1aR only requires β-arrestin 2, whilst PAR2 only necessitates β-arrestin 1; chemokine receptors activate ERK phosphorylation via both G protein and β-arrestins (Ahn et al., 2004, Ge et al., 2003, Sun et al., 2002). Ligands which bind the same receptor and preferentially activate either the G protein- or the β-arrestin-mediated pathway are known as biased ligands (Galandrin and Bouvier, 2006).

However, pivotal studies on the $G\alpha_s$-coupled receptors the thyrotropin-stimulating hormone receptor (TSHR) and the parathyroid hormone receptor (PTHR) provided evidence of persistent cAMP signalling after internalisation, demonstrated by the use of cAMP biosensors (Calebiro et al., 2009, Ferrandon et al., 2009). For PTHR, endosomal cAMP signalling is enhanced by β-arrestin and attenuated by the retromer complex, essential for its recycling, whereby endosomal acidification plays a key role in this deactivating switch ((Ferrandon et al., 2009, Feinstein et al., 2011, Gidon et al., 2014, McGarvey et al., 2016) and Figure 1.5 A). In a recent study, β-arrestins were shown to simultaneously associate with $G\alpha_s/\beta\gamma$ heterotrimer and GPCRs that exhibit persistent arrestin associations via C-terminal serine/threonine phosphorylation sites, forming 'megaplexes' (Thomsen et al., 2016). However, the first direct evidence of active heterotrimeric G proteins at endosomes came from development of nanobody (Nb) biosensors that detect the active, nucleotide-free form of $G\alpha_s$ (Nb37) or, in the case of B2AR, the ligand-activated conformation of the receptor (Nb80) (Irannejad et al., 2013, Pardon et al., 2014). Recent studies on B2AR endosomal $G\alpha_s$/cAMP reveal signalling may be highly organised at the level of the individual endosome. The α-arrestin ARRDC3 associates with B2AR on EEs where it negatively regulates receptor entry into sequence-directed recycling tubules characterised by SNX27 and retromer and thus results in increased endosomal signalling (Tian et al., 2016a). Interestingly a subsequent study on B2AR endosomal signalling revealed a further layer of complexity in control of endosomal B2AR signalling. While B2AR/Nb80 was detected throughout the endosome membrane, microdomains of B2AR/Nb37 were observed specifically in regulated SNX27/WASH recycling tubules ((Bowman et al., 2016) and Figure 1.5 B). Considering the role of ARRDC3 in promoting endosomal signalling, and that it was found to alter associations of B2AR with SNX27, perhaps the functional site of ARRDC3 is at microdomains

within the regulated recycling tubule ((Tian et al., 2016a) and Figure 1.5 B). Whether there are other pathways in addition to $G\alpha_s$/cAMP that B2AR may activate, as revealed by the diffuse endosomal distribution of Nb80, is still unknown.

Endosomal G protein signalling is also relevant for heterotrimeric G protein pathways in addition to $G\alpha_s$; it has been demonstrated that $G\alpha_{q/11}$-coupled receptors, such as the kisspeptin receptor and the neurokinin 1 receptor, exhibit persistent calcium signalling profile entirely dependent on receptor internalisation (Min et al., 2014, Bianco et al., 2011, Jensen et al., 2017). Moreover, Gβγ was shown to have a key role in regulating endosomal signalling from the PTHR; in this study Gβγ released from $G\alpha_i$ coupled to B2AR can stimulate endosomal AC to prolong PTHR cAMP response (Jean-Alphonse et al., 2016), demonstrating for the first time receptor crosstalk between plasma membrane and endomembrane signalling.

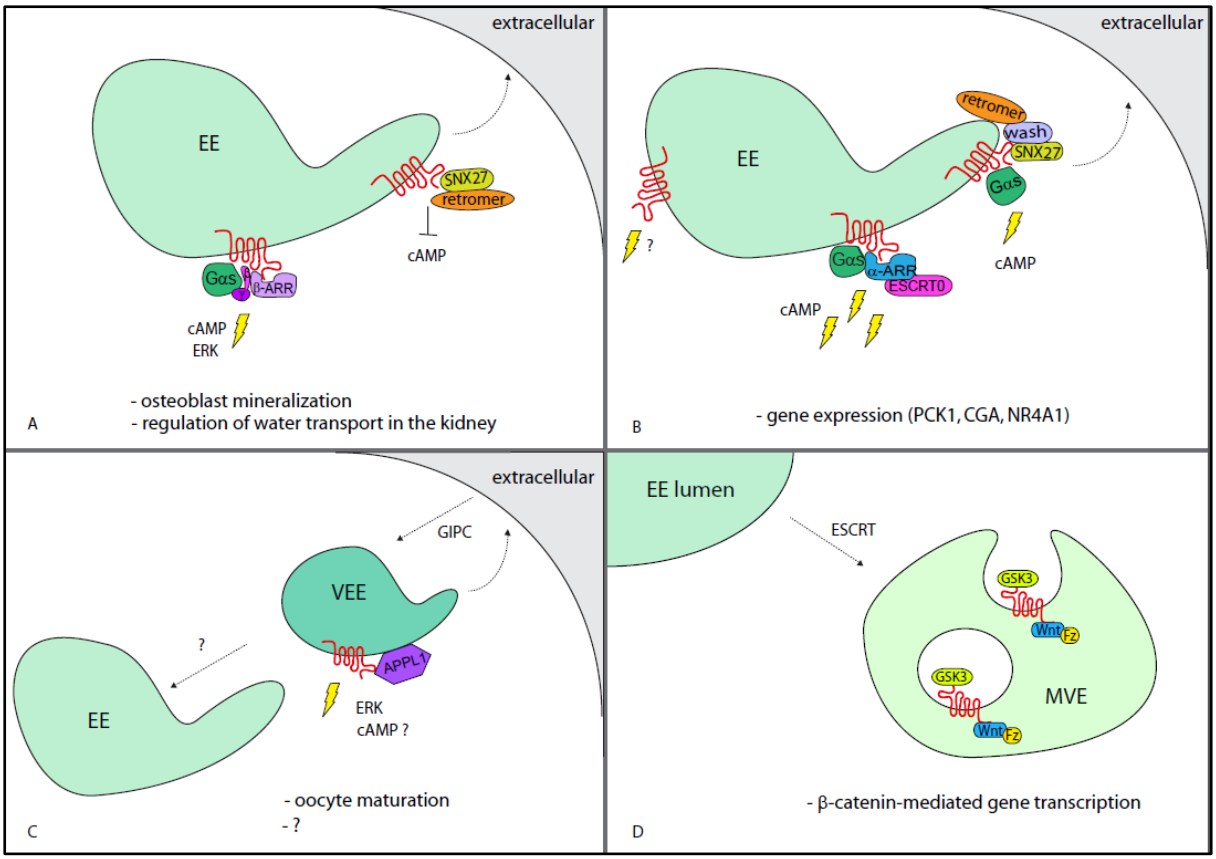


Figure 1.5 GPCR intracellular signalling is achieved and by different endosomal compartments. (A) Class B GPCRs (e.g. V2R, PTHR) are targeted to the EE as well, where they sustained cAMP and ERK responses through the interaction with both the G protein heterotrimer and β-arrestin; receptor's interaction with retromer and SNX27 dictates its localisation into recycling tubules and prevents endosomal signalling. (B) Class A GPCRs (e.g. B2AR) are internalised into the EE where active receptors are present throughout the endomembrane; $G\alpha_s$ mediated-cAMP production is elicited by receptors localised in recycling tubules marked by SNX27, retromer and WASH complexes and increased by retention of receptors outside these tubules by interaction with α-arrestin and ESCRT-0. (C) A subset of class A GPCRs (e.g. LHR) are internalised into VEE marked by APPL1 through the interaction with GIPC where they sustain ERK and, possibly, cAMP production. (D) Class C GPCRs are sorted through ESCRT from the EE to the multi vesicular endosome (MVE), where they invaginate together with glycogen synthase kinase 3 (GSK3).

1.2.4.2 Signalling from other endosomal and non-endosomal compartments

Currently there are few studies to date that address the question whether endomembrane signalling takes place from endosomes other than the EE. The recently reported VEE, an

endosomal compartment distinct from the EE where a subset of GPCRs (LHR, B1AR and the follicle stimulating hormone receptor (FSHR)) traffic to for their post-endocytic sorting (Jean-Alphonse et al., 2014), has been shown to play a key role in direct spatial control of receptor signalling. For the LHR, agonist activation induced a sustained temporal profile of ERK signalling that requires both internalisation and targeting to the correct endosomal compartment, the VEE ((Jean-Alphonse et al., 2014) and Figure 1.5 C). Loss of association with the PDZ protein GIPC, re-routes the receptor to EEs resulting in a transient ERK profile, while inhibition of internalisation completely inhibits ERK activation. Furthermore, addition of the LHR C-tail sequence to an EE localised GPCR, a C-tail truncated form of the vasopressin receptor 2 (V2R), re-routes the receptor to the VEE and results in sustained endosomal ERK signalling (Jean-Alphonse et al., 2014). However, the downstream role of VEE targeting and ERK signalling of LHR remains to be determined, as well as the involvement of the VEE in the signalling of other GPCRs.

Late endosomes, or MVBs, may also represent GPCR signalling platforms. Indeed, a signalling role for late endosomes, defined by Rab7 localisation, has been demonstrated for certain growth factor and nutrient signals such as the epidermal growth factor (EGF), insulin and amino acids, via endosomal signalling through Ras GTPases and mTORC-1 (Flinn and Backer, 2010, Lu et al., 2009). The strongest evidence to date for GPCRs is the requirement of MVBs for Wnt signalling. Wnts activate members of the GPCR superfamily, Frizzled. Rather than attenuate receptor signalling, the sequestration/involution of the enzyme glycogen synthase kinase 3 with the Wnt/Frizzled receptor was essential for sustained Wnt signalling, illustrating a signalling role of MVBs independent of receptor degradation (Figure 1.5 D). This results in a decrease of active GSK3 causing the accumulation of newly synthesized β-catenin and its subsequent transportation to the nucleus to activate gene transcription (Dobrowolski et al., 2012, Taelman et al., 2010).

GPCRs might activate signalling responses also from other cellular compartments other than the endosomes, namely the trans-Golgi network. The first study to report such intracellular signalling was conducted on a $G\alpha_i$-coupled receptor, the sphingosine-1-phosphate 1 receptor (S1P1R), although persistent signal is delivered only by the synthetic agonist FTY720P and not the natural ligand SP1 (Mullershausen et al., 2009). More recently, studies

on B1AR, which poorly internalises to the EE compared to the B2AR, showed that it is able to stimulate $G\alpha_s$-mediated cAMP signalling from the TGN and the potency of its cAMP response depends on the accessibility of different ligands to the Golgi-pool of this receptor (Irannejad et al., 2017).

Thus, in addition to the compartmental bias in heterotrimeric G protein signalling between the plasma membrane and EEs described in the studies above, there is also compartmental bias in GPCR signalling across distinct endosomes and intracellular compartments in general.

1.2.4.3 Cellular and physiological significance of endosomal signalling

While it is clear that the endocytic system can shape the plasma membrane signalling and permit endomembrane signalling to control and diversify GPCR activity, the downstream significance of GPCR plasma membrane *versus* endosomal signalling and how cells mechanistically decode similar signals (e.g. $G\alpha_s$-cAMP, ERK) from plasma membrane *versus* endomembrane, or even between different endosomes, remains largely unanswered. Some of the seminal studies proposing persistent endosomal cAMP signalling also address its physiological relevance. Sustained endosomal cAMP signalling was demonstrated for TSHR in 3D cultures of thyroid follicles (Calebiro et al., 2009). TSH-mediated cAMP signalling in the thyroid is known to reorganise the actin cytoskeleton, implicated in the reuptake of thyroglobulin and in the induction of thyroid-specific genes. Accordingly, chemical inhibition of TSHR internalisation impaired the ability of TSH to modulate actin depolymerisation (Calebiro et al., 2009). For the PTHR, which has critical roles in regulating Ca^{2+} homeostasis, sustained signalling induced by distinct PTH ligands impacts on trabecular bone volume yet induces greater increases in cortical bone turnover by its actions on the bone and kidney, with clinical implications for using PTH analogues in osteoporosis due to their sustained signalling properties (Ferrandon et al., 2009, Okazaki et al., 2008). The endosomal source of cAMP is also required for: glucose-stimulated insulin secretion mediated by glucagon-like peptide 1 receptor (GLP1R) (Kuna et al., 2013), regulation of renal water and sodium transport achieved by the V2R though the binding of vasopressin or oxytocin (Feinstein et al., 2013), rapid dopaminergic neurotransmission through dopamine-D1 receptor interaction

(Kotowski et al., 2011), excitability of cardiac neurons accomplished by the pituitary adenylate cyclase activating polypeptide type 1 receptor in guinea pigs (Merriam et al., 2013). Additionally an interesting *in vivo* study on neurokinin 1 receptor, which mediates nociception in response to substance P through $G\alpha_{q/11}$-calcium pathway, has revealed the importance of targeting antagonists to sites of endosomal signalling to mitigate chronic pain (Jensen et al., 2017).

The mechanisms used by the cells to decode the differential sources of cAMP have just started to be elucidated using the prototype GPCR the B2AR. Optogenetic targeting of AC probes to either the plasma membrane, the EE or the cytoplasm resulted in distinct downstream transcriptional responses (Tsvetanova and von Zastrow, 2014), demonstrating that endosomally generated cAMP is differentially translated by the cell. In order to achieve endosomal cAMP-mediated activation of gene transcription, B2AR must reside in regulated, and not default, EE recycling tubules, consistent with previous findings that active B2AR-mediated $G\alpha_s$ signalling occurs exclusively in these microdomains ((Bowman et al., 2016) and Figure 1.5 B). This is just an example of how location can bias GPCR activity; other mechanisms are likely to be discovered to include ligand-dependent differences and/or other cellular functions in addition to gene expression.

1.3 The endosomal adaptor protein APPL1

1.3.1 The endosomal pathway

As mentioned above, the endosomal pathway is composed of multiple endosomal compartments, which identity is defined by phospholipids and members of the Rab family of small GTPases (Elkin et al., 2016). During their maturation from the periphery towards the centre of the cell, vesicles undergo changes in both their phospholipid composition, Rabs and luminal pH, regulated by phosphoinositide (PstIns) phosphatases and kinases, GAP and GEF for Rabs, and the proton pumping vacuolar H^+-ATPase and the proton leaking endosomal $Na^+(K^+)/H^+$ exchangers, respectively (Huotari and Helenius, 2011). Along the endosomal pathway, luminal pH gradually acidifies from ~7.0 of the newborn endosome, to ~6.5 of the EE and ~6 of the late endosome (LE). This enables the endosomal lumen to

perform tasks, including dissociation of receptor-ligand and protease activity (Scott and Gruenberg, 2011). At the same time, the pinching off of endocytic vesicles from the plasma membrane, composed mainly by PtdIns(4,5)P2, start to be enriched in PtdIns(3)P, which are characteristic of the EE. Through the action of phosphoinositide kinases, the EEs mature into MVBs/late endosomes, PtdIns(3,5)P2 rich-vesicles (Vicinanza et al., 2008). It is important to note that endosomes are not fixed units in space nor time and that, throughout their maturation, they cross intermediate states with mixed PstIns composition. Their lipid content serves to recruit adaptor proteins that lack enzymatic activity, but do contain interacting domains that recognise phospholipid binding domains. In addition, adaptor proteins are recruited to endosomes also by Rabs. There are 66 Rabs in the human genome, most of which are associated to the endosomal membranes through prenylation of their C-terminal cysteines. They cycle between their GDP-bound/inactive and GTP-bound/active form due to the action of GEFs and GAPs, allowing them to function as molecular switches. GTP-Rabs can recruit a huge variety of effectors including PstIns kinases/phosphatases and adaptor proteins like A kinase anchoring proteins (AKAPs), PDZ-binding proteins, arrestins, through which they mediate protein sorting, vesicle budding, motility, tethering and fusion, and cytoskeletal translocation (Rink et al., 2005). The assembly of Rabs, scaffolding proteins and effectors permits the formation of defined signalling microenvironments at the endosomal membrane, where adaptors play pivotal roles in orchestrating this process in both time and space.

1.3.2 APPL1 structure and its interacting proteins

APPL is a ~80 kDa protein present only in eukaryotes and highly conserved in vertebrates (Liu et al., 2017). In humans there are two APPL isoforms, APPL1 and APPL2, which share ~54% of sequence homology and the same structure; APPL1 is by far the most studied of the two isoforms, for this reason since now on I will only refer to APPL1, unless otherwise stated (Cheng et al., 2014). APPL1 expression has been detected in a number of cell lines including HEK 293 and HeLa (Mao et al., 2006). High expression levels of APPL1 were reported in brain, skeletal muscle, fat, liver, heart, and spleen of mice and in fat, brain, liver, muscle and pancreas of humans (Mao et al., 2006, Kim et al., 2016). High expression levels of APPL2

were observed in kidney and pancreas, moderate levels in fat and brain and, importantly, very low levels in HEK 293 cells (Wang et al., 2009, Rashid et al., 2009).

APPL1 lacks catalytic activity but is composed by several binding domains which allow it to interact with a variety of proteins and membrane components; specifically, APPL1 has a N-terminal Bin1-Amphiphysin-Rvs167 (BAR) domain, originally identified as Leucin-zipper motif, that senses and induces membrane curvature; a Pleckstrin Homology (PH) domain that recognises phosphoinositides and increases the lipid specificity of the BAR domain; a BPP (between PH and PTB) domain; a PTB domain that binds phosphorylated tyrosines of other proteins to scaffold them; a C-terminal coil-coiled domain. APPL1 BAR domain is atypical as it is composed by 4 α helices instead of 3 and provides a dimerisation interface and it has been proven to work with the PH domain as one functional unit. The PTB domain is unusual as well as it binds unphosphorylated tyrosines, conversely to the canonical PTB domain property ((Li et al., 2007) and Figure 1.6).

The first interacting protein of APPL1 to be identified was the Ser/Thr kinase Akt2 and soon after the tumour suppressor Deleted in Colorectal Cancer (DCC) (hence the APPL1 alternative name DIC13α) (Mitsuuchi et al., 1999, Liu et al., 2002). So far, APPL1 has been shown to interact with more than 30 proteins: Rabs, receptors like EGF receptor (EGFR), FSHR, insulin receptor, adiponectin receptor, androgen receptor, kinases and phosphatases like protein kinase B (PKB), PtdIns(3)P kinase (PI3K) and OCRL, adaptor proteins like GIPC, thus acting as linker between the cellular components of different trafficking and signalling pathways ((Diggins and Webb, 2017) and Figure 1.6).

APPL1 is subjected to various post-translational modifications mainly in the BAR, PH and PTB domains (Liu et al., 2017). Ser/Thr phosphorylation is the most common, but also Tyr phosphorylation has been reported (Gant-Branum et al., 2010). Phosphorylation at Ser410 acts as molecular switch to regulate APPL1 interaction with OCRL (Erdmann et al., 2007), whilst phosphorylated Ser430 appears to be connected to cellular stress (Liu et al., 2012). Except for these examples, the significance of most of these modifications remains unknown.

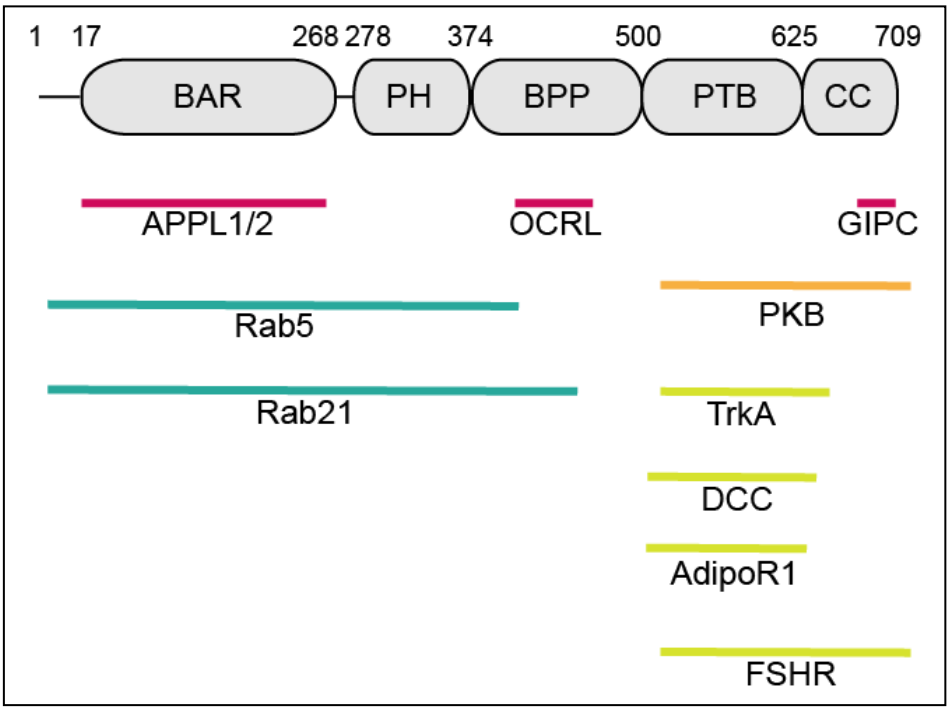


Figure 1.6 Schematic representation of APPL1 domains and interacting proteins. Magenta: proteins involved in both trafficking and signalling; orange: signalling protein; blue: trafficking proteins; yellow: receptors.

1.3.3 APPL1 and trafficking

Different groups have provided abundant evidence of the presence of APPL1 in endosomes located at the cell periphery. Whether APPL1 endosomes represent exclusive vesicles along the endosomal pathway or are intermediates, which after endocytic formation of intracellular vesicles develop to classic EEs, is still under debate. Imaging of endosomal compartments via a range of microscopy techniques (multifocal plane, confocal, TIR-FM, structured illumination) have provided evidence for both: newly formed APPL1-decorated structures fuse to larger, Rab5-positive sorting endosomes and, during this fusion, APPL1 is lost whilst other Rab5-competing effectors are acquired, namely WD repeat and FYVE domain-containing protein 2 (WDFY2) and early endosome antigen 1 (EEA1) (Miaczynska et al., 2004, Zoncu et al., 2009). Similarly, it has been shown that incoming FcRn-loaded vesicles are marked by APPL1/Rab4/SNX4 and that APPL1 dissociates when these fuse with

the larger and more static EE; all vesicles originating from the EE, either crossing the EE compartment or recycling, remain APPL1-negative (Gan et al., 2013). More recently, APPL1 vesicles have been recognized as a non-essential intermediate of the endosome maturation pathway. In fact, a subpopulation of APPL1 endosomes are stable and can sort cargo to the recycling pathway without targeting it to the EE (Kalaidzidis et al., 2015). Additionally, targeting to the EE does not necessarily involve an APPL1-intermediate step as shown for the EGFR, which traffics to the EE via an ESCRT-0-positive compartment devoid of APPL1 (Flores-Rodriguez et al., 2015).

Although more work is required to better define the APPL1 compartment, it is highly possible that both models presented above are plausible, consistent with the fact that APPL1 endosomes are extremely heterogeneous and according to the cell type and cargo under investigation, APPL1 might play slightly different roles thus can exhibit varied cellular organisation.

1.3.4 APPL1 and signalling

Thanks to the strategic position of its BAR-PH and PTB domain, APPL1 is able to couple trafficking and signalling of its interacting proteins, in most cases resulting in the propagation, translocation and integration of signalling pathways from and at the endosome. APPL1 has been associated with positive regulation of signalling; it tethers GIPC to the nerve grow factor receptor TrkA, necessary for MAPK activation (Lin et al., 2006); through the interaction with Rab5, it stabilizes the epidermal grow factor receptor EGFR at the protein level, thus promoting its signalling activity (Lee et al., 2011); it unlocks Wnt/β-catenin stimulated transcription via direct interaction with both a transcription repressor and activator (Rashid et al., 2009); it mediates the formation of a complex composed by FSHR, APPL2, Akt and FOXO1, possibly involved in the propagation of follicle stimulated hormone (FSH)-triggered G protein signalling pathways (Thomas et al., 2011, Dias et al., 2010, Nechamen et al., 2007, Nechamen et al., 2004). Extensive research has been conducted on the relationship between APPL1 and the regulation of glucose homeostasis, achieved by the action of two metabolic hormones, insulin and adiponectin. After

stimulation with insulin, APPL1 in pre-formed complex with insulin receptor substrate 1/2 is recruited to the plasma membrane-localised insulin receptor, where it is now coupled to its effectors and can transduce the signal through PI3K, PKB and MAPK pathways to inhibit glucogenesis and promote lipogenesis and glucose uptake. Similarly, upon binding to their ligand, adiponectin receptors interact with APPL1 which, by recruiting cell-specific effectors, mediates AMPK-pathway leading to the anti-inflammatory, anti-proliferative, anti-obesity and insulin-sensitising actions of adiponectin in hepatocytes, skeletal muscle and endothelial cells (Mao et al., 2006). Interestingly, APPL2 has opposite effects on adiponectin signalling by competing for adiponectin receptor binding and heterodimerising with APPL2 (Wang et al., 2009).

1.3.5 Physiological and pathological implications of APPL1

Most of the research on the physiological implications of APPL1 action has been focused on the insulin and adiponectin pathways. Studies conducted on rodent models reported lower levels of APPL1 in both diabetic and obese animals and that APPL1 deficiency results in glucose intolerance and impaired insulin secretion in APPL1 knock-out mice; on the other hand, overexpression of APPL1 alleviates obesity- and diabetes-related glucose and insulin dysfunctions (Cheng et al., 2012, Ryu et al., 2014, Liu et al., 2017). The beneficial effects of APPL1 rise from its modulating activity of both Akt and adenosine-5'-monophosphate activated protein kinase (AMPK) which lead to insulin-sensitisation, suggesting that both adiponectin and insulin signalling cascades converge on APPL1, which in turn confers the synergistic effects of these two hormones in maintaining glucose levels within the physiological range (Cheng et al., 2014). This model is also corroborated by accumulated evidence that genetic variations and mutations of APPL1 and 2 genes are associated with abnormal body fat distribution, coronary artery disease, non-alchoholic fatty liver disease and familial forms of diabetes (Liu et al., 2017). Recent studies have connected APPL1 to Alzheimer's disease (AD) and Down's syndrome (DS). In neurons from both AD and DS mice, APPL1 endosomal localisation, triggered by the interaction with the C-terminal fragment of the β-Amyloid precursor protein (APP), is increased in addition to its elevated stabilizing function of active Rab5 (Kim et al., 2016). Furthermore, malignant tissues from prostate

cancer patients were found to have altered RNA levels of endosomal markers, including APPL1 (Johnson et al., 2015).

1.4 The luteinizing hormone receptor

1.4.1 Receptor structure

Together with FSHR and TSHR, LHR belongs to the glycoprotein hormone receptors, a sub-group of the rhodopsin family of GPCRs.

The human LHR is encoded by a single gene on chromosome 2 composed by 10 introns and 11 exons (Rousseau-Merck et al., 1990). The mature protein has 675 amino acids and molecular weight of ~90 kDa, higher than the predicted one as a result of the extensive glycosylation of LHR N-terminal domain (Minegishi et al., 1990). LHR protein can be divided into three portions: a large N-terminal domain (340 aa) typical of glycoprotein hormone receptors, a transmembrane core and a C-tail (Dufau, 1998). Surprisingly, the whole core and C-terminal regions are encoded by exon 11, whilst the N-terminal domain is generated by alternate splicing of the remaining introns and exons (Ascoli, 2005). Across different species, the highest dissimilarity is in the C-tail, if we consider that human and rat LHR display 90% sequence identity across the N-terminus and core portions, and only 70% in their C-tails (Ascoli et al., 2002). The LHR N-terminal domain can be divided into three regions according to its amino acid composition: a N-terminal cysteine-rich domain, a leucine-rich motif region involved in the interaction with the ligand, and a hinge region, with a possible role in linking ligand binding with receptor activation (Caltabiano et al., 2008, Ascoli and Segaloff, 1989, Mueller et al., 2010). Additionally, it contains six sites of N-linked glycosylation that ensure correct folding of the protein and interaction with chaperones during the biosynthetic pathway (Davis et al., 1997). The C-tail undergoes palmitoylation and phosphorylation, both involved in the internalisation and recycling of the receptor, although palmitoylation occurs during biosynthesis and phosphorylation after ligand binding (Kawate and Menon, 1994, Munshi et al., 2001, Lazari et al., 1998). Interestingly, FSHR is characterised by highly similar structural/functional features (Menon and Menon, 2012).

In addition to PTMs, receptor activity can be modulated also by interaction with its splice variants. LHR splice variants have been reported in sheep, cow, horse and human. In humans there are three alternative LHR splice variants in addition to full length LHR (LHR-A) in corpus luteum and ovary; one lacking exon 9 (LHR-B), one lacking part of exon 11 (LHR-C) and one lacking both exon 9 and part of exon 11 (LHR-D) (Minegishi et al., 1997). LHR-B is signalling deficient and reduces the expression of both LHR-A and FSHR (Yamashita et al., 2005, Nakamura et al., 2004); LHR-D alters LHR-A signalling activity (Dickinson et al., 2009). Aberrant splice variants have been observed in individuals suffering from Leydig cell hypoplasia, hypospadias or micropenis, where LHR is depleted of exon 7, 8 or 10 respectively, resulting in impaired ligand binding (Han et al., 2012).

Finally, it has been shown that LHR and FSHR form heterodimers in HEK 293 cells and that this association results in attenuation of cAMP signalling through each receptor (Feng et al., 2013).

1.4.2 Glycoprotein hormones

In addition to LH, FSH and TSH, the glycoprotein hormones also include chorionic gonadotrophin (CG); this is the only member not produced by the pituitary but from the placenta (initially secreted by embryonic syncytiotrophoblasts), it only binds to LHR (thus also called LHCGR) and is only present in primate and equine species (Cahoreau et al., 2015). All glycoprotein hormones are composed by one common α-subunit and one specific β-subunit; only when structured as heterodimers glycoprotein hormones preserve their biological activity (Combarnous, 1988). They bind their receptors according to the "negative specificity" model, where the α-subunit contributes to the high-affinity of the binding and the β-subunit inhibits binding to non-specific receptors (Caltabiano et al., 2008, Jiang et al., 2014). Focusing on hLHR ligands, they are often considered as interchangeable but, although they share 82% sequence homology, they are distinct molecules with different patterns of expression and physiological functions (Choi and Smitz, 2014). While CG retains all 145 aa of its β-subunit, LH leading sequence is cleaved generating a 121 aa β-subunit. This confers to CG a higher stability, greater receptor binding affinity and longer half-life

(hours instead of minutes) compared to LH (Rahman and Rao, 2009). It is also important to note that a variety of post-translationally modified isoforms of both LH and CG exist, as well as naturally occurring genetic variations, which influence ligand bioactivity (Stanton et al., 1993). As a consequence, LH, CG and their variants result in unique LHR signalling cascades.

1.4.3 LHR signalling

After binding to the horseshoe shaped ectodomain of their receptors, gonadotrophins also interact with the TM regions to transmit the signal to the intracellular effectors through conformational changes of the receptor's core (Moyle et al., 1995, Cosowsky et al., 1995). These are transmitted to the cognate G protein which, for all glycoprotein receptors, is classically known to be $G\alpha_s$/cAMP/PKA (Puett et al., 2007). LHR was also one of the first GPCRs shown to independently bind to another type of G protein, $G\alpha_{q/11}$, although only in presence of high concentrations of ligand and/or receptor (Gilchrist et al., 1996, Breen et al., 2013). Similarly, receptor levels seem to regulate LHR coupling to $G\alpha_i$ in Leydig cells (Moraga et al., 1997). Furthermore, LHR is also able to activate the MAPK pathway, even if the molecular mechanisms triggering ERK 1/2 phosphorylation are still under debate. As described in previous paragraphs, most GPCRs activate the MAPK pathway in a G protein-independent manner, using β-arrestin not only to mediate their desensitisation and internalisation, but also as a scaffold to propagate signalling, both at the plasma membrane and from endosomes (Cahill et al., 2017). In fact, β-arrestin recruitment for scaffolding purposes requires receptor phosphorylation in its C-terminal Ser/Thr cluster, which is lacking in LHR (Bhaskaran et al., 2003b). Considering this and that LHR exhibits sustained MAPK activation only if it reaches its correct endosomal localisation at the VEE (Jean-Alphonse et al., 2014), one intriguing question is which signalling effectors are contributing to the generation of the endosomal MAPK if this is not mediated by β-arrestin. Interestingly, the closely related FSHR does possess the C-terminal phosphorylation barcode and, as expected, displays β-arrestin mediated MAPK activation, though the Ser/Thr cluster is only required for the internalisation of the receptor (Kara et al., 2006). Moreover, low levels of FSHR at the plasma membrane determine FSHR signalling to be dependent on β-arrestin only (Tranchant et al., 2011).

Since LHR is able to bind two hormones and ligand bias is an established concept in the GPCR field, studies have been carried out to test whether LH and CG could selectively engage or preferentially activate some signalling pathways among those mediated by the same receptor. Experiments conducted in COS7 and human primary granulosa cells reported hCG is five times more potent than hLH in stimulating cAMP production at equimolar doses, though cAMP accumulation is faster in cells treated with hLH compared to hCG used at ED_{50} doses. On the contrary, hLH is more potent than hCG in activating ERK and AKT pathways (Casarini et al., 2012). More recently it has been shown that when measuring cAMP production *versus* β-arrestin recruitment induced by the two ligands, hCG was found more potent than hLH in stimulating both pathways, in both HEK 293 cells transfected with hLHR and in mouse Leydig tumour cells (mLTC-1) endogenously expressing mLHR (Riccetti et al., 2017). Interestingly, both ligands were acting as full agonists in regard to cAMP, but hLH effects were only partial in β-arrestin recruitment compared to hCG (Riccetti et al., 2017).

1.4.4 LHR trafficking

Following ligand binding, hLHR internalises in a clathrin-dependent manner (Ghinea et al., 1992). Conversely to most GPCRs, LHR recruits β-arrestin independently of GRK phosphorylation, engaging the ADP-rybosylation factor 6 (Arf6) and its ICL3 (Min et al., 2002). Specifically, inactive Arf6-GDP is anchored at the plasma membrane and bound to β-arrestin; upon ligand stimulation, activated LHR recruits the ARF nucleotide binding site opener (ARNO), which triggers the GDP-GTP exchange on Arf6 and subsequent release of β-arrestin; this binds the receptor via its ICL3 and mediates its desensitisation and internalisation (Mukherjee et al., 2000). Specific residues in the LHR sequence have been identified that dictate higher rates of hLHR internalisation and β-arrestin sensitivity compared to rat LHR; these include 4 aa in ICL3 and additional 3 aa in ICL2 and 1 aa in the C-tail (Nakamura et al., 2000).

Once internalised, LHRs from different species are directed to two distinct sorting fates: rat, mouse and porcine LHR are predominantly directed to the lysosomes for degradation, whilst hLHR is mainly recycled back to the plasma membrane (Kishi et al., 2001). Extensive research conducted in Ascoli's laboratory identified a number of residues responsible for the divergent behaviour of different LHRs. The first sequence to be recognized was the GTALL in LHR C-tail, highly similar to the DSLL sequence in B2AR already known to direct receptor recycling via interaction with NHERF, although this was not the case for LHR (Kishi et al., 2001). Soon after, it was demonstrated that $G^{687}T^{688}$ residues in the GTALL sequence are sufficient to sustain hLHR recycling as well as showing that phosphorylation of Thr^{688} or other serines present in hLHR C-tail is not required to sort LHR to the recycling pathway (Galet et al., 2003). Moreover, the adaptor protein GIPC was found to interact with LHR in a yeast two-hybrid screen and further immunoprecipitation analysis confirmed that it was the PDZ domain of GIPC to selectively bind LHR C-tail; specifically, Cys^{699} and more marginally Leu^{683} mediate LHR recycling via GIPC (Hirakawa et al., 2003). Although these findings appear contradictory when considering the previously identified GT motif, it has been proposed that the whole 17 residue C-tail of LHR determines its tendency to recycle via interaction with an array of proteins including the main player GIPC (Galet et al., 2004). More detailed information about the role of GIPC on LHR sorting was provided by studies conducted in Hanyaloglu's laboratory. The evidence that LHR internalises into a much smaller endosomal compartment, the VEE, compared to the prototype rhodopsin family member B2AR, led to the hypothesis that other endosomes exist in addition to the EE and that different receptors might be directed to divergent endocytic compartments, whose specificity is determined by the adaptor proteins recruited by each receptor. As previously mentioned, in the case of LHR, it is the interaction between GIPC and its C-tail that dictates the internalisation to the VEE; truncation of LHR C-tail or GIPC depletion determine re-routing of LHR to the EE, from where the receptor is unable to recycle nor fully activate its MAPK response (Jean-Alphonse et al., 2014).

Some similarities and differences between LHR and FSHR trafficking have been reported. As for LHR, FSHR internalisation relies on β-arrestin and clathrin recruitment, although β-arrestin requires both receptor and activation to associate with FSHR (Krishnamurthy et al., 2003a). FSHR follows the same sorting fate as LHR, with most of the internalised receptors

being recycled to the plasma membrane still in conjunction with their ligands (Krishnamurthy et al., 2003b). Furthermore, FSHR displays VEE localisation and GIPC requirement for a sustained ERK phosphorylation, suggesting GIPC has the same role in targeting FSHR to VEE, as for LHR (Jean-Alphonse et al., 2014). Lastly, the recently discovered VEE marker APPL1 is a known interactor of FSHR (Nechamen et al., 2007, Jean-Alphonse et al., 2014), strengthening the hypothesis that both LHR and FSHR could share both sorting platform and machinery.

1.5 Gonadotrophin receptors: physiological roles and clinical perspectives

1.5.1 The hypothalamic-pituitary-gonadal axis

Glycoprotein hormone receptors respond to hormones secreted by the anterior pituitary gland: specifically, TSH is produced by thyrotrope cells, whilst LH and FSH by gonadotrope cells; for this reason, FSHR and LHR are also known as gonadotrophin receptors (Jiang et al., 2014). The production of gonadotrophins is triggered by a series of hormonal signalling events that start in the hypothalamus and terminate in the gonads: the hypothalamic kisspeptin neurons produce kisspeptin, which binds its receptors expressed in gonadotrophin releasing hormone (GnRH) neurons. This triggers the pulsatile generation of GnRH, which binds GnRH receptors (GnRHR) expressed in the pituitary gland (Roseweir and Millar, 2009). This, in turn, induces secretion of FSH and LH into the circulation, which then bind their receptors expressed in both male and female gonads to regulate reproduction, including steroid hormone production (Waters and Conn, 1991). Testosterone, inhibin, oestradiol and progesterone feed back to the pituitary and hypothalamus to regulate kisspeptin and GnRH production (Wildt et al., 1981, Rivier et al., 1986). This integrated system is considered a whole functional unit termed the HPG axis and governs the endocrine control of reproduction ((Plant, 2015) and Figure 1.7).

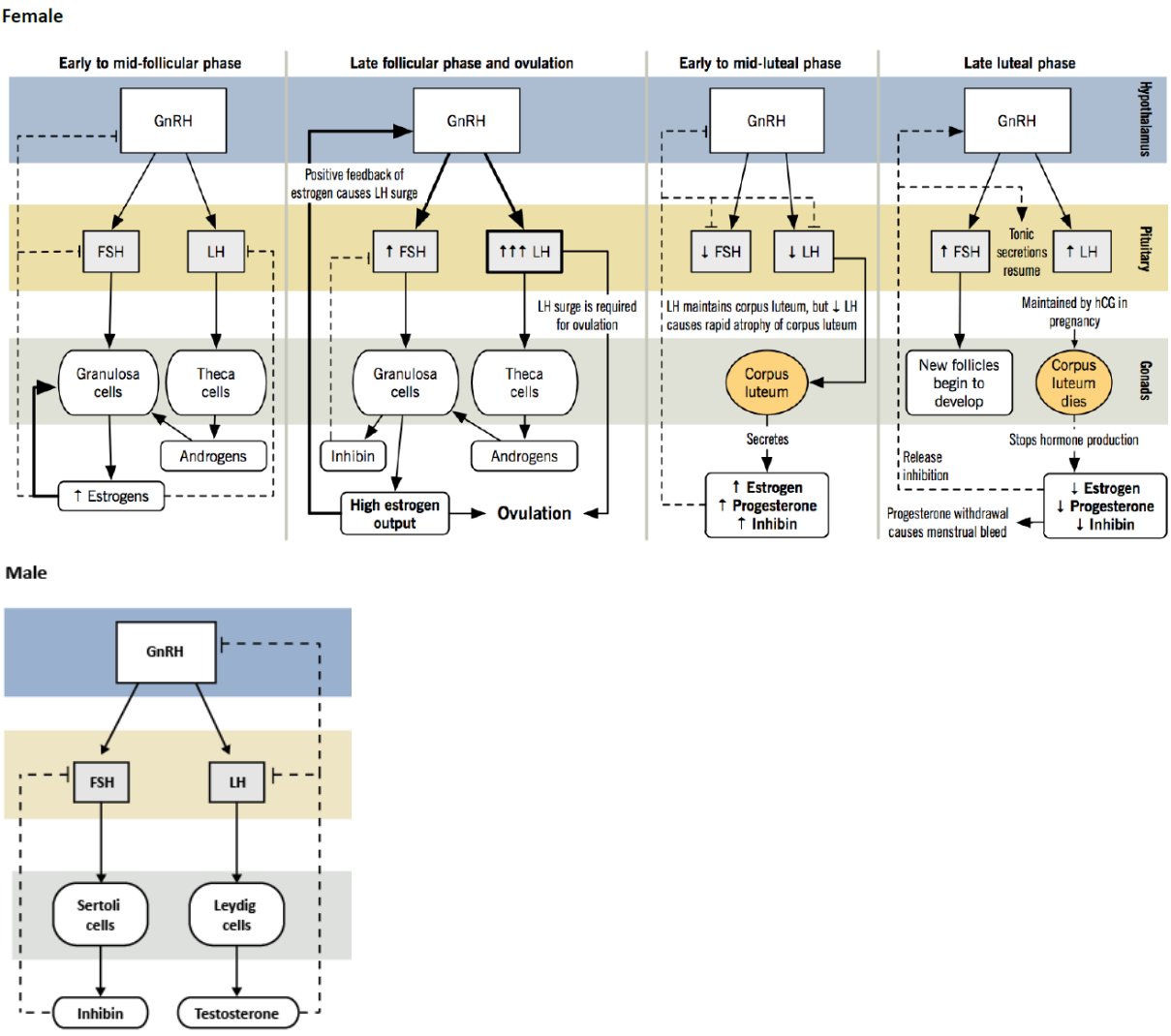


Figure 1.7 HPG axis in females and males. GnRH produced by the hypothalamus triggers the production of gonadotrophins by the anterior pituitary gland. In turn, LH and FSH act on the gonads. In females, in the follicular phase of the cycle FSH acts on granulosa cells and LH on theca cells which produce estrogens and androgens, respectively. After ovulation, the luteal phase of the cycle starts and the corpus luteum appears, taking over the ovary in the production of sex hormones; it is maintained for a short period of time in non-pregnant individuals by LH and throughout the pregnancy by hCG. In males FSH acts on Sertoli cells and LH on Leydig cells which release inhibin and testosterone, respectively. Negative feedback: dotted lines; positive feedback: full lines.

In adult males, the testis contains spermatogenic, Sertoli and Leydig cells. Spermatogenic cells develop into spermatozoa, Sertoli cells support and nourish the spematogenic cells, and Leydig cells are interstitial cell which lie between the seminiferous tubules. Leydig cells

respond to LH producing testosterone, which stimulates spermatogenesis and negatively feeds back to the pituitary and the hypothalamus to stop LH and GnRH production, respectively ((Ramaswamy and Weinbauer, 2014, Jarow and Zirkin, 2005) and Figure 1.7). Sertoli cells are responsive to FSH and, as a consequence, they secrete inhibin which blocks pituitary FSH production ((Ramaswamy and Weinbauer, 2014) and Figure 1.7).

In adult females, hormonal regulation is more complicated than in males because the effects of different hormones vary with the stage of the menstrual cycle (Figure 1.8). Similarly to male gonads, the female ovary is composed by the maturing oocytes and two types of endocrine cells, granulosa and theca cells. Granulosa cells have the same function of Sertoli cells, supporting follicles during their development, whilst theca cells surround the follicle and are the source of androgens. Granulosa cells respond to FSH and, when the follicle reaches the mature stage (Graafian follicle) they become responsive to both LH and FSH, producing inhibin and estrogens; theca cells, instead, respond only to LH generating androgens, which are converted to oestradiol by aromatases produced by granulosa cells (Figure 1.7). While inhibin always exerts its negative feedback action on FSH and GnRH production, oestradiol can serve as a positive or negative feedback hormone according to the stage of the menstrual cycle (Figure 1.7). The menstrual cycle can be divided into 3 phases: the follicular phase, the peri-ovulatory phase and the luteal phase. In the follicular phase (day 0-12), primary follicles mature to secondary ones, with increasing amount of oestradiol being produced until reaching a maximum on day 11-12 ((Mihm et al., 2011) and Figure 1.8). High levels of oestradiol positively act on the pituitary determining a robust production of LH (LH surge); this is the peri-ovulatory phase (day 12-14) when only one dominant follicle is present in the ovary and, in addition to LH, also FSH levels suddenly increase in preparation for ovulation (day 14). After ovulation, the ovary enters the luteal phase that last until menstruation (day 15-28) ((Reed and Carr, 2000) and Figure 1.8). In the early luteal phase, the follicle develops into the corpus luteum, which secretes progesterone, responsible for keeping LH and FSH levels low. In the late luteal phase, in the circumstance of a non-fertilised oocyte, the corpus luteum regresses and stops producing progesterone, thus LH and FSH levels start to rise again ((Mihm et al., 2011) and Figure 1.8).

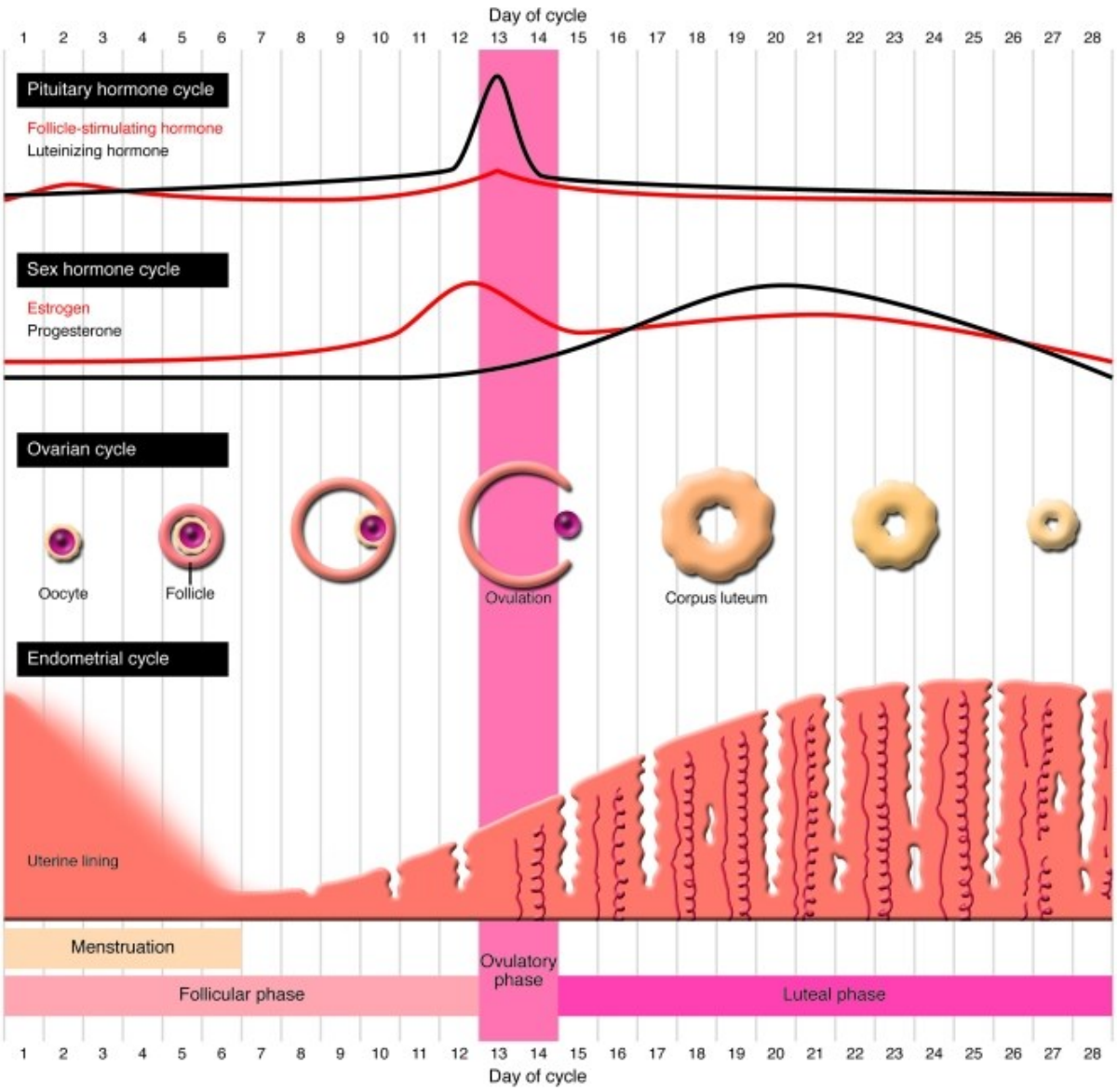


Figure 1.8 The menstrual cycle. The menstrual cycle is a series of ovarian hormone-driven changes of the ovary (ovarian cycle) and the endometrium (endometrial cycle) that occurs on a monthly basis. The ovarian cycle is comprised of phases characterised by changes in the ovarian follicles: follicular phase, ovulatory phase, and luteal phase. The endometrial cycle is comprised of phases characterised by changes in the endometrial lining of the uterus: menstruation, proliferative phase and secretory phase. Adapted from (Aitken et al., 2008).

1.5.2 Physiological roles of gonadotrophins and their receptors in the normal female cycle

Expression of both receptors and their ligands undergoes dynamic changes during the menstrual cycle. Early antral follicles express FSHR in granulosa cells and small amounts of

LHR in theca cells. Both LHR and FSHR levels rise with follicle growth, with LHR expression also in granulosa cells, reaching their maximum in the pre-ovulatory phase. From here, whilst FSHR decreases constantly after ovulation, LHR levels are temporarily down-regulated in response to the LH surge, they rise again in the mid-luteal phase and decrease with the corpus luteum regression. LHR expression pattern is inverse compared to the expression of bioactive LH isoforms: they reach their peak in the middle of the cycle (LH surge) and are their minimum during the luteal phase (Jeppesen et al., 2012).

According to their timing of appearance and to the receptor expression pattern, gonadotrophins play different roles. Initially FSH acts on FSHR-expressing granulosa cells to stimulate follicular growth and aromatisation of androgens to oestradiol; at the same time, LH stimulates androgen production from LHR-expressing theca cells (Miro and Aspinall, 2005). FSH also promotes LHR expression in granulosa cells and, in the mid follicular phase of the cycle, synergises with LH to trigger further follicular development and oocyte maturation (Miro and Aspinall, 2005). Concomitantly, oestradiol levels increase causing FSH levels to fall, decidualisation of the endometrium and stimulation of LH production (March et al., 1979). The subsequent LH surge provokes ovulation, resumption of meiosis in the oocyte and corpus luteum formation and early steroidogenic activity (Kumar and Sait, 2011). If the oocyte is fertilised, the trophoblastic cells of the early embryo, and the placenta later on, produce hCG, essential for maintaining progesterone production by the corpus luteum in early pregnancy, the initiation of the implantation, inhibition of LH and FSH secretion and steroidogenesis in fetal gonads (Csapo and Pulkkinen, 1978, Cole, 2010). Recent evidence that hCG levels increase even after hCG is no longer needed for progesterone production raise the possibility that hCG plays additional roles during pregnancy, including avoidance of fetal rejection, development of fetal organs and coordination of fetal-uterine growth (Cole and Muller, 2010, Cole, 2009). In the absence of pregnancy menstruation takes place, the corpus luteum regresses and a new pre-antral follicle cohort is recruited.

LHR expression has been observed in a number of extra-gonadal tissues including oviduct, cervix, myometrium, endometrium, uterus, breast and brain of various species, namely primates, cow, turkey, rodents, pig and human (Rao and Lei, 2007). The function of extra-gonadal LHR is still under debate and results are often controversial; as an example, two

different groups transplanted LHR knock-out mice with wild-type ovaries and in one case those mice could get pregnant, in the other they were infertile (Pakarainen et al., 2005, Chudgar et al., 2005). In the uterus of more than one species, LHR expression is correlated with enhanced prostaglandin-endoperoxide synthase levels and prostaglandins levels (Fields and Shemesh, 2004, Reshef et al., 1990, Ziecik et al., 1992). Although these results are intriguing, more research needs to be conducted to unveil the significance of LHR expression in each of these tissues.

1.5.3 Gonadotrophin receptors in pathology

Consistent with gonadotrophin receptors' pivotal role in reproduction, mutations affecting LHR or FSHR result in diseases which affect sexual development or fertility (Themmen and Huhtaniemi, 2000). Both activating and loss-of-function mutations have been reported. Mutations leading to constitutive activation of LHR are all single base substitutions in exon 11 affecting TM6 or ICL3 and, in most cases, specifically Asp^{578} (Shenker et al., 1993). Gain-of-function mutations have only been observed in males, where the most common phenotypes are hyperplasia and precocious puberty. Loss of function mutations are restricted to exon 10 and caused by single base substitution, partial gene deletion or insertion causing, in the majority of cases, intracellular retention of the receptor (Newton et al., 2016). Male phenotypes include micropenis, absence of puberty, Leydig cells hypoplasia and pseudohermaphoditism (Laue et al., 1995); although more rarely, inactivating mutations affect also females: amenorrhea or oligomenorrhea, infertility, polycystic ovary syndrome (PCOS) and empty follicle syndrome are the reported phenotypes (Arnhold et al., 2009, Yariz et al., 2011). Mutations in FSHR have been identified as well, although fewer than in LHR (Huhtaniemi and Themmen, 2005). Activating mutations determine ovarian hyperstimulation syndrome in females; inactivating mutations cause amenorrhea and infertility in females and less severe spermatogenic defects in males, suggesting FSHR is not an absolute requirement for spermatogenesis (Tao and Segaloff, 2009, Hugon-Rodin et al., 2017).

Gonadotrophin receptors have also been linked to breast and ovarian cancers in women and testicular and prostate cancer in men (Zhang et al., 2017a). The insertion of 2 amino acids (Leu-Gln) in the signal peptide of LHR encoded by exon 1 results in a mutant receptor characterised by increased surface expression and activity and, importantly, correlates with decreased probability of breast cancer-free survival of patients (Piersma et al., 2006). Increased migration and invasion of breast cancer cells has been observed upon LH and FSH treatment, which, acting through LHR and FSHR, stimulate PI3K and PKB pathways leading to cytoskeletal rearrangements required for breast cancer progression (Sanchez et al., 2016). The involvement of gonadotrophin receptors in ovarian cancer has been hypothesized following the evidence that they are expressed in ovarian surface epithelial cells (Parrott et al., 2001); for LHR, its expression in carcinoma cells decreases with ovarian cancer progression suggesting a role for LHR in influencing the tumour growth (Lu et al., 2000). LHR expression at the surface of ovarian cancer cells has encouraged the development of LH- and FSH-linked nanoparticles to target and enhance cell death in the tumour as well as the search for biomarkers based on genes up- or down-regulated by LHR in these cells (Shah et al., 2013, Zhang et al., 2009, Cui et al., 2011). Moreover, gonadotrophins appear to induce cell migration and invasiveness in ovarian cancer cells through gonadotrophin receptor-activated pathways, including PKA and PI3K (Choi et al., 2006, Lau et al., 2010).

1.5.4 Polycystic ovary syndrome (PCOS)

PCOS is the main cause of infertility, affecting up to 10% of the total female population of reproductive age (Polson et al., 1988). It is a metabolic and hormonal disorder characterised by at least two of these clinical observations: ovarian dysfunction (oligo-ovulation or anovulation) or menstrual irregularity, polycystic ovary at ultrasound examination and hyperandrogenism. Hyperandrogenism results from insulin resistance and subsequent hyperinsulinemia, typical of PCOS patients. Insulin directly stimulates theca cells, more sensitive to insulin in PCOS patients, to produce higher amount of androgens. Notably, the insulin resistance observed in PCOS women predisposes to the development of type 2 diabetes mellitus, especially if patients are obese (Pasquali et al., 2002). Compellingly, APPL1 seems to be downregulated at RNA level in PCOS granulosa cells and APPL1 protein levels

are lower in the endometrium from obese PCOS women compared to both lean and obese women with normal ovaries (Dehghan et al., 2016, Garcia et al., 2015). Ovulatory dysfunctions might be caused by altered levels of gonadotrophins and their receptors as a consequence of the increased frequency of GnRH release observed in PCOS patients (Arroyo et al., 1997). The involvement of gonadotrophic hormones and their receptors has been suggested by a number of different studies; granulosa cells from polycystic ovaries display premature sensitivity to LH and higher levels of LHR have been found in both polycystic ovarian theca and granulosa cells (Willis et al., 1998, Jakimiuk et al., 2001); in addition, the FSHR variant $Asn^{680}Ser$ correlates with more severe forms of PCOS (Valkenburg et al., 2009). Further evidence of the implication of LHR and FSHR in the aetiology of the disease comes from genome-wide association studies conducted in Chinese, Egyptian and European women, where risk loci for PCOS were found in chromosome 2, in close proximity of the genes encoding LHR and FSHR, as well as another locus associated with altered gonadotrophin secretion and PCOS (Tian et al., 2016b, Hayes et al., 2015, El-Shal et al., 2016).

1.5.5 Small allosteric modulators of gonadotrophin receptors

As previously stated, GPCRs have been among the most successful drug targets, but there is evidence of side effects due to cross-reactivity of synthetic agonists and antagonists with other highly similar GPCR members; in addition, the development of effective drugs is made more challenging by the fact that orthosteric molecules, which bind to the endogenous ligand binding site, require to enter the highly lipophilic transmembrane core of the receptor to activate it (Conn et al., 2009). To overcome these problems, allosteric molecules, that bind the receptor in sites different from the ligand-binding orthosteric one, have been designed in the last few decades and shown to modulate receptor activity positively, in the case of positive allosteric modulators (PAMs), or negatively (NAMs) (Wootten et al., 2013). The development of allosteric molecules targeting gonadotrophin receptors will overcome a number of problems: the fact that orthosteric modulators cannot be developed for gonadotrophin receptors considering the size of the endogenous ligands; patient convenience as in assisted reproductive technology (ART) recombinant hormones are still

administered to patients by injections; the fact that allosteric modulators could synergise with the gonadotrophins physiologically produced by the patient; the use of NAMs as oral contraceptives with less side effects compared to the currently available steroid-based drugs (Arey, 2008). Most of the allosteric modulators developed so far are PAMs for FSHR, as FSH is the only hormone able to achieve ovarian hyperstimulation required for ART. Different classes of FSHR PAMs exist according to their chemical structure but, although some showed oral bioactivity in rodents, this has not been confirmed in humans (van Koppen et al., 2013, Yu et al., 2014). Among LHR PAMs, Org43553 is characterised by remarkable features: high oral bioavailability, short half-life, biased agonism towards the cAMP pathway and low risk of ovarian hyperstimulation syndrome (van de Lagemaat et al., 2011, van de Lagemaat et al., 2009, van Koppen et al., 2008); notably, it is able to induce ovulation in healthy, pituitary-suppressed women (Gerrits et al., 2013). NAMs for both LHR and FSHR have been generated, although none of them are currently used for clinical purposes (Nataraja et al., 2015). Two NAMs originally created to antagonise FSHR action, ADX68692 and ADX68693, have also been tested on LHR; the effect of these molecules differed from their pharmacological profile on FSHR and between distinct pathways analysed, highlighting the complexity of the regulation of gonadotrophin receptors signalling (Ayoub et al., 2016).

1.6 Hypotheses and aims

As described in this chapter, GPCR functions are highly regulated in both time and space via a bidirectional control of trafficking and signalling. The requirement for precise control of both these activities is fundamental in the achievement of correct cellular and physiological functions, as highlighted by pathological conditions with perturbed GPCR signalling or trafficking.

The profound effects of membrane trafficking on GPCR signalling output has been emphasised by recent evidence that in addition to the plasma membrane, GPCRs can signal also from intracellular locations. Cells decode differential sources of signal activation which, as a consequence, results in specific cellular outputs. Compellingly, post-endocytic signalling is not restricted to the EE, but can be activated from other endosomal compartments, namely the VEE, where LHR, FSHR and B1AR are internalised to. LHR trafficking to the VEE has been demonstrated to depend on the interaction between LHR PDZ-ligand and the adaptor protein GIPC; additionally, localisation of LHR at the VEE is essential to drive its recycling back to the plasma membrane and to fully activate the MAPK pathway. Experiments aimed at the characterisation of the VEE led to the identification of the adaptor protein APPL1 as marker of this compartment, yet its functions are still unknown, as well as the mechanisms regulating LHR recycling and signal activation from the VEE.

Therefore, the overall goal of this thesis is to determine the regulatory mechanisms dictating LHR post-endocytic sorting from the VEE and its influence on LHR signalling pathways, and to unveil how adaptations in spatial-temporal control of receptor signalling specifically correspond to a physiological or patho-physiological outcome.

Specifically, my aims are:

I. To unveil the role of APPL1 in LHR trafficking and/or signalling at the VEE.

II. To characterise the molecular mechanisms dictating LHR recycling.

III. To identify LH-induced signalling pathways, in addition to MAPK, that are regulated in a location-dependent manner and if they are differently activated at the plasma membrane or from specific endosomal compartments.

IV. To characterise the LH-induced MAPK activation via the identification of its contributors.

V. To elucidate the role of VEE-localised LHR under physiological and patho-physiological conditions.

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Primary antibodies

Antibody	Isotype	Supplier	Catalogue number	Diluition WB	Diluition IF (FACS/confocal)	Diluition IF (TIRF/SIM)
APPL1	rabbit	Cell Signalling	3858	1:500	1:500	1:500
Clathrin heavy chain	rabbit	Cell Signalling	4794	N/A	N/A	1:150
EEA1	rabbit	Cell Signalling	3288S	N/A	1:500	1:400
Flag M1	mouse	Sigma	F3040	N/A	1:1000	1:2000
GAPDH	mouse	Millipore	AB2302	1:2000	N/A	N/A
GFP	rabbit	Thermo Fisher	A11122	1:1000	N/A	N/A
GIPC	rabbit	Santa Cruz	sc-25556	N/A	N/A	1:150
OCRL	rabbit	Thermo Fisher	PA5-27844	1:500	N/A	N/A
Phospho-ERK1/2	rabbit	Cell Signalling	9101	1:1000	N/A	N/A
Phospho-serine	rabbit	Millipore	AB1603	1:1000	N/A	N/A
Total-ERK1/2	rabbit	Cell Signalling	9102	1:1000	N/A	N/A

2.1.2 Secondary antibodies

Antibody	Isotype	Reactivity	Supplier	Catalogue number	Diluition WB	Diluition IF
AlexaFluor 488	goat	mouse	Thermo Fisher	A11001	N/A	1:1000
AlexaFluor 488	goat	rabbit	Thermo Fisher	A11008	N/A	1:1000
AlexaFluor 555	goat	mouse	Thermo Fisher	A21422	N/A	1:1000
AlexaFluor 555	goat	rabbit	Thermo Fisher	A21428	N/A	1:1000
AlexaFluor 568	goat	mouse	Thermo Fisher	A11004	N/A	1:1000
AlexaFluor 594	goat	mouse	Thermo Fisher	A11005	N/A	1:1000
AlexaFluor 647	goat	mouse	Thermo Fisher	A21235	N/A	1:1000
AlexaFluor 647	goat	rabbit	Thermo Fisher	A21245	N/A	1:1000
Horseradish Peroxidase HRP conjugated IgG	goat	mouse	Life Technologies	626520	1:1000	N/A
Horseradish Peroxidase HRP conjugated IgG	goat	rabbit	Life Technologies	656120	1:1000	N/A

2.1.3 Plasmids

DNA construct	Source
APPL1-Rluc8	Frederic Jean-Alphonse
Flag-B1AR	Addgene Ref. 14698
Flag-B2AR	Mark von Zastrow
Flag-FSHR	Ilpo Huhtaniemi
Flag-LHR	Ilpo Huhtaniemi
Gαs-Rluc8	Eric Reiter
Gαs-Venus	Eric Reiter
Gβ	Eric Reiter
Gγ-Venus	Eric Reiter
GFP-Nb37	Mark von Zastrow
GFP-Rab31	Chiara Recchi
GFP-Rab35	Chiara Recchi
GFP-S410A APPL1	Frederic Jean-Alphonse
GFP-WT APPL1	Pietro De Camilli
HA-Gαs	Mark von Zastrow
LHR-Venus	Stanford Chen
mCherry-OCRL	Pietro De Camilli
mCherry-S410A APPL1	Silvia Sposini
mCherry-S410D APPL1	Silvia Sposini
mCherry-WT APPL1	Frederic Jean-Alphonse
SEP-B2AR	Manoj Puthenveedu
SEP-LHR	Silvia Sposini
SEP-TfR	Manoj Puthenveedu

2.1.4 Primers and siRNAs

Primer sequence	fw 5' - 3'
APPL1 S410A	GCAGAGGCACGAGGCCCTGCGGCCAGCAGC
APPL1 S410D	GCAGAGGCACGAGGACCTGCGGCCAGCAGC
LHR AgeI	GTGTGGTCTCCGATTACACCGGTGATGATGATAAGCGAGC
OCRL W739A	GGTTCCCAAGGAGATCGCGCTTCTAGTAGATCAC
siRNA sequence	**fw 5' - 3'**
scramble	AATTCTCCGAACGTGTCACG
APPL1	GACAAGGTCTTTACTAGGTGTATTT
OCRL	GGGTCTCATCAAACATATCTT

2.1.5 Inhibitors and activators

Inhibitor	Target	Supplier	Catalogue number	Working concentration
2-HE	sAC	Sigma	H3131	20 µM
Cilostamide	PDE3	VWR	BML-PD-125-0005	400 nM
Cyclohexamide	protein syntesis	Sigma	C104450	10 µg/mL
Cytochalasin B	actin	Sigma	C6762	2 µM
ddA	tmAC	Calbiochem	288104	100 µM
Dyngo-4a	Dynamin	Abcam	ABI20689	30 µM
Gallein	Gβγ	Tocris	2103-64-2	25 µM
IBMX	PDEs	Sigma	I5879	0.5 mM
KT5720	PKA	Abcam	ABI41818	10 µM
Nocodazole	microtubules	Sigma	31430-18-9	10 µM
Phenylmethylsulfonyl fluoride	serine proteases	Sigma	P7626	1 mM
Protease inhibitor tablets	proteases	Roche	4693159001	1 in 10 mL
Roflumilast	PDE4	Sigma	SML1099	50 nM
Sodium fluoride	pSer/pThr phosphatases	NEB	P0759S	1 mM
Sodium orthovanadate	pTyr phosphatase	Sigma	S6508	1 mM
UBO-QIC	$G\alpha_{q/11}$	University of Bonn	NA	1 µM

Activator	Target	Supplier	Catalogue number	Working concentration
Forskolin	tmAC	Sigma	F3917	3 µM
8-Br-cAMP	PKA	Sigma	B7880	0.5 mM

2.1.6 Reagents

Reagent	Supplier
Absolute ethanol	VWR
AgeI restriction enzyme	New England Biolabs
30% Acrylamide/Bis solution (37.5:1)	Bio-Rad
Ampicillin	Fisher Scientific
Agarose powder	Appleton Woods
Ammonium persulfate (APS)	Sigma
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Sigma
Chloroform	Sigma
Coelenterazine-h	Promega
Deoxynucleotide triphosphate (dNTPs)	Life Technologies
Dminethyl sulphoxyde (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Estadiol	Sigma
Ethylenne diamine tetraacetic acid (EDTA)	Sigma
Glycerol	Sigma
Hydrochloric acid	Sigma
Insulin	Sigma
Isopropanol	Sigma
Kanamycin	Sigma
Lysogeny broth or Luria Bertani broth (LB)	MP Biomedicals
Magnesium chloride	Sigma
Magnesium sulphate	Sigma
Medroxyprogesterone (MPA)	Sigma
Methanol	VWR
Nonylphenyl polyethylene glycol (NP-40)	Calbiochem
Paraformaldehyde powder	Sigma
Phosphate buffered saline (PBS)	BD Healthcare
PBS with Ca^{2+}	Sigma
SDS solution 10% w/v	Bio-Rad
Sodium azide	Sigma
Sodium chloride	Sigma
Sodium dodecyl sulphate (SDS)	BD Healthcare
Tris base	Sigma
Tris HCl	Sigma
Triton X-100	Sigma
Tween 20	BD Healthcare
β-mercapotethanol	Sigma

2.1.7 Cell culture reagents

Reagent	Supplier
Antibiotic/antimycotic (x100)	Invitrogen
Collagenase type IA	Sigma
Deoxyribonuclease I (Dnase I)	Roche
Dextran-coated charcoal	Sigma
Dulbecco's modified eagles medium (DMEM) ± phenol red	Sigma
DMEM/F12 (1:1) 1x nutrient mixture, with L-glutamine, 15 mM HEPES ± phenol red	Invitrogen
Fetal bovine serum (FBS)	Sigma
Geneticin G418	Thermo Fisher
Heat-inactivated FBS	Thermo Fisher
L-glutamine, 200 mM (x100)	Thermo Fisher
Lipofectamine 2000	Thermo Fisher
Lipofectamine RNAimax	Thermo Fisher
Opti-MEM I reduced serum media	Thermo Fisher
Opti-MEM I reduced serum media + HEPES	Thermo Fisher
Penicillin/Streptomycin	Thermo Fisher
Trypsin-EDTA solution	Thermo Fisher

2.1.8 Kits

Kit	Supplier
cAMP Dynamic 2	Cisbio
Fluo-4 Direct Calcium Assay	Thermo Fisher
HiSpeed Plasmid Maxi	Qiagen
QIAprep Speen Miniprep	Qiagen
QIAquick Gel Extraction	Qiagen
Quick Ligation	NEB
QuickChange Site-Directed Mutagenesis	Agilent Technologies

2.1.9 Miscellaneous

Miscellaneous	Supplier
0.22 µm syringe filters	Millipore
1 µ-slide 8 well glass bottom chambered slide	Ibidi
ECL Hyperfilm	GE Healthcare
Fluoromount G	Thermo Fisher
GFP-Trap_A beads	Chromotek
Glass coverslips	Menzel-Glazer
Hybond blotting paper	GE Healthcare
Hyerladder DNA marker	Bioline
Luminata Classico HRP substrate	Millipore
Luminata Forte HRP substrate	Millipore
Nitrocellulose blotting membrane	GE Healthcare
PageRuler Plus prestained protein ladder	Fermentas
Plasticware	Corning
Primers	Thermo Fisher
RNaseZap	Ambion
Skimmed milk powder	Sigma
SYBR green agarose gel stain	Thermo Fisher
Vectashield	Vector

2.1.10 Buffers and solutions

Solutions were stored at room temperature, unless otherwise stated. All buffers were made with deionized and distilled water.

Phosphate buffered saline (PBS)

140 mM NaCl

2.5 mM KCl

1.5 mM KH_2PO_4

Tris buffered saline (TBS)

130 mM NaCl

20 mM Tris, pH 7.6

TBS-Tween 20 (TBS-T)

0.1% Tween 20 in TBS

FACS buffer

2% (v/v) FBS in PBS with Ca^{2+}

Acid wash buffer

50 mM glycin

100 mM NaCl

pH 3.0

Cell lysis buffer

10 mM Tris-HCl, pH 7.5

0.5 mM EDTA

150 mM NaCL

1% (v/v) Triton X-100

1 mM PMSF

1 mM NaF

1 mM $NaVO_3$

Stored at +4°C (maximum 1 week).

For immunoprecipitation, 0.5% NP-40 was used instead of TritonX-100.

Protein loading buffer (Laemmli buffer) (2X)

4% (v/v) SDS

20% (v/v) Glycerol

10 mM Tris-HCl, pH 6.8

10% (v/v) β-mercaptoethanol

0.04% Bromophenol Blue

4% PFA

4% (w/v) PFA in PBS

pH 7.2-7.4

Store at -20°C (long storage) or at +4°C (1 week).

Flow cytometry buffer

2% (v/v) FBS in PBS

Western blot SDS running buffer (10X)

250 mM Tris Base

1.9 M Glycine

1% (w/v) SDS

Western blot Transfer Buffer

250 mM Tris Base

192 mM glycine, pH 8.3

20% (v/v) Methanol

Used cold.

Western blot Stripping Buffer

100 mM β-mercaptoethanol

2% (v/v) SDS

62.5 mM Tris HCl, pH 6.8

Western blot Blocking Solution / Primary Antibody Dilution Solution

5% (w/v) skimmed milk or BSA in TBS-T

Stored at +4°C.

Immunofluorescence Primary Mouse Anti-FLAG Antibody Stripping Solution

0.04 M EDTA in PBS

Immunofluorescence Blocking Solution / Primary and Secondary Antibody Dilution Solution

2% (v/v) FBS in PBS with Ca^{2+}

Immunofluorescence Permeabilisation Solution

2% (v/v) FBS in PBS with Ca^{2+}

0.2% (v/v) Triton X-100

SDS Polyacrylamide Gels

Separating Gels

380 mM Tris HCl, pH 8.8

0.1% (v/v) SDS

12% (v/v) Acrylamide Bis Solution

0.03% (w/v) APS

TEMED added at 1:2000

Stacking Gels

130 mM Tris HCl, pH 6.8

0.1% (v/v) SDS

5% (v/v) Acrylamide Bis Solution

0.06% (w/v) APS

TEMED added at 1:1000

2.1.11 Bacterial media

LB Broth

1% (w/v) Bacto Tryptone

0.5% (w/v) Yeast Extract

0.5% (w/v) NaCl

LB Agar

LB Broth + 1.5% (w/v) Agar

2.1.12 Cell culture media

HEK 293 cells

Maintenance medium

DMEM (phenol red)

+ 10% (v/v) FBS

+ 100 U/ml penicillin/streptomycin

Transfection medium

DMEM (phenol red)

+ 10% (v/v) FBS

Assay medium

DMEM (phenol red free)

hESCs

Maintenance medium

DMEM/F12 (phenol red)

+ 10% (v/v) DCC-FBS

+ 2 mM L-Glutamine

+ Antibiotic-Antimycotic

100 U/ml penicillin

10 µg/ml streptomycin

0.25 µg/ml Fungizone antimycotic

+ 2 µg/ml Insulin

+ $1*10^{-6}$ M Oestradiol

Decidualising medium

DMEM/F12 (phenol red free)

+ 2% (v/v) DCC-FBS

+ 2 mM L-Glutamine

+ Antibiotic-Antimycotic

100 U/ml penicillin

10 µg/ml streptomycin

0.25 µg/ml Fungizone antimycotic

+ 0.5 mM 8-Br-cAMP

+ 10 µM MPA

Transfection medium

DMEM/F12 (phenol red free)

+ 5% (v/v) DCC-FBS

+ 2 mM L-Glutamine

2.2 Methods

2.2.1 Bacteria handling

Preparation of chemically competent bacteria for transformations

Propagation of all plasmids in this work was carried out using the Escherichia *coli* (E.*coli*) DH5α strain. Bacteria were thawed on ice, plated onto L-agar plates and incubated at 37°C overnight. A single colony was then picked and grown in 5 ml of LB broth. This colony was grown at 37°C overnight on a rocking platform shaker. The following day, 200 µl of the bacterial culture were added to 200 ml of LB broth and placed on a rocking platform shaker. Aliquots were taken at regular intervals and the OD was measured. When the OD600 reached 0.2-0.4 the culture was chilled on ice for 10 min before centrifugation at 1000 rpm for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 30 ml of ice-cold solution containing 100 mM $CaCl_2$ and 10% glycerol. The cells were then aliquoted and snap-frozen with dry ice. This procedure was carried out near a flame to ensure sterility.

Transformation of bacteria and purification of plasmid DNA

DH5α cells (usually 50 or 100 µl aliquots) were thawed on ice and ~1 µg of plasmid DNA to be amplified was added and gently mixed. The cells were incubated on ice for 20 min and then heat-shocked on a 42°C heat block for 30 sec before being placed on ice for a further 2 min. 800 µl of LB broth was then added and the mix was placed on a rocking platform shaker for 45 min at 37°C. Subsequently, 200 µl of the cells were spread on appropriate antibiotic-containing (100 µg/ml of ampicillin or 50 µg/ml of kanamycin) LB agar plates and placed in a 37°C incubator overnight. The following day, a single colony was picked and inoculated in 10 ml (mini-prep) or 200 ml (maxi-prep) of LB broth containing 100 µg/ml of ampicillin or 50 µg/ml of kanamycin, as appropriate, and grown at 37°C on a rocking platform overnight. Cells were then centrifuged at 3000 rpm at 4°C and the supernatant was removed. Amplified DNA was purified from this pellet using the QIAprep Speen Miniprep (Qiagen) or the HiSpeed Maxi Plasmid kits (Qiagen) and instructions were followed as per the kit manual. All DNA was eluted in DEPC-treated water, purity concentration was determined using the ND-2000 spectrophotometer (NanoDrop) at A260/A280 and A260 respectively. The ideal A260/A280 ratio is ≥ 1.8, which indicates pure DNA.

2.2.2 Cloning

SEP-LHR

Human FLAG LHR plasmid has been previously described (Jean-Alphonse et al., 2014). In order to N-terminally tag this receptor with SEP for TIR-FM imaging, I used the SEP-B2AR. SEP was digested from SEP-B2AR contruct using AgeI restriction enzyme and ligated into a FLAG-LHR construct where I created an AgeI restriction site (LHR-AgeI) by QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). The design of the primers (see Materials, paragraph 2.1.4) was guided by the specification given by the Site Directed Mutagenesis kit (Agilent Technologies) and the protocol provided in the kit was followed to carry out the mutagenesis. Briefly, a mix was made of the following: 5 µl of 10 X reaction buffer, 10 ng of Flag-LHR plasmid (in pcDNA 3.1), 125 ng of LHR-AgeI fw primer, 125 ng LHR-AgeI rev primer and 1 µl of dNTP. DEPC-treated water was then added to a final volume of 50 µl. Finally, 1 µl

of PfuUltra HF DNA polymerase (2.5 U/µl) was added and gently mixed. The following cycling conditions were then used for the reaction: 95°C for 30 sec, followed by 18 cycles of: 95°C for 30 seconds, 55°C for 60 seconds, and finally 68°C for 7.5 min (the latter time calculated according to plasmid size, 7500 bp). This reaction was then put on ice to cool it to 37°C. After the reaction was cooled, 1µl of the restriction enzyme DpnI (10 U/µl) was added and thoroughly mixed by gentle pipetting, this was then left at 37°C for 2 hours to digest the non-mutated DNA. Next, 50 µl of XL1-Blue supercompetent cells were thawed on ice and 5 µl of the DpnI digest was added and gently mixed. Following this, the protocol for transforming bacteria as described above was followed. Once LHR-AgeI was obtained (confirmed by sequencing and restriction digests), both LHR-AgeI and SEP-B2AR were digested using the same endonuclease AgeI in order to obtain the vector and insert for the next ligation, respectively. This was done by incubating 4 µg of each of the plasmids with 5 µl of CutSmart Buffer, 2 µl of AgeI restriction endonuclease and DEPC treated water to make a final volume of 50 µl. The reactions were incubated for 2 hours at 37°C and the reaction was then stopped by chilling on ice. Digested products were separated by electrophoresis on a 1% (w/v) agarose gel containing SYBR Safe DNA Gel stain (1:10000). The electrophoresis process was run for 1 hour at 100 V. A molecular weight marker, HyperLadder, was loaded alongside the samples in order to determine size. The gel containing the separated digested products and the molecular weight marker was exposed to UV illumination and the insert (approximately 700 bp) and linearised plasmid (approximately 7500 bp) were excised. The products were purified with QIAquick Gel Extraction Kit (Qiagen) and concentration was determined using a ND-2000 spectrophotometer (NanoDrop). Ligation was then carried out using the Quick Ligation Kit (NEB). 50 ng of linearised plasmid was used and combined with the insert in a 1:10 ratio. The volume was then adjusted to 10 µl using DEPC-treated water, then 10 µl of 2 X Quick Ligation Buffer was added, followed by 1 µl of T4 DNA Ligase and further mixing. This reaction was incubated for 5 min at room temperature and stopped by chilling on ice before bacterial transformation as described above. Colonies were picked and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was then carried out by Beckman Coulter Genomics to confirm if the cloning was successful.

mCherry-S410A APPL1 and mCherry-S410D APPL1

In order to generate the S410 phospho-deficient and phospho-mimetic mCherry-APPL1 mutants, Serine in position 410 was mutated to an Alanine (S/A) or an Aspartate (S/D) via site directed mutagenesis. The primer sequences I designed and used are listed in Materials, paragraph 2.1.4. The protocol used is the same as the one above.

2.2.3 Human samples recruitment

Ethical approval

This study was permitted by Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (Ref: 1997/5065). All participants in this study read the patient information form and written informed consent was obtained from all patients prior to endometrial sampling.

Patient details

Patients were recruited from women undergoing treatments for infertility, of which the primary causes were male factor infertility, tubal or filling defects, PCOS or unexplained infertility. Women who had a history of endometriosis were excluded from the study.

Identifier	Age	Live Births	1st Trimester Losses	Notes
INF176	35	0	0	Tubal factor infertility.
INF177	N.A.	0	0	Possible male factor infertility
INF187	29	0	0	Possible male factor infertility
INF204	37	0	0	1 x miscarriage at 39 weeks. Male factor infertility. PCOS.
INF205	36	0	0	never had period in her life
INF206	N.A.	0	0	unexplaied
INF207	35	0	0	unexplained, male factor, PCOS
INF208	31	0	0	unexplained
INF209	30	0	1 <3 months	PCOS unovulatory
INF210	29	0	1 <3 months	mild PCOS
INF211	24	0	0	1 tube ectopic pregnancy
INF214	39	0	0	unexplained
INF216	33	0	0	male factor, mild PCOS

Endometrial tissue samples for primary cell culture

Endometrial biopsies were obtained by pipelle or curettage from women aged 18-42 years at the time of diagnostic hysteroscopy or hysterectomy. Biopsies were collected in PBS.

2.2.4 Cell culturing

Preparation of dextran coated charcoal stripped fetal bovine serum (DCC-FBS)

FBS contains endogenous steroid hormones that mask the effect of some exogenous ligands and therefore FBS used for hESC culture was stripped of small molecules by DCC treatment. 500 ml of FBS had 1.375 g of DCC added and was heated in a water bath to 56°C for 2 h, with regular mixing. The solution was then centrifuged at 3000 x *g* for 30 min and the supernatant was then filter-sterilised using a 0.2 µm filter system into a sterile bottle before aliquoting and stored at -20°C until use. DMEM/F12 was supplemented with 10, 5 or 2% (v/v) DCC-FBS for cell culture.

Isolation of hESCs from tissue samples

hESCs were isolated using a standard protocol, the purity of which has been well-established. Endometrial biopsies were placed in a Petri dish and finely minced using sterile scalpels. The tissue was then suspended into 10 ml of digest media and added into a T-25 cm² flask. The digest media consisted of phenol-red free DMEM/F12 supplemented with antibiotic/antimycotic, 0.5 mg/ml collagenase type IA broke up the extracellular matrix and 0.1 mg/ml of DNAse I, which removed the viscous DNA released during the digestion when cell apoptosis takes place. The endometrial digest mix was left to digest at 37°C with vigorous shaking every 10-20 min. After 1 h (or 1.5 h for larger samples), the digested sample was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The pellet, containing stromal, epithelial, glands and blood cells, was then resuspended in 20 ml of 10% DCC-DMEM/F12 and, depending on biopsy size, the suspension was transferred into a T-25 cm² flask or a T-75 cm² flask and placed for 1 h in a 37°C incubator. The media was then removed and fresh 10% DCC-DMEM/F12 was added. This allowed for the separation of

epithelial and stromal cells and was necessary as stromal cells adhere more rapidly than epithelial cells.

Maintenance of hESC cultures

Proliferating stromal monolayers were maintained in 10% DCC-DMEM/F12 in a cell culture incubator which had a humid atmosphere of 5% (v/v) CO_2 and was at 37°C. When confluent, stromal cells were passaged by removing media and adding 5 ml 0.05% trypsin/EDTA in PBS (at 37°C) to wash and remove excess media. A further 5 ml 0.05% trypsin/EDTA was added to the flask and the cells were placed at 37°C for 5 min and gently tapped to ensure all cells were lifted from the surface of the flask. Trypsin activity was inhibited by adding 15 ml of 10% DCC-DMEM/F12, and the suspension was centrifuged at 1000 rpm for 5 min. Cells were then resuspended and reseeded out into flasks or plates at a suitable dilution. Experiments were performed in 6-, 12- or 24-well plates. Experiments were conducted on cells at passage 3 or 4 and were performed when cells reached confluency $\geq$ 80 %.

Decidualisation of hESCs

When cultures were decidualised, this was carried out in 2% DCC-DMEM/F12 media (phenol-red free). Cells were grown to confluency, and either left untreated in 2% DCC-DMEM/F12 alone, or were cultured in 2% DCC-DMEM/F12 media containing 0.5 mM 8-Br-cAMP and 1 µM medroxyprogesterone acetate (MPA).

Maintenance of HEK 293 cell cultures

HEK 293 cells were grown in a monolayer in a T-75 cm^2 flask at a humid atmosphere of 5% (v/v) CO2 at 37°C. Cells were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. Depending on growth, cell cultures were passaged 1-2 times per week. Briefly, the medium was removed, cells were washed with 5 ml PBS and then 1 ml 0.05% trypsin/EDTA in PBS (at 37°C) was added. The cells were incubated for 1 min at 37°C

and the flask was tapped to ensure cell detachment. Trypsin activity was inhibited with the addition of 9 ml of 10 % DMEM and cells were diluted and seeded out as appropriate. Experiments were normally carried out in 6-, 12- or 24 well plates.

Transfections

Transient transfection of plasmid DNA in hESCs or HEK 293 cells

Both hESCs and HEK 293 cells were routinely transfected using Lipofectamine 2000. The following method describes transfection method for a 6-well plate but cells were also transfected in 12- and 24-well plates and 10 cm dishes, and amounts were adjusted according to surface area. Cell confluency was 85-95% for hESCs and 70-80% for HEK 293 cells for transfection. Prior to transfection, the media was changed to 2 ml of the appropriate transfection medium (see Materials). For each well, a transfection mix was first made as follows: in a 1.5 ml eppendorf, 5 µl of Lipofectamine 2000 was added to 250 µl of Opti-MEM and in a separate 1.5 ml eppendorf, the appropriate amount of plasmid DNA was added (typically 0.5-2 µg) with 250 µl of Opti-MEM. These mixes were incubated for 5 min and then gently mixed together and incubated for a further 20 min at room temperature to allow the complexes to form. The 500 µl mix was then added in a drop-wise manner to the cells and incubated at 37°C with 5% CO_2. Depending on the experiment being carried out, the medium was then changed back to general culture medium or decidualisation medium after 6 h (hESCs). hESCs were not passaged following transfection due to increased cell death. The day after transfection, HEK 293 cells were split in the appropriate format. Transfections were generally carried out 48 hours prior to the experiment endpoint.

Transfection of siRNA in hESCs or HEK 293 cells

hESCs and HEK 293 cells were routinely transfected using Lipofectamine RNAiMAX. The following method describes transfection method for a 6-well plate but cells were also transfected in 12 and 24-well plates and amounts were adjusted according to surface area. Cell confluency was 85-95% for hESCs and 40-60% for HEK 293 cells for transfection. As

above, the media was changed to the appropriate transfection media (see Materials). In a 1.5 ml eppendorf, 150 µl of Opti-MEM was mixed with 7.5 µl of Lipofectamine RNAiMAX and in a separate eppendorf, 150 µl of Opti-MEM was mixed with 20 µl of siRNA (at 20 µM stock). The two were incubated for 5 min and then mixed together and incubated for a further 20 min at room temperature. This mix was then added in drop-wise manner to each well and cells were incubated at 37°C with 5% CO_2. The media was then changed back to general culture or decidualisation media (hESCs) or cells were split in the appropriated format (HEK 293).

Generation of SEP-LHR stable HEK 293 cell line

HEK 293 cells were plated in 10 cm dishes and grown until 70-80% confluent. Prior to transfection, the media was changed to 10 ml of transfection medium. For each well, a transfection mix was first made as follows: in a 15 ml tube, 25 µl of Lipofectamine 2000 was added to 1250 µl of Opti-MEM and in a separate 15 ml tube, 10 µg of DNA with 1250 µl of Opti-MEM. These mixes were incubated for 5 min and then gently mixed together and incubated for a further 20 min at room temperature to allow the complexes to form. The 2.5 ml mix was then added in a drop-wise manner to the cells and incubated at 37°C with 5% CO_2. Two days after transfection the medium was changed to general culture medium supplemented with geneticin (1:100, added on the day) and then replaced with fresh general culture medium supplemented with geneticin every other day. The addition of geneticin to the medium caused the death of all those cells that did not acquire the plasmid containing the geneticin resistance gene. After 2 weeks, almost all cells were died and only a few remained. In ~1 week, these individual cells grew into bigger colonies which were picked from the plate under the microscope in sterile conditions and each colony transferred to a well of a 48-well plate. Once confluent, each clone was transferred to a well of 24-, then 12-, then 6-well plate. Confluent cells were transferred from the 6-well plate to T75 flasks. Each clone was tested via flow cytometry for the expression of SEP-LHR. Both monoclonal and polyclonal cell lines were identified and only clones with a good amount of GFP fluorenscence were retained. Those clones were imaged by confocal microscopy to

verify SEP-LHR was expressed at the plasma membrane. Clones with correct cellular localisation of SEP-LHR were retained, expanded and froze in several vials kept at -80°C.

2.2.5 Protein analysis

Protein extraction from cells for Western Blot analysis

hESCs or HEK 293 cells were grown in 10 cm dishes or 6 well plates and treated as appropriate. Cells were washed with ice-cold PBS and lysed on ice by adding 50-100 µl of lysis buffer (see Materials, section 2.1.11). Cells were scraped to ensure maximum protein retrieval, transferred into pre-chilled 1.5 ml eppendorf tubes and centrifuged at 4°C at 13,000 rpm for 10 min. The supernatants were transferred to new tubes on ice and either stored at -20°C or analysed for protein concentration, normalised and combined with Laemmli buffer, then heated to 95°C for 10 min and loaded on SDS-polyacrylamide gels.

Protein immunoprecipitation

Lysates from HEK 293 cells transfected with GFP-APPL1 constructs were subjected to immunoprecipitation in order to concentrate our protein of interest, APPL1, using GFP-Trap A beads (Chromotek) following the protocol provided. Briefly, after the appropriate treatment, cells were washed with ice-cold PBS three times, collected and homogenized with lysis buffer (see Materials) for 30 min. Lysates were centrifuged, and the supernatant was incubated for 2 h with 25 µL of GFP-Trap agarose beads/sample. Beads were washed three times with 500 µL of wash buffer (same as lysis buffer but without NP-40) and proteins eluted by resuspension in 2X Laemmli buffer and heating at 95°C for 10 min. Samples were stored at -20°C or separated on a 12% SDS-PAGE gel for Western blot analysis.

Measurement of protein concentration

In order to ensure the same amount of protein for Western blot analysis across samples, the concentration of each sample was determined using the Bradford Protein Assay kit (Thermo), which contains the Coomassie Blue dye. When protein is added, the reagent binds to the arginine and hydrophobic amino acid residues of the protein, causing a colour change that can be read using a spectrophotometer at 595 nm wavelength. The darker the colour, the more concentrated is the sample. To determine exact concentration, a set of standards were made using bovine serum albumin (BSA) provided with the kit.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved on polyacrylamide gels using the Mini-PROTEAN Tetra Cell system (Bio-Rad). Gels were prepared using the Bio-Rad glass casting modules to form the stacking and resolving parts of the gel. The resolving gels were normally 12% acrylamide, 1% SDS, 375 mM Tris-HCl pH 8.8. The stacking gel was 5% acrylamide, 1% SDS, 125 mM Tris-HCl pH 6.8. These mixes were polymerised using both TEMED and ammonium persulphate (APS). An equal amount of each sample was loaded. Pre-stained molecular weight markers were loaded alongside samples. Gels were run with SDS running buffer at 100 V for 20 min. This was to ensure that all samples aligned together before entering the separating gel. The gel was subsequently run at 140 V for ~2 hours, depending on the size of the protein of interest and the degree of separation required. Lastly, the gels were removed from the glass modules, ready for transfer.

Protein transfer to nitrocellulose membranes

Gels were transferred onto nitrocellulose blotting membranes using a wet transfer system. All parts of the transfer were pre-wetted with transfer buffer (TB). A ‘sandwich’ was created – sponge, filter paper, nitrocellulose membrane, the gel, filter paper and sponge and tightly sealed. This was then placed perpendicular to a voltage gradient of 100 V at 4°C

for 1 hour. The negatively charged proteins on the gel then migrated towards the anode and were deposited on the nitrocellulose membrane.

Following transfer, the membranes were removed and briefly washed with Tris-Buffered Saline with 0.5% Tween20 (TBS-T). The membrane was then blocked in either 5% (w/v) skimmed milk or BSA in TBS-T for 30 min at room temperature. The membrane was then washed briefly with TBS-T, placed in the primary antibody at appropriate dilution (see Materials) and incubated overnight on a roller at 4°C. The following day, the membrane was washed (3 X 15 min washes) in TBS-T, and the appropriate HRP-conjugated secondary (diluted in TBS-T or blocking buffer) was added for 1 h at room temperature. Further 3 X 15 min washes in TBS-T were carried out, and Luminata Forte or Classico Western HRP substrate (according to the expected strength of the signal) was spread onto the membrane. The membrane was subsequently exposed to autoradiography films in dark room or imaged using the ImageQuant Las 4000 chemi-imager (GE Healthcare).

Stripping membranes

When two proteins of the same or similar molecular weights needed to be visualised on the same membrane, primary and secondary antibodies were stripped off the membrane using Western Stripping buffer (see Materials). The buffer was first heated to 60°C, fresh β-mercaptoethanol was added and membranes were incubated with this mix for 30 min on a roller at room temperature. The membrane was washed extensively with TBS-T (typically 4 washes of 15 min each) to remove any remaining stripping buffer and was re-blocked with the appropriate blocking buffer, primary antibody was then added as above.

2.2.6 Signalling assays

Calcium assay

For Calcium assays, HEK 293 cells were seeded in 35 mm glass-bottomed dishes. The experiment was conducted following the Fluo-4 Direct Calcium Assay Kit (Thermo Fisher) protocol. Cells were pre-incubated with 1 mL of a mixture composed by 50% of Calcium

assay reagent, probenecid and Calcium assay buffer (as detailed in the manufacturer's manual) and 50% of DMEM for 30 min at 37°C followed by an additional 30 min at room temperature. When inhibitors were used, these were added at the appropriate time before the imaging started and within the 1 h of incubation with the dye. Cells were then imaged using a Leica SP5 confocal microscope using a 20x dry objective and a 488 excitation laser. Movies were recorded at 1 fps speed for 1 min before ligand addition and further 10-20 min after ligand addition to allow for the calcium to lower to almost basal levels. All experiments were conducted in duplicate and repeated at least 3 times. The intensity of each cell was measured using the ImgeJ plugin Time Series Analyser. The maximal intensity was obtained after subtracting the average background intensity (recorded before LH addition) for each cell and averaged across 10 cells per condition.

cAMP accumulation assay

For cAMP assays, HEK 293 cells and hESCs were grown in 24- or 12-well plates, respectively. Prior to each experiment conducted on hESCs, cells were pre-treated for 5 min with DMEM/F12 (0% FBS) containing IBMX (0.5 mM). IBMX was then kept for the whole length of the experiment. For experiments conducted on HEK 293 cells IBMX was not used for two reasons: because cAMP measured in LHR expressing cells was very high and to be consistent with the experimental conditions used during imaging experiments. After the appropriate treatment, cells were quickly washed with ice-cold PBS and then lysed in Cisbio cAMP lysis buffer (supplemented with 0.2% Triton X-100 for hESCs cells) (100µl/well) before being incubated at room temperature for 15 min on a shaking platform. Plates were then scraped and lysates transferred to 1.5 ml eppendorf tubes and centrifuged at 14000 rpm for 10 min. The supernatant was transferred into a fresh eppendorf and were either stored at -20°C or analysed following the protocol provided by the kit (cAMP dynamic 2, Cisbio). In each well of a 384 white bottomed well plate I added 10 µl of each sample, 5 µl of dye d2 and 5 µl of anti-cAMP cryptate conjugate. The reaction was incubated at room temperature in the dark for 1 h, then readings were taken using a PHERAstar FSX plate reader (BMG Labtech) equipped with a Homogenous Time Resolved Fluorescence (HTRF) 337 optic module. Results are calculated from the 665nm/620nm fluorescence ratio and were normalised to protein

concentration which was determined as described previously. All experiments were conducted in triplicate and repeated at least 3 times.

MAPK assay

To assess the levels of ERK 1/2 phosphorylation following LH stimulation, HEK 293 cells stably expressing FLAG-LHR were grown into 6-well plates until 80-90% confluent and serum starved for 16-18 h before the experiment. On the day of the experiment, medium was replaced with fresh DMEM and cells were pre-treated with inhibitors/DMSO for the appropriate time before stimulation with LH (10 nM) at different time points (0, 5, 10, 15, 30, 60 min). Cells were then put on ice, washed with ice-cold PBS for 3 times and Lysis buffer (see Materials, section 2.1.11) was added (100 uL/well). Protein extraction, SDS-gel loading for Western blot and protein transfer to nitrocellulose membrane were the same as described above (see Methods, section 2.2.5). Once proteins were transferred to the nitrocellulose membrane, this was blocked in Western blot blocking solution (5% BSA in TBS-T) for 1 h at room temperature and incubated overnight at 4°C with primary Phospho-ERK 1/2 antibody solution. The following day the membrane was incubated with HRP-anti rabbit secondary antibody for 1 h, imaged, stripped, blocked and incubated overnight with Total-ERK 1/2 antibody solution. The following day the following day the membrane was incubated with HRP-anti rabbit secondary antibody for 1 h and imaged. The intensity of the bands was quantified using ImageJ and Phospho-ERK 1/2 over Total-ERK 1/2 ratio was calculated.

2.2.7 Microscopy-based techniques

All procedures were the same for both hESCs and HEK 293 cells, unless otherwise stated.

Fixed sample preparation

Cells were grown onto glass-coverslips in 12-well plates or in 35-mm glass bottomed dishes until 60-70% confluent. On the day of the experiment, medium was replaced with fresh DMEM without serum. Cells were incubated with FLAG M1-antibody (1:1000) for 30 min at 37°C (in addition to the appropriate inhibitor, when required), and for the last 5 or 15 min LH was added. Cells were washed 3 times with ice-cold PBS+Ca^{2+}, $\pm$ washed with stripping buffer to remove surface bound FLAG M1-antibody and fixed with 4% PFA for 20 min at room temperature. Cells were then incubated with blocking buffer for 1 h at room temperature followed by incubation with permeabilisation buffer for 15 min at room temperature. After 3 quick washes with blocking buffer, cells were incubated with 1^{ary} antibody (e.g. APPL1 antibody) solution (diluted as described in table x.x in blocking buffer) at 4°C overnight. The day after cells were washed 3 times with blocking buffer and incubated with AlexaFluor secondary antibodies (1:10000 in blocking buffer) for 30 min at room temperature in the dark. Cell were then washed with PSB+Ca^{2+} and either imaged straight after (35-mm glass bottom dishes) for TIR-FM experiments or coverslips were mounted on glass slides using Fluoromount-G (confocal) or Vectashield (SIM), sealed using clear non quick dry nail polish to prevent dye fading and kept in the dark at 4°C until imaged. Samples kept at 4°C were put at room temperature 30 min before the imaging session to reduce the drift of the microscope focus due to differences in temperature between the sample and the microscope chamber (~21°C).

Confocal microscopy

Receptor imaging in live cells was monitored by “feeding” cells with AlexaFluor488 or 555 conjugated-M1 FLAG antibody (15 min, 37 °C) in phenol red-free DMEM prior to agonist treatment. Both live and fixed cells were imaged using a TCS-SP5 confocal microscope (Leica) with a 63× objective and 1.4 numerical aperture (NA). Leica LAS AF image acquisition software was utilised. All subsequent raw image files were analyzed using ImageJ or LAS AF Lite (Leica) to measure level of co-localisation.

Total Internal Reflection-Fluorescence Microscopy (TIR-FM)

Cells were imaged using a Elyra PS.1 AxioObserver Z1 motorized inverted microscope with a sCMOS or EMCCD camera and an alpha Plan-Apochromat 100x/1.46 Oil DIC M27 Elyra objective (Zeiss), with solid-state lasers of 488 nm, 561 nm and/or 642 nm as light sources. For live cell imaging, cells were imaged live for 1 min at a frame rate of 10 fps at 37°C in phenol red free Opti-MEM supplemented with HEPES (Life technologies). ZEN lite image acquisition software was utilised to collect time-lapse movies and analyzed as tiff stacks using ImageJ plugin Time series analyzer. The number of recycling events was counted and normalised by the cell area.

Structured Illumination Microscopy (SIM)

Cells were imaged using an Elyra PS.1 AxioObserver Z1 motorized inverted microscope with a EMCCD camera and Plan-Apochromat 63x/1.4 Oil DIC M27 Elyra objective (Zeiss) with solid-state lasers of 488 nm and 561 nm as light sources. ZEN lite software was used for both acquisition of Z-stacks (5 phases and 3 rotations grating) and reconstruction. Quality of raw and reconstructed data was determined using the ImageJ plugin SIM check (Ball et al., 2015).

2.2.8 Bioluminescence Resonance Energy Transfer (BRET)

To assess the interaction between twp proteins in live cells, BRET was used. HEK293 cells were plated into 6-well plates and transfected with a constant amount of a donor (Rluc8-tagged protein) and increasing amounts of acceptor (Venus-tagged protein) per well. Transfections were conducted as described in section 4.2.4.4 and cells were assayed 48 h post-transfection. On the day of the assay, cells were washed and harvested in PBS and seeded at a density of approximately 200,000 cells/well into white flat bottom 96-well plates. The Rluc8 substrate Coelentrazine-h (Promega) was added at a final concentration of 5 µM, and BRET readings at 475 nm and 535 nm emission wavelenghts were immediately taken for a total of 10 cycles using a luminescence plate reader (LUMIstarOPTIMA, BMG Labtech). In parallel cells, fluorescence of the Venus-tagged protein was determined at

485 nm ex/540 nm em. The BRET ratio was calculated by dividing the signal at 535 nm over that emitted at 475 nm, and net BRET values calculated by subtracting from all readings the basal BRET ratio obtained when Rluc8-tagged protein was expressed alone. Net Venus were also calculated by subtracting from all readings the basal fluorescence signal obtained when Rluc8-tagged protein was expressed alone. Net BRET values were plotted as a function of Net Venus/Rluc8 values. Data were fitted using a nonlinear regression equation (GradPad Prism V5). All experiments were conducted in duplicate and at least three times.

2.2.9 Flow cytometry

HEK 293 cells were grown in 12-well plates to ~80% confluency subject to analysis by flow cytometry to assess receptor surface expression under condition of APPL1 knock-down. In order to do this, cells were pre-treated with mouse Flag M1 antibody (1:500) diluted in DMEM with 0% FBS at 37°C for 15 min. Cells were then washed 3 times with ice-cold PBS and harvested in 500 µl of FACS buffer which consisted of ice-cold PBS with 2% (v/v) FBS. Cells were transferred to round bottomed polypropylene FACS tubes, centrifuged at 1000 rpm at 4°C and the supernatant was then removed and the pellet resuspended in FACS buffer containing AlexaFluor 488-anti mouse antibody. The cells were then incubated for 1 hour on ice in the dark. Subsequently, the cells were centrifuged again as before, and resuspended in 1 ml of FACS buffer. The latter washing step was carried out 3 times in total and the cells were then resuspended in a final volume of 300 µl. These cells were analysed on a FACS Calibur Flow Cytometer. Cells that had not been exposed to any antibodies or only secondary antibodies alone were used as controls, the latter allowing normalisation to basal cell fluorescence intensity.

2.2.10 Statistical analysis

Data represent mean $\pm$ SE. Statistical significance was determined using unpaired Student's *t* test, One-way ANOVA followed by Dunnett post-test, or Two-way ANOVA followed by Bonferroni post-test, using GraphPad Prism. Differences were considered significant at $p<0.05$.

Chapter 3:

Tracking LHR post-endocytic sorting from the VEE with single-event resolution

3.1 Introduction

Recently in our laboratory, a novel endosomal compartment has been identified where GPCRs such as LHR internalise to, termed VEE. Compared to the classic EE were other receptors like B2AR traffic to after internalisation, the VEE is smaller in size (~400 nm), closer to the plasma membrane (~100-200 nm) and appears to be distinct from the EE, as only a small proportion of LHR endosomes are marked by EEA1, Rab5 and PI3P, classic markers of the EE. Targeting of LHR to the VEE is dictated by the interaction of the PDZ ligand within LHR's C-tail with the PDZ protein GIPC. Truncation of the distal portion of the LHR C-tail or knock-down of GIPC targets the receptor to the EE and prevents its recycling back to the plasma membrane (Jean-Alphonse et al., 2014, Hirakawa et al., 2003, Galet et al., 2003). Whilst the importance of this newly identified GPCR sorting station is remarkable, a deeper knowledge on which elements characterise the VEE, as well as the mechanisms regulating LHR trafficking, is needed.

Until recently, post-endocytic sorting of GPCRs has been studied using traditional methods such as confocal microscopy, biochemical techniques or flow cytometry. These techniques fail to achieve the temporal and spatial resolution to capture and appreciate such dynamic processes. The combination of TIR-FM, which allows the visualisation of fluorescent molecules only present within a range of 100 nm from the glass/sample interface, and SuperEcliptic GFP, which is quenched at pH<6.5 thus making visible only those molecules in a neutral environment, enabled these limitations to be overcome and measure recycling in live cells, in real time, at single event resolution.

This chapter will focus on the research I have carried out in regard to the optimisation of a TIR-FM live cell imaging protocol for the detection, quantitation and characterisation of LHR recycling.

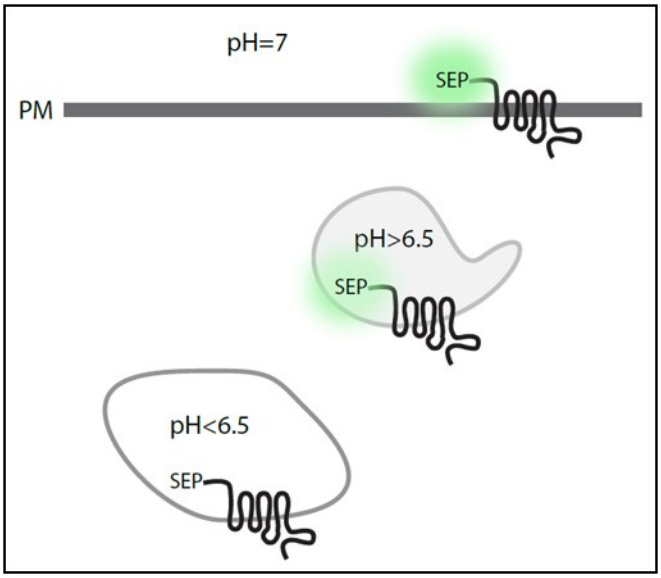
3.2 Results

3.2.1 LHR Recycling from the VEE

After ligand stimulated internalisation, human LHR is sorted to a regulated recycling pathway (Kishi et al., 2001). We thus focused our attention on receptor recycling from the novel endosomal compartment, the VEE. One method which has been widely used to study GPCR recycling in live cells in real time is TIR-FM (detailed in Methods, paragraph 2.2.7.3) and employing a pH sensitive-GFP tagged receptor (Yudowski et al., 2009b, Yudowski et al., 2007). SuperEcliptic pHluorin (SEP) is a GFP variant whose fluoresce is quenched at a pH lower than 6.5 (Miesenböck et al., 1998). GPCRs that are N-terminally tagged with this protein are visible, through fluorescence microscopy, only when they are located in cellular compartments characterised by neutral pH, such as at the plasma membrane that exposes SEP to the extracellular environment (Figure 3.1 A). When studying events that happen at the cell surface, the use of SEP-GPCRs highly increases the signal to noise ratio, enhancing the quality and specificity of TIR-FM imaging. Taking advantage of this tool, I N-terminally tagged hLHR with SEP generating a construct I called SEP-LHR (detailed in Materials, paragraph 2.2.2). The correct localisation of SEP-LHR at the plasma membrane of HEK 293 cells was confirmed via confocal microscopy, epifluorescence and TIR-FM (Figure 3.1 B). I then established a HEK 293 cell line stably expressing SEP-LHR.

Clones were assessed for the cell surface expression of SEP-LHR via flow cytometry and for their ability to activate cAMP and MAPK pathways in response to LH at a comparable level to FLAG-tagged LHR. The clone I selected (clone 4) showed very similar LH-induced cAMP generation (Figure 3.2 A) and ERK1/2 phosphorylation to those measured using a FLAG-LHR stable cell line (Figure 3.2 B-C).

A.



B.

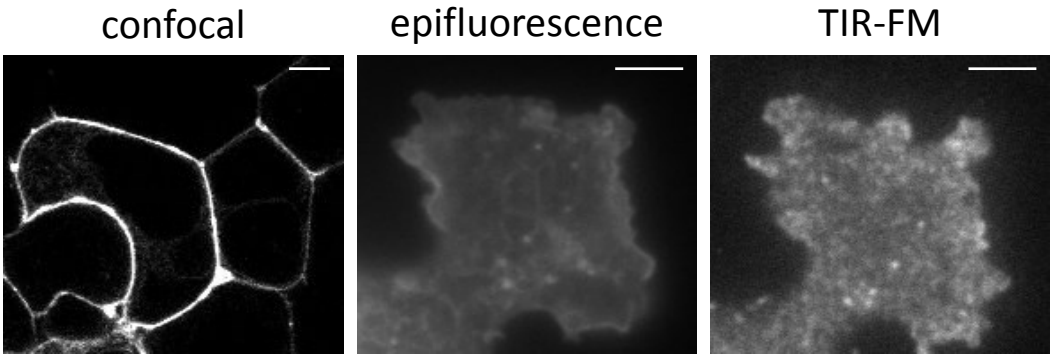
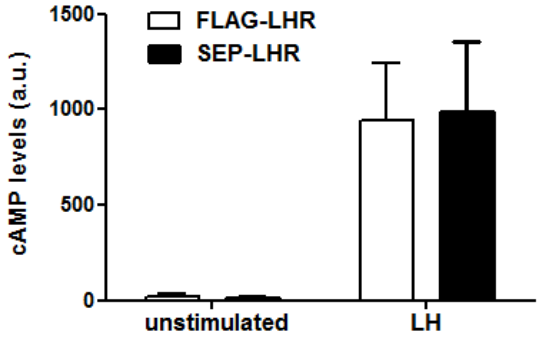
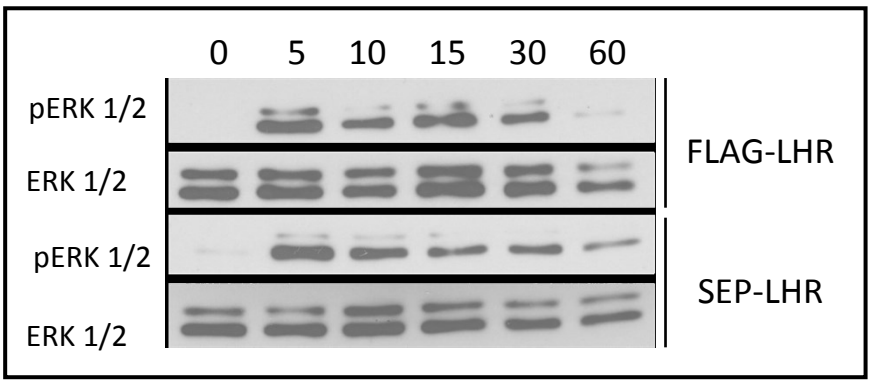


Figure 3.1 Visualisation of SEP-tagged GPCRs. (A) Cartoon depicting pH dependence of SEP in relation to GPCR cellular localisation. (B) HEK 293 cells expressing SEP-LHR were imaged by confocal, epifluorescence or TIR-F microscopy; scale bar=5 µm.

A.



B.



C.

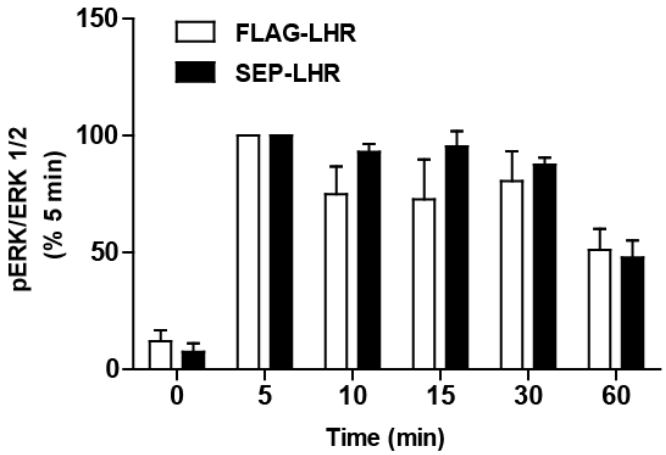
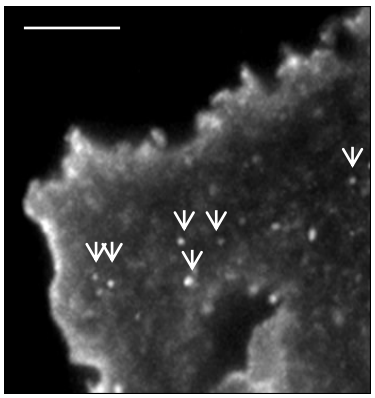


Figure 3.2 SEP-LHR activates LH-induced cAMP generation and ERK phosphorylation in a similar manner. (A) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR or SEP-LHR prior to and after stimulation with LH (10 nM) for 5 min; n=3. (B) Phosphorylation of ERK 1/2 at stated time points after LH (10 nM) stimulation was determined by Western blotting in HEK 293 cells stably expressing FLAG-LHR or SEP-LHR. Total ERK was used as a loading control. A representative immunoblot is shown. (C) Densitometry analysis of ERK 1/2 phosphorylation from B was normalised to the 5 min stimulation; n=3.

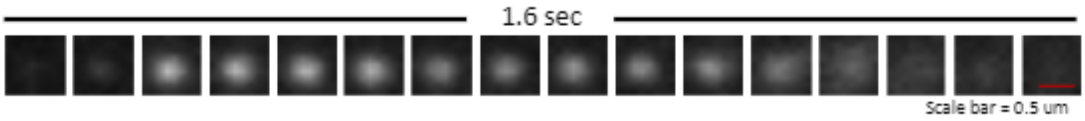
To study LHR recycling in real time, SEP-LHR cells were imaged live before and after stimulation with the ligand. After agonist addition, movies were recorded every 5 minutes over a period of 30 minutes. To be able to capture recycling events that happen within fractions of a second and to not photobleach or induce cell toxicity with prolonged laser exposure, I opted for an acquisition time of 1 minute with frames recorded every 0.1 seconds, for a total of 600 frames per movie. When recorded live, recycling events appeared as fast bursts of fluorescence over the background upon reinsertion of the receptor in the plasma membrane and are referred to as “puffs” (Figure 3.3 A-B) (Yudowski et al., 2007).

Although the average duration of these puffs at the plasma membrane (1.47 $\pm$ 0.11 sec) was not affected by ligand addition or removal (Figure 3.3 C), they could be divided in groups according to the kinetics of cargo release. I have identified three categories I termed “transient”, “pre-tail” and “persistent”, taking into account the time spent at the plasma membrane, the intensity profile and the kymograph of each puff. Most events (~94%) fall into the transient category with maximum duration of 2 seconds and a single well-defined spot as intensity profile; ~5% of LHR puffs appear in the TIR-FM field and remain docked at the plasma membrane for a few seconds before releasing the cargo; persistent events are those where the fluorescence at the plasma membrane is sustained for >5 seconds and release their content slowly compared to the other categories of puffs (Figure 3.3 D).

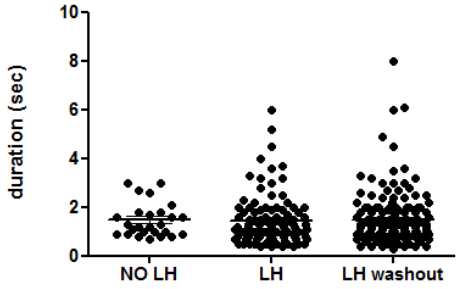
A.



B.



C.



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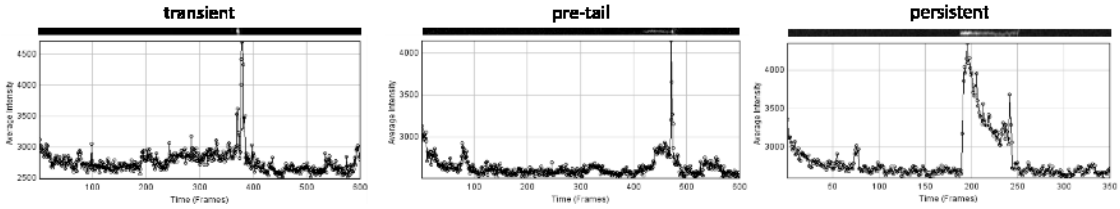


Figure 3.3 Characterisation of SEP-LHR recycling events via TIR-FM. (A) Representative Maximum Intensity Projection of a representative TIR-FM movie of a HEK 293 cell stably expressing SEP-LHR stimulated with LH (10 nM). Recycling events are marked by arrows. Scale bar= 5 µm. (B) Series of TIR-FM images of a single SEP-LHR recycling event following stimulation with LH (10 nM). (C) Duration of SEP-LHR recycling events in different conditions. (D) Representative Intensity profile and Kymograph of the three categories of SEP-LHR recycling events.

Although recycling events exhibit distinguishing features that allow their recognition over other events happening in the cell, such as vesicles moving along the y axis and appearing and disappearing from the TIR-FM field, it has not been possible to develop in house, nor access the few non-open source newly developed analysis software for automated puff detection. For this reason, all the data showed in this thesis are originated from manual visual inspection and counting of puffs, using Time Series Analyzer plugin of ImageJ to record them.

Arguments could be raised against the fact that the plasma membrane insertion events observed by TIR-FM are not recycled receptors but newly synthesized ones. I excluded this possibility by showing that pre-treatment of cells with the protein synthesis inhibitor cyclohexamide had no effect on the number of puffs (Figure 3.4).

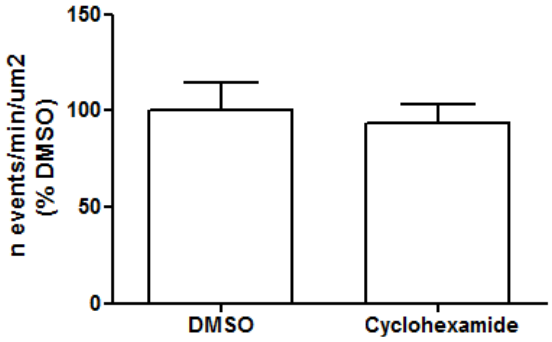
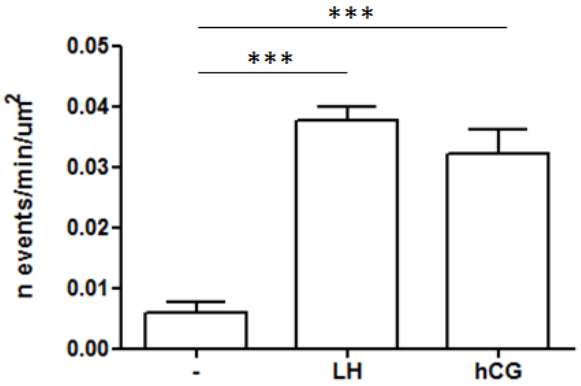


Figure 3.4 LHRs delivered at the plasma membrane are not newly synthesized receptors but recycled ones. Number of recycling events measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR, with or without cyclohexamide treatment (10 µg/mL 1.5 h) prior to LH stimulation (10 nM); n=6 cells per condition.

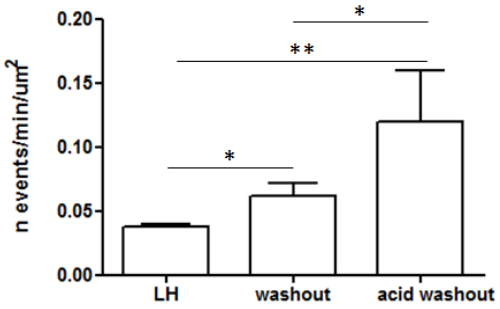
As reported in paragraph 1.4.4 of Chapter 1, ligand bias has been observed for LHR signalling pathways. To test if hCG and LH exhibited any differences in the plasma membrane targeting of LHR, I compared SEP-LHR recycling in the presence of either hormone. Both LH and hCG induced a significant increase in the number of puffs over basal condition to a similar level (Figure 3.5 A). To assess whether the constant presence of ligand was necessary

to drive LHR recycling, TIR-FM movies were also recorded after ligand washout. Cells stimulated with LH or hCG were washed with fresh ligand-free media and imaged immediately. LH, but not hCG, removal increased recycling frequency (Figure 3.5 B-C). The difference between LH and hCG washout could be explained by the fact that the two ligands bind the receptor with different affinities and have different K_{off} (Galet and Ascoli, 2005, Jia et al., 1991), thus washing cells with media could not be sufficient to remove all the ligand molecules. To increase the efficiency of the washout, I washed the cells with an acid buffer (detailed in Materials), which has been shown to detach almost all ligand molecules from the receptor (Ascoli, 1982). Both LH and hCG acid washout increased the number of LHR puffs compared to media washout, but only for the acid washout following LH stimulation this increase was significant compared to when recycling was measured in the presence of ligand (Figure 3.5 B-C). Comparing the two ligands by each condition revealed that there is a significant difference in SEP-LHR recycling after ligand washout (both media and acid wash) between LH and hCG (Figure 3.5 D). In addition, plotting the number of recycling events over time after ligand addition or removal, there were distinct differences between LH and hCG in their kinetic profiles. Whilst the number of puffs after LH addition remained constant over the imaging period, it increased in the continued presence of hCG. Further, LHR recycling rates increased in the 5 minutes after LH withdrawal and decreaed over time, but remained moderately constant following hCG removal (Figure 3.5 E).

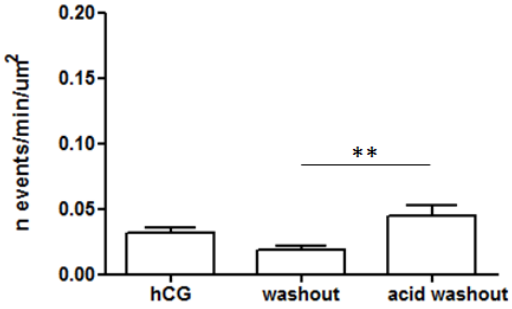
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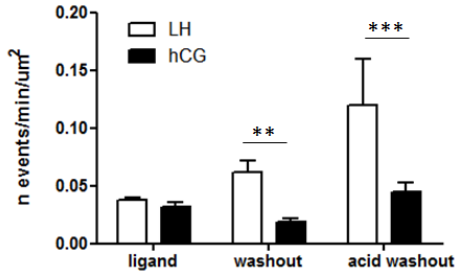
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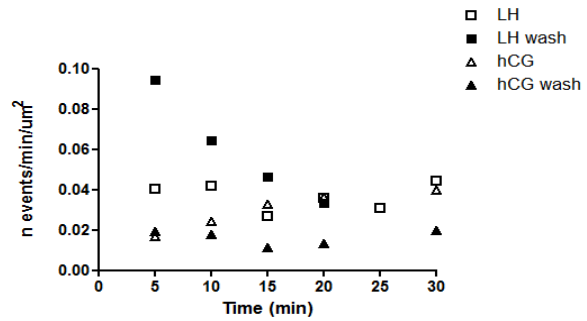


Figure 3.5 LHR recycling frequency is modulated by the presence of agonist. (A) Number of recycling events measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR ± LH or hCG (10 nM); n=16 cells per condition; One-way ANOVA: ***p<0.001. (B-D) Number of recycling events measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR stimulated with LH or hCG (10 nM) ± ligand washout using media (wash) or acid wash buffer (acid wash); n≥ 11 cells per condition; One-way ANOVA (B-C) or two-way ANOVA (D): *p<0.05, **p<0.01, ***p<0.001. (E) Number of recycling events over time of ligand stimulation measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR, stimulated with LH or hCG (10 nM) ± ligand washout; n≥ 3 cells per time point.

I then investigated the underlying mechansims mediating LHR recycling by first analysing which cytoskeletal components could serve as a "railway" for transporting LHR vesicles to the plasma membrane. Disruption of either tubulin or actin fibres, using Nocodazole or Cytochalasin b respectively, demonstrated a significant reduction in the number of LHR puffs to 30.86 ± 5.62 % and 35.76 ± 6.47 %, respectively (Figure 3.6), suggesting both components are required for LHR recycling.

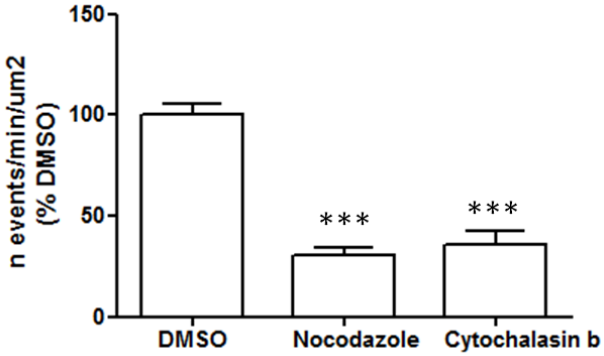


Figure 3.6 LHR recycles via a pathway that requires both microtubules and actin filaments. Number of recycling events measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR, pre-treated with either DMSO, the microtubule disrupting agent Nocodazole (10 µM, 1 h) or the actin depolymerising agent Cytochalasin b (2 µM, 30 min) and stimulated with LH (10 nM). n=16 cells per condition; One-way ANOVA: ***p<0.001.

Considering APPL1 localises to a subpopulation of LHR within the VEE (Jean-Alphonse et al., 2014), I asked whether APPL1 co-trafficked with LHR to the cell surface. To assess this, I conducted dual colour TIR-FM on cells co-expressing SEP-LHR and mCherry-APPL1 and monitored the presence of APPL1 with LHR puffs. Only 4.11 ± 2.12 % of SEP-LHR recycling events also displayed presence of APPL1, suggesting APPL1 may not be necessary for LHR release at the plasma membrane (Figure 3.7).

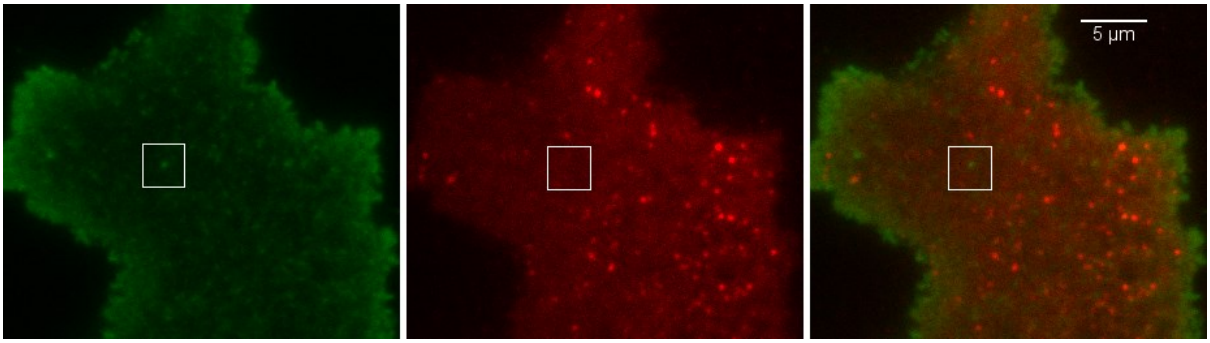


Figure 3.7 APPL1 does not recycle to the plasma membrane with LHR. HEK 293 cells stably expressing SEP-LHR were transfected with mCherry-WT APPL1 and imaged live by TIR-FM after LH (10 nM) addition. The image shown is taken from a representative movie and depicts a single LHR recycling event (square) lacking APPL1. n=66 recycling events analyzed across 6 different cells imaged across different experiments.

3.3 Discussion

The discovery of the VEE as specialised compartment for signalling and trafficking of a subset of GPCRs has added a new layer of complexity to the endosomal network (Jean-Alphonse et al., 2014). The post-endocytic fate of GPCRs is not restricted to either the recycling or degradative pathway from the EE, but includes additional sorting steps and cellular fates, which are made possible by receptor trafficking to endosomes other than the EE; these might represent both precursors of the EE or new endosomal compartments serving as distinct signalling and sorting stations.

The optimisation of TIR-FM imaging of SEP-LHR has provided tools to analyse in live cells and in real time LHR recycling from the VEE. Using this approach, I have been able to demonstrate that LHR recycling is supported by both components of the cytoskeleton, microtubules and actin. The involvement of both cytoskeletal components for GPCR recycling has been observed for MOR (Roman-Vendrell et al., 2012). B2AR recycling, instead, has been shown to depend on receptor entry in actin-rich microdomains of the recycling tubules where phosphorylated cortactin, an actin polymerision stimulating protein, promotes the scission of B2AR-containin recycling vesicle from the EE (Bowman et al., 2016). Other GPCRs have been proposed to depend on cytoskeleton-associated proteins to

recycle; these include filamin A binding to calcitonin receptor, calcium sensing receptor, MOR and dopamine receptors (Lin et al., 2001, Onoprishvili et al., 2003, Seck et al., 2003, Huang et al., 2006), and Myosin Vb interaction with MOR and M4 muscarinic acetylcholine receptor (Volpicelli et al., 2002, Roman-Vendrell et al., 2012). These studies suggest that the same cytoskeletal components are differentially assembled into recycling machinery by distinct GPCRs, delineating a broad spectrum of pathways receptors could exploit to recycle, according to their physiological requirements. It would be interesting to investigate which molecular motors and which anchoring proteins are exploited by LHR vesicles during their journey to the plasma membrane.

I also introduced, for the first time, the concept that LHR recycling may be subjected to ligand bias. Albeit both LH and hCG trigger LHR recycling as early as 5 minutes after ligand exposure, the frequency of recycling is ligand-specific. This difference could be due to the affinity of binding to LHR, as K_i is 8.90 $\pm$ 1.20 nM for hLH and 4.15 $\pm$ 0.75 for hCG (Galet and Ascoli, 2005), but not to the different efficicacy the two hormones stimulated cAMP production as at saturating doses, both LH and hCG promoted full activation of cAMP pathway and with similar kinetics (Riccetti et al., 2017). Interestingly, ligand removal had opposite effects on LHR recycling depending on which ligand the receptor had been previously stimulated with; hCG removal dampened, whilst LH removal increased, LHR recycling. One possibility is that these differences rely on the fact that different ligands direct the receptor to different intracellular sorting fates; this cannot be excluded, as LHR trafficking to APPL1-positive VEEs upon stimulation with hCG has not been assessed in this study. It is also important to note that ligand removal does not have the same effect on different GPCRs; for example, removal of the MOR specific agonist DAMGO induces a decrease in MOR recycling; isoproterenol removal, instead, enhances B2AR recycling (Roman-Vendrell et al., 2012, Yudowski et al., 2009b).

The analysis of LHR recycling events also revealed the existence of more than one type of recycling vesicle, according to the time spent by the vesicle between its docking at the plasma membrane and the release of its cargo. The fact that the majority are transient, may highlight the need for LHR to be quickly re-exposed to the extracellular environment or, conversely, to spend as little time as possible inside the cell. The pre-tail events may

represent a group of vesicles which are packed, or fused with other vesicles, while they are docked at the plasma membrane or, from another perspective, require prolonged localisation at precise locations to allow the receptor, and its interacting partners, to perform certain cellular functions. The characteristic slow opening and cargo-release of the persistent vesicles could be a reflection of the fact that receptors carried by these endosomes are released in a specific area or domain of the plasma membrane where the density is higher, thus preventing LHR from fast diffusion (West and Hanyaloglu, 2015). Indeed, LHR clustering in plasma membrane microdomains has been previously reported (Wolf-Ringwall et al., 2014, Wolf-Ringwall et al., 2011). Interestingly, persistent events could represent specialised signalling microdomains, as proposed for B2AR in neurons (Yudowski et al., 2006). Unexpectedly, the proportion of each recycling event category was not affected by the different conditions LHR cells were exposed to (e.g. inhibitors, ligand washout, etc), possibly reflecting an intrinsic property of exocytic vesicles as similar categories have been observed for non-GPCR recycling proteins like the insulin-regulated aminopeptidase (Yuan et al., 2015).

Overall, the data presented in this chapter establish a single-event resolution approach to assess in live cell, in real time, TIR-FM imaging of LHR.

Chapter 4:

Spatially-directed signalling from the VEE

4.1 Introduction

Membrane trafficking has contributed to an understanding of how GPCR signalling is both specified and diversified, explaining how receptors that activate common pathways can mediate the vast array of biological functions they are involved in. Endosomes have been recognized not only as sorting stations, but they are now also considered as platforms from where GPCRs can signal. In fact, more and more receptors have demonstrated that they require internalisation to sustain or re-activate a specific signalling pathway (detailed in Introduction, paragraph 1.2.4).

This emerging concept of GPCR intracellular signalling has triggered the development of new tools that allow the study and visualisation of signalling events within specific subcellular compartments, including pharmacological inhibitors of dynamin or clathrin, BRET/FRET biosensors of targets of second messenger pathways, such as EPAC, that can be targeted to specific membranes. More recently, direct visualisation of G protein activation has been developed via nanobody technology. Although only restricted to $G\alpha_s$, Nb37 allows the localisation of activated G protein. This single chain antibody was first developed to stabilize GPCR-G protein complex for crystallography studies (Manglik et al., 2017, Ring et al., 2013, Rasmussen et al., 2011); after GFP fusion and transfection in cells, it successfully binds active, the nucleotide-free form of $G\alpha_s$ (Irannejad et al., 2013). In addition to Nb37, a number of receptor-specific nanobodies have been recently developed, including those against MOR and M2 muscarinic receptor (Huang et al., 2015, Kruse et al., 2013).

Published research from our laboratory has shown that LHR required internalisation to trigger a MAPK response and localisation to the VEE, via GIPC, to maintain a sustained MAPK profile (Jean-Alphonse et al., 2014). However, little is known on how MAPK is activated by LHR from this compartment or if other signalling pathways are spatially regulated in a similar manner. Similarly, the other pathways activated by LHR, namely $G\alpha_{q/11}$-calcium and $G\alpha_s$-cAMP, also lack detailed mechanistic information.

Another interesting concept in GPCR signalling is crosstalk between different signalling pathways activated even by the same receptor. Signalling crosstalk is a well described

feature of GPCRs and perhaps not surprising when considering the magnitude of functions elicited by GPCRs and the relatively smaller number of effectors. Crosstalk between MAPK and cAMP pathways has been documented at different levels in a variety of cell type, with cAMP acting as negative or positive regulator of MAPK phosphorylation to mediate, for example, gene expression, cell differentiation and proliferation (Bhat et al., 2007, Sengupta et al., 2007, Maymo et al., 2010). Studies on the prototype GPCR B2AR have denoted that G protein-dependent regulation of MAPK is mediated by both $G\alpha_s$-cAMP-PKA and Gβγ, and that these could have stimulatory or inhibitory roles according to the cell type B2AR is expressed (Schmitt and Stork, 2000, Crespo et al., 1995). Interestingly, MAPK can be regulated by βγ associated with $G\alpha_s$ or $G\alpha_i$, after a PKA-driven G protein switch (Daaka et al., 1997).

Conversely to cAMP-MAPK, the crosstalk between $G\alpha_s$ and $G\alpha_{q/11}$ pathways is bidirectional. Calcium transporters that determine calcium release are activated by cAMP-PKA by phosphorylation, whilst calcium can directly or indirectly regulate cAMP synthesis, either stimulating or inhibiting different types of ACs, including mitochondrial sAC (Ahuja et al., 2014, Halls and Cooper, 2011, Katona et al., 2015). Another example of mutual control is represented by phosphodiesterase (PDE) 1, whose activation is triggered by the binding of calmodulin/calcium, which affinity is dictated by PKA (Goraya and Cooper, 2005).

In this chapter I have identified the LHR signalling pathways that require receptor internalisation and investigated the mechanisms underlying spatially-regulated LH-LHR signalling. I also identified cellular components that contribute to cAMP and MAPK activation. Finally, I present evidence for the possibility of crosstalk between the $G\alpha_{q/11}$-calcium and $G\alpha_s$-cAMP pathways activated by LH.

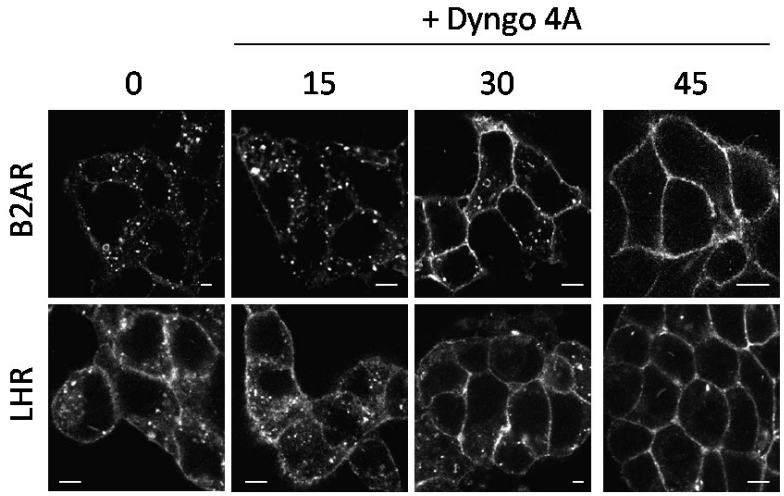
4.2 Results

4.2.1 Characterisation of LH-induced $G\alpha_s$-cAMP-PKA pathway

The $G\alpha_s$-cAMP-PKA pathway is the main signalling cascade activated by LH-LHR. I assessed the proportion of cAMP generated from internalized LHR in cells treated with the dynamin

inhibitor Dyngo-4a, which blocks internalisation of multiple GPCRs (Jean-Alphonse et al., 2014, Tsvetanova and von Zastrow, 2014, Bowman et al., 2016, McCluskey et al., 2013). Work conducted by other groups revealed that B2AR activates a second wave of cAMP signalling after internalisation, although the endosomal cAMP pool is only marginal compared to that generated at the plasma membrane (McCluskey et al., 2013, Bowman et al., 2016, Tsvetanova et al., 2016, Tsvetanova and von Zastrow, 2014). Thus, B2AR was employed as a reference tool to compare the effect of Dyngo-4a on LHR-mediated cAMP production. After 45 minutes of Dyngo-4a pre-treatment, internalisation of both FLAG-B2AR and FLAG-LHR was completely inhibited (Figure 4.1 A); nonetheless, dynamin inhibition had different effects on cAMP levels generated by the two receptors after acute stimulation with their respective ligands. Consistent with prior published data (Bowman et al., 2016), Dyngo-4a did not significantly attenuate isoproterenol-induced intracellular cAMP production in B2AR-expressing cells (Figure 4.1 B left panel). However, LH-induced cAMP signalling was strongly reduced by Dyngo-4a pre-treatment (~95%) (Figure 4.1 B right panel), implying that for LHR, cAMP production is largely dependent on receptor internalisation.

A.



B.

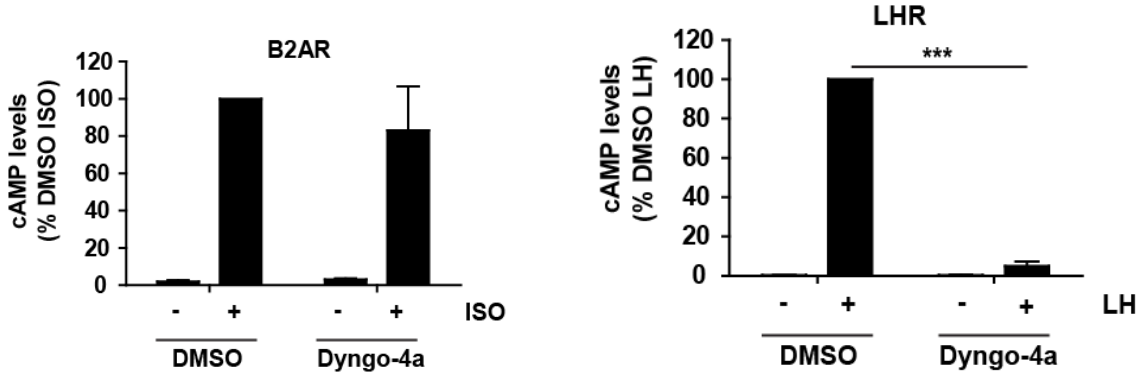


Figure 4.1 LHR stimulates cAMP production mainly from intracellular compartments. (A) Representative confocal images showing the effect of Dyngo-4a on B2AR and LHR internalisation. HEK293 cells stably expressing FLAG-B2AR or FLAG-LHR were ± pre-treated with Dyngo-4a (30 µM) for 0, 15, 30 or 45 minutes, fed live with M1-488 conjugated antibody and stimulated with ISO (10 µM) or LH (10 nM), respectively. (B) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG- B2AR or FLAG- LHR ± stimulation with LH (10 nM) or isoproterenol (ISO, 10 µM) for 5 min ± pre-treatment with either DMSO or Dyngo-4a (30 µM) for 45 min. Data represent mean ± SE, n=5 for B2AR, n=3 for LHR. Two-way ANOVA: ***p<0.001.

To spatially capture the sub-cellular location of $G\alpha_s$ activation, I used a GFP-tagged Nb37 (Nb37-GFP), a biosensor that captures the activated but highly transient, nucleotide free state of $G\alpha_s$ (Irannejad et al., 2013). To test whether Nb37-GFP binding to active $G\alpha_s$ could

alter LHR signalling by either locking the receptor-G protein in an active or inactive conformation, I measured cAMP production in FLAG-LHR cells expressing Nb37-GFP and found no difference compared to untransfected cells (Figure 4.2 A). When FLAG-LHR cells transfected with Nb37-GFP were imaged by confocal microscopy, I observed that a proportion of LHR endosomes were positive for Nb37, highlighting the presence of LHR endosomes signalling through $G\alpha_s$ (Figure 4.2 B). Due to the restrictive size of VEEs (~400 nm) and the diffraction limit of visible light is ~200 nm, I employed a super-resolution microscopy technique, structured illumination microscopy (SIM), on fixed cells to increase the resolution of the imaging and uncover the structural organisation of LHR signalling endosomes. As shown in Figure 4.2 C, a fraction of FLAG-LHR endosomes also contained Nb37-GFP, consistent with the confocal imaging data (Figure 4.2 B). Due to the increase in both lateral and axial resolution that SIM affords, I noted that Nb37 is not uniformly distributed within LHR endosomes, but localises to sub-domains (Figure 4.2 C i-ii).

A.

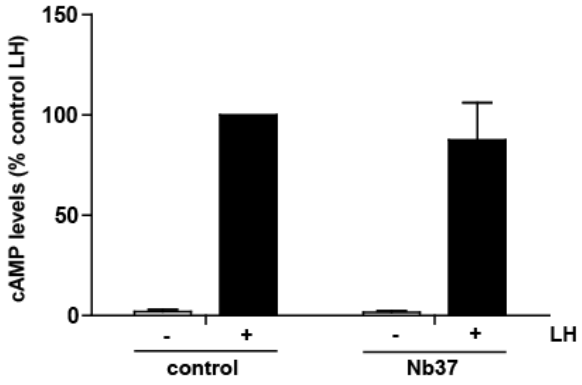
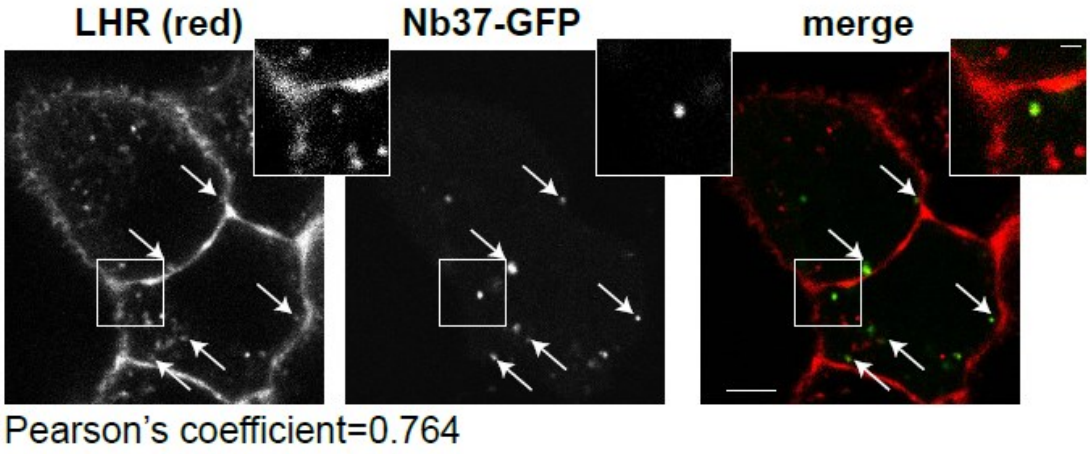


Figure 4.2 continued on the following page.

B.



C.

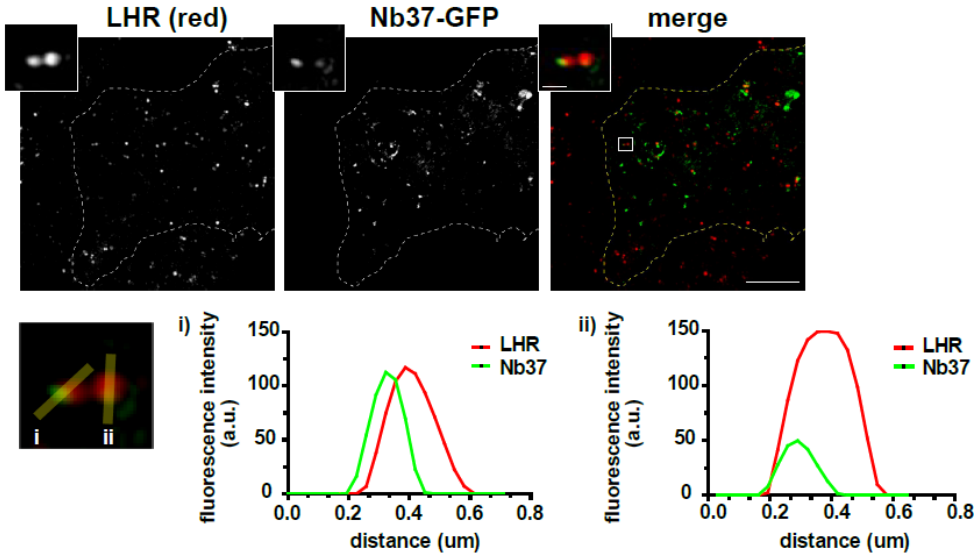


Figure 4.2 Active $G\alpha_s$ is detected in LHR endosomal microdomains by Nb37. (A) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR ± transfected with Nb37-GFP. Data represent mean ± SE, n=3 independent experiments. (B) HEK 293 cells stably expressing FLAG-LHR were transfected with Nb37-GFP and imaged live via confocal microscopy following stimulation with LH (10 nM, 30 min). Arrows indicate LHR endosomes which are also positive for Nb37-GFP. Scale bar=5 µm. Inset scale bar=1 µm. (C) HEK 293 cells stably expressing FLAG-LHR, and co-expressing Nb37-GFP, were stimulated with LH (10 nM, 30 min) following incubation with FLAG antibody. Surface FLAG antibody was stripped and cells fixed and permeabilised; representative SIM images are shown. Scale bar=5 µm. Inset shows the microdomain organisation of Nb37 within individual LHR endosomes (scale bar=500 nm). Line intensity analysis is shown for two endosomes (i, ii).

cAMP levels generated after receptor activation are controlled at different levels, including cAMP formation through AC activity, and degradation, accomplished by PDEs. ACs have been recently discovered to exist not only as transmembrane enzymes (tmAC), but also in a soluble, G protein- and forskolin-independent form localised in the cytoplasm (sAC) (Zippin et al., 2013); the latter has been proposed, for the first time, to contribute to endosomal cAMP generation by GPCRs, specifically by corticotropin-releasing hormone receptor 1, but not B2AR, in hippocampal neurons (Inda et al., 2016). Measurement of cAMP levels in FLAG-LHR cells stimulated with LH and pre-treated with either the tmAC inhibitor 2'-5'-dideoxyestradiol (ddA) or the sAC inhibitor 2-hydroxyestradiol (2-HE) resulted in a significant reduction of cAMP levels to 16.5 $\pm$ 9.6% and 54.7 $\pm$ 4.5%, respectively (Figure 4.3 A), indicating that both AC types are involved in LH-induced cAMP generation.

To investigate which PDE contributes to the cAMP profile produced by LHR, FLAG-LHR cells were pre-treated with either the non-specific PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX), the PDE4-specific inhibitor roflumilast or the PDE3-specific inhibitor cilostamide, as PDE4 and PDE3 have been previously reported to be the dominant PDEs to degrade cAMP in the cytosol and in the membrane fraction of HEK 293 cells, respectively (Matthiesen and Nielsen, 2011). After stimulation with LH, IBMX- and roflumilast-treated cells produced cAMP which was 96.81 $\pm$ 15.65 % and 224.13 $\pm$ 20.14 % higher than in DMSO-treated cells, respectively. On the contrary, PDE3 inhibition did not significantly affect cAMP values (Figure 4.3 B).

A. B.

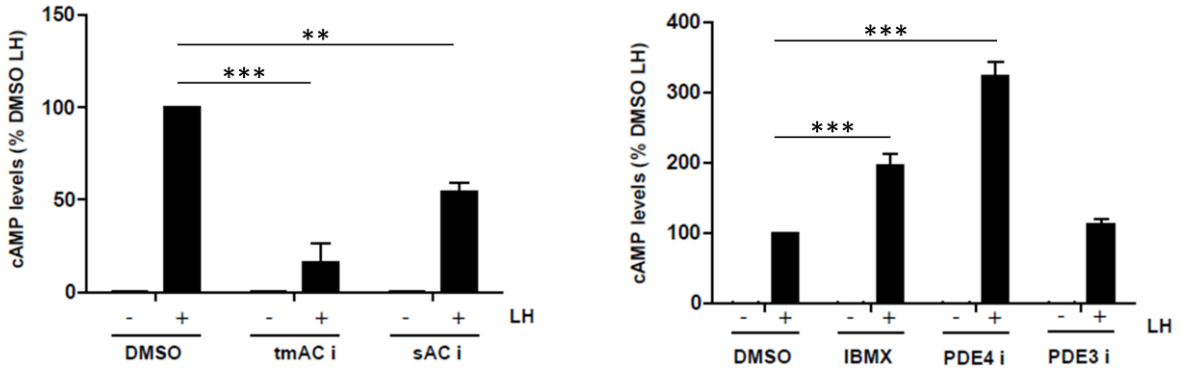


Figure 4.3 LH-induced cAMP levels are regulated by both transmembrane AC and soluble AC, and PDE4. Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR ± stimulation with LH (10 nM) for 5 min. In (A) cells were ± pre-treatment with either DMSO, the transmembrane AC inhibitor (tmAC i) ddA (100 µM) or the soluble AC inhibitor (sAC i) 2-HE (20 µM) for 5 min. In (B) cells were pre-treated with either IBMX (0.5 mM), the PDE4 inhibitor roflumilast (50 nM) or the PDE3 inhibitor cilostamide (400 nM). Data represent mean ± SE, n=3 (A), n=5 (B); Two-way ANOVA: ** $p<0.005$, *** $p<0.001$.

4.2.2 LHR activates the $G\alpha_{q/11}$-calcium pathway also from intracellular compartments

LHR also couples to $G\alpha_{q/11}$ under high ligand and receptor levels, conditions that are physiologically relevant in ovulatory follicles during the LH surge (Breen et al., 2013). To test whether $G\alpha_{q/11}$ pathway was activated by surface or intracellular LHR, I measured calcium release in FLAG-LHR cells under conditions where receptor internalisation was inhibited by Dyngo-4a pre-treatment. LH-dependent increases in intracellular calcium was significantly inhibited in these cells compared to DMSO-treated cells (Figure 4.4), suggesting that also this pathway is also activated by intracellular LHR.

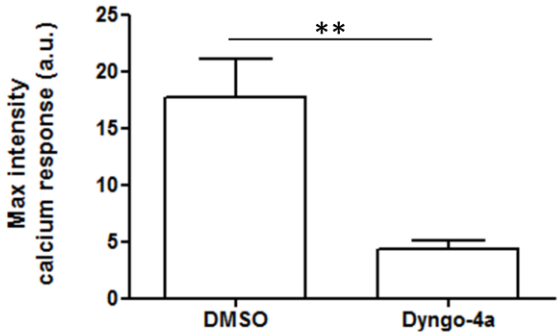


Figure 4.4 LHR activates calcium signalling mainly from intracellular compartments. Calcium release was measured in HEK 293 cells stably expressing FLAG-LHR, incubated with the fluorescent calcium indicator dye Fluo4-AM for 1 h, pre-treated with either DMSO or Dyngo-4a (30 nM) for 45 min and imaged live via confocal microscopy from 1 min before LH (100 nM) addition. n=4 independent experiments; t-test: **p<0.01.

4.2.3 Crosstalk of spatially-controlled signalling from the LHR

I investigated whether the primary second messenger signalling pathways activated by LHR were regulating each other by measuring either calcium release or cAMP accumulation in the presence of the PKA inhibitor KT5720 or the $G\alpha_{q/11}$ inhibitor UBO-QIC, respectively. As shown in Figure 4.5 A, inhibition of PKA activation greatly affects LH-induced calcium response. Similarly, cAMP levels in cells stimulated with LH are strongly reduced following $G\alpha_{q/11}$ inhibition (Figure 4.5 B). Taken together, these data suggest that LH-induced cAMP and calcium responses have a mutual synergistic effect.

A. B.

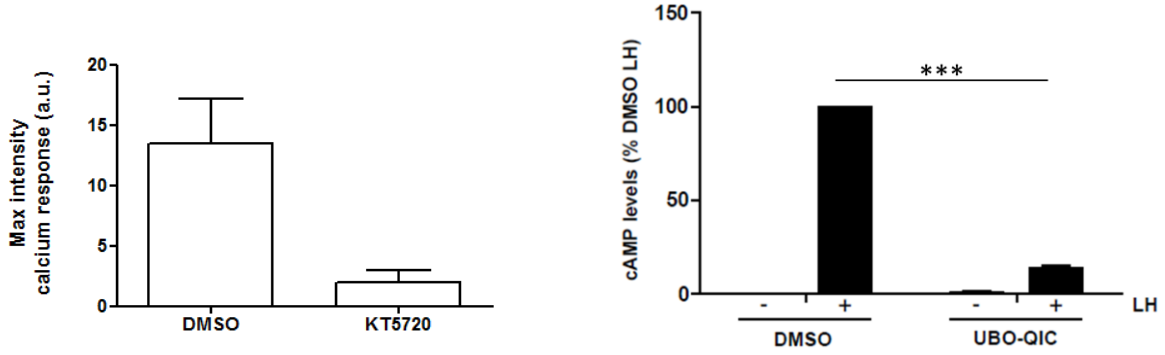


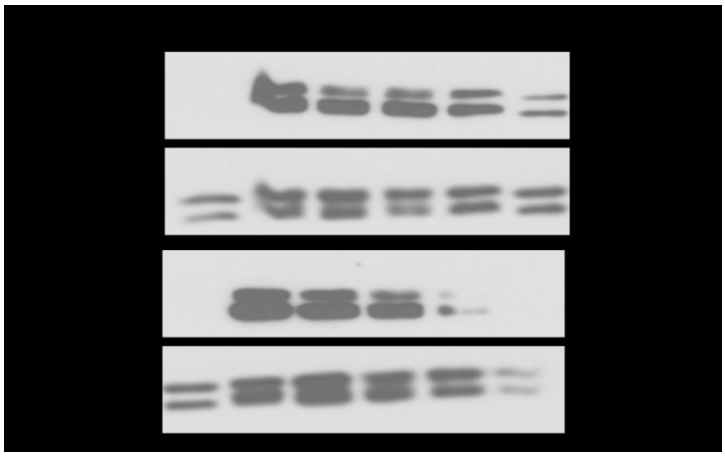
Figure 4.5 Crosstalk of $G\alpha_{q/11}$-calcium - $G\alpha_s$-cAMP-PKA pathways activated by LHR. (A) Calcium release was measured in HEK 293 cells stably expressing FLAG-LHR, incubated with the fluorescent calcium indicator Fluo4-AM for 1 h, pre-treated with either DMSO or KT5720 (10 µM) for 15 min and imaged live via confocal microscopy from 1 min before LH (100 nM) addition; n=2 independent experiments. (B) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR stimulated with LH (10 nM) and pre-treated with either DMSO or the $G\alpha_{q/11}$ inhibitor UBO-QIC (1 µM) for 2 h. n=3 independent experiments; Two-way ANOVA: ***p<0.001.

4.2.4 Characterisation of LH-induced MAPK pathway

As briefly mentioned above, ligand-induced LHR internalisation determines the activation of signalling cascades that lead to ERK 1/2 phosphorylation (Jean-Alphonse et al., 2014). It has been shown that the Ras-Raf-ERK pathway can be activated by different stimuli, thus representing a convergence of signals from distinct heterotrimeric G protein subunits. In the case of LHR, $G\alpha_s$, $G\alpha_{q/11}$ and/or Gβγ could contribute to ERK 1/2 phosphorylation. Using specific G protein subunit inhibitors, I measured phosphorylation of ERK 1/2 at different time points after LH addition and quantified the effect of each inhibitor. Interestingly, cells pre-treated with the Gβγ inhibitor, Gallein, exhibited a more transient pERK 1/2 profile, suggesting that Gβγ participates in generating a sustained MAPK response (Figure 4.6) and mimics the profile obtained when LHR is re-routed from the VEE to the EE (Jean-Alphonse et al., 2014). Therefore, I tested the possibility that Gβγ inhibition was having a similar effect on endosomal targeting of LHR. LHR co-localisation with both the VEE marker APPL1 and the EE marker EEA1 was not altered by Gallein pre-treatment (Figure 4.7), indicating that the

transient pERK profile observed under Gβγ inhibition was not a consequence of receptor being trafficked to the EE.

A.



B.

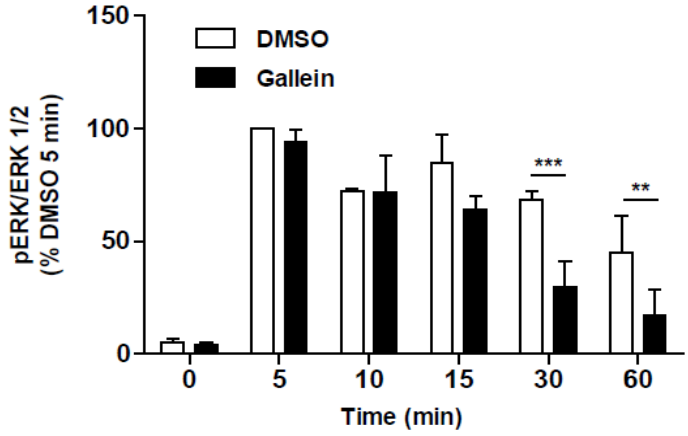
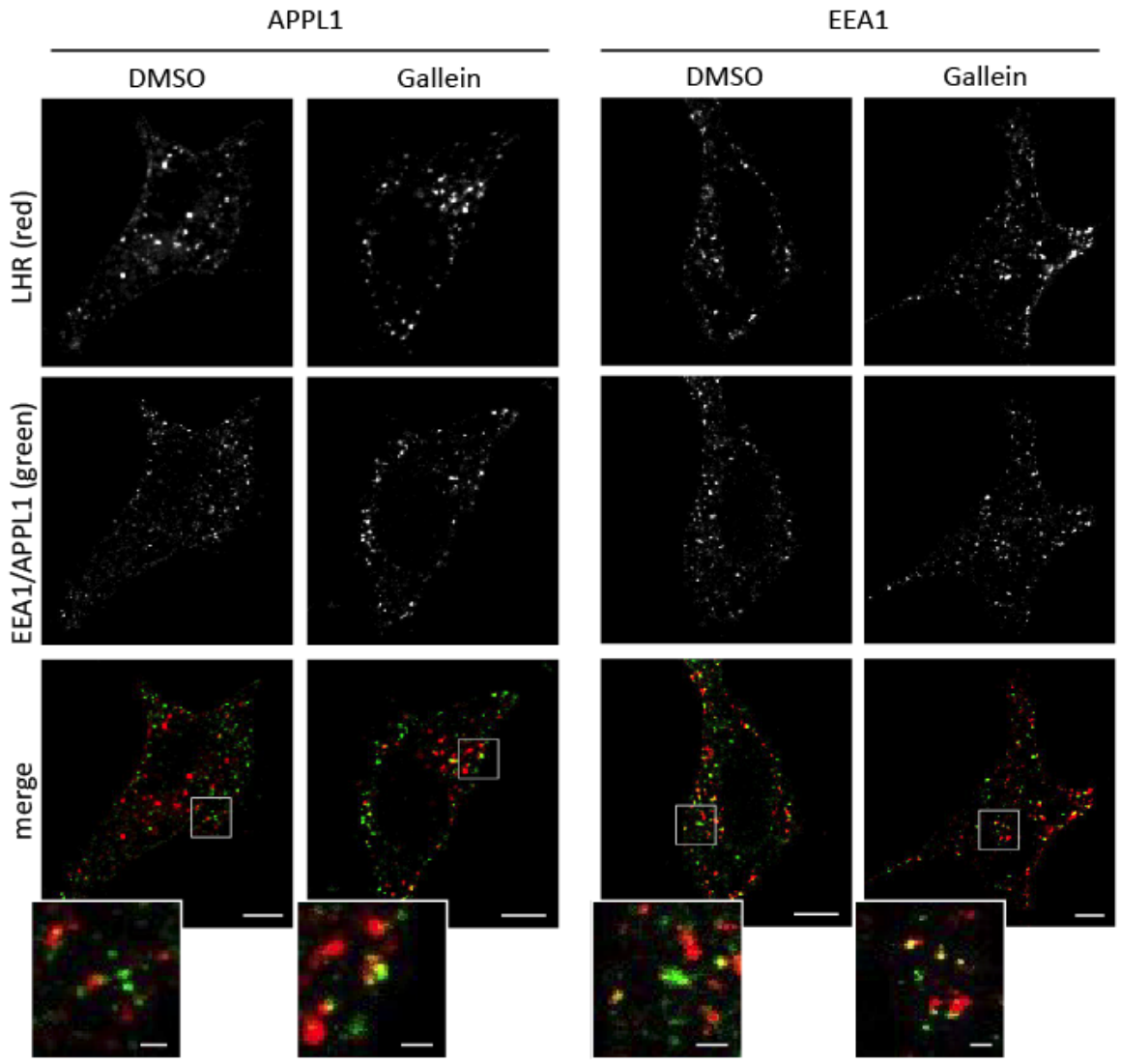


Figure 4.6 Gβγ inhibition determines a more transient LH-induced ERK 1/2 phopshorylation. (A) HEK 293 cells stably expressing FLAG-LHR were pre-treated with either DMSO or the Gβγ inhibitor Gallein (25 μM) for 1 h and phosphorylation of ERK 1/2 at stated time points after LH (10 nM) stimulation was determined by Western blotting. Total ERK was used as a loading control. A representative immunoblot is shown. (B) Densitometry analysis of ERK 1/2 phosphorylation from A. n=3; Two-way ANOVA: **p<0.01, ***p<0.001.

A.



B.

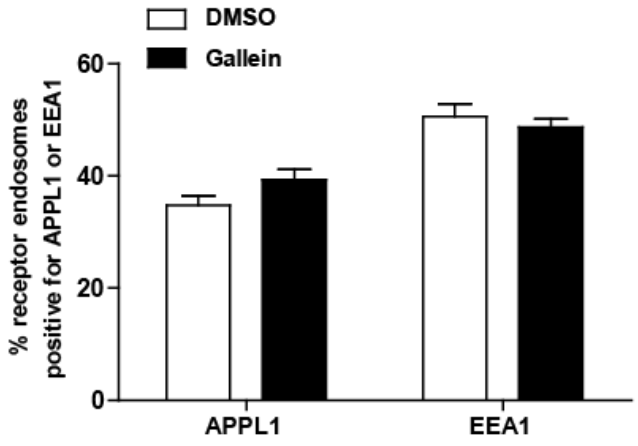
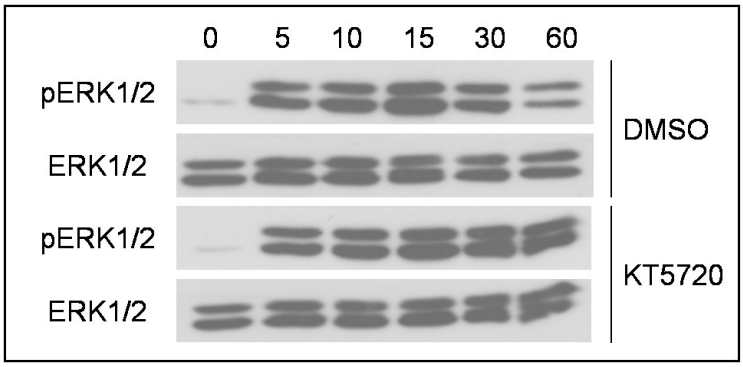


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Figure 4.7 Gβγ inhibition does not affect LHR endosomal localisation. (A) Representative confocal images showing the effect of Gallein on the co-localisation between LHR and either endogenous APPL1 or EEA1. HEK 293 cells stably expressing FLAG-LHR were pre-treated with either DMSO or Gallein (25 μM) for 1 h, fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 or anti-EEA1 antibodies followed by anti-rabbit AlexaFluor488 and anti-mouse AlexaFluor555 antibodies labelling, and imaged with confocal microscopy; scale bar=5 μm, insert=1 μm. (B) Quantification of LHR endosomes positive for endogenous APPL1 or EEA1 from A; n=23-28 cells per condition, collected across 3 independent experiments.

I used a similar approach to assess $G\alpha_s$ contribution to ERK 1/2 phosphorylation. Due to unavailability of a selective $G\alpha_s$ inhibitor, I used KT5720 to inhibit PKA, a downstream effector of the $G\alpha_s$-cAMP pathway. Inhibition of PKA significantly increased the levels of ERK 1/2 phosphorylation at the later time points of LH stimulation (Figure 4.8). Furthermore, when PKA was inhibited the levels of co-localisation with APPL1 also significantly increased, while co-localisation EEA1 was significantly reduced (Figure 4.9). This suggests that PKA inhibition increases retention of LHR into VEE and, together with previous findings that LHR trafficking to VEE is required for MAPK (Jean-Alphonse et al., 2014), is consistent with the more sustained ERK 1/2 phosphorylation observed under PKA inhibition.

A.



B.

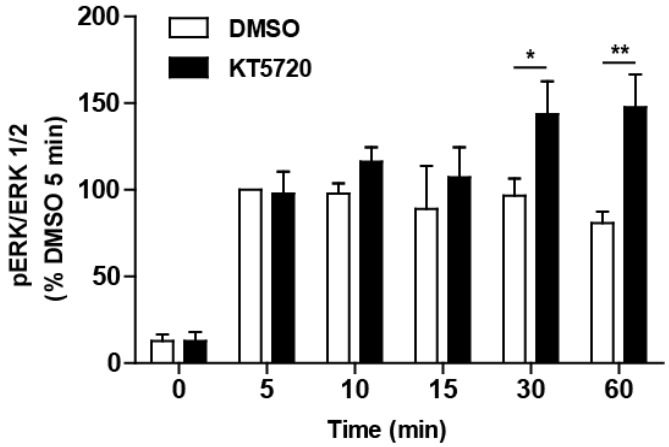
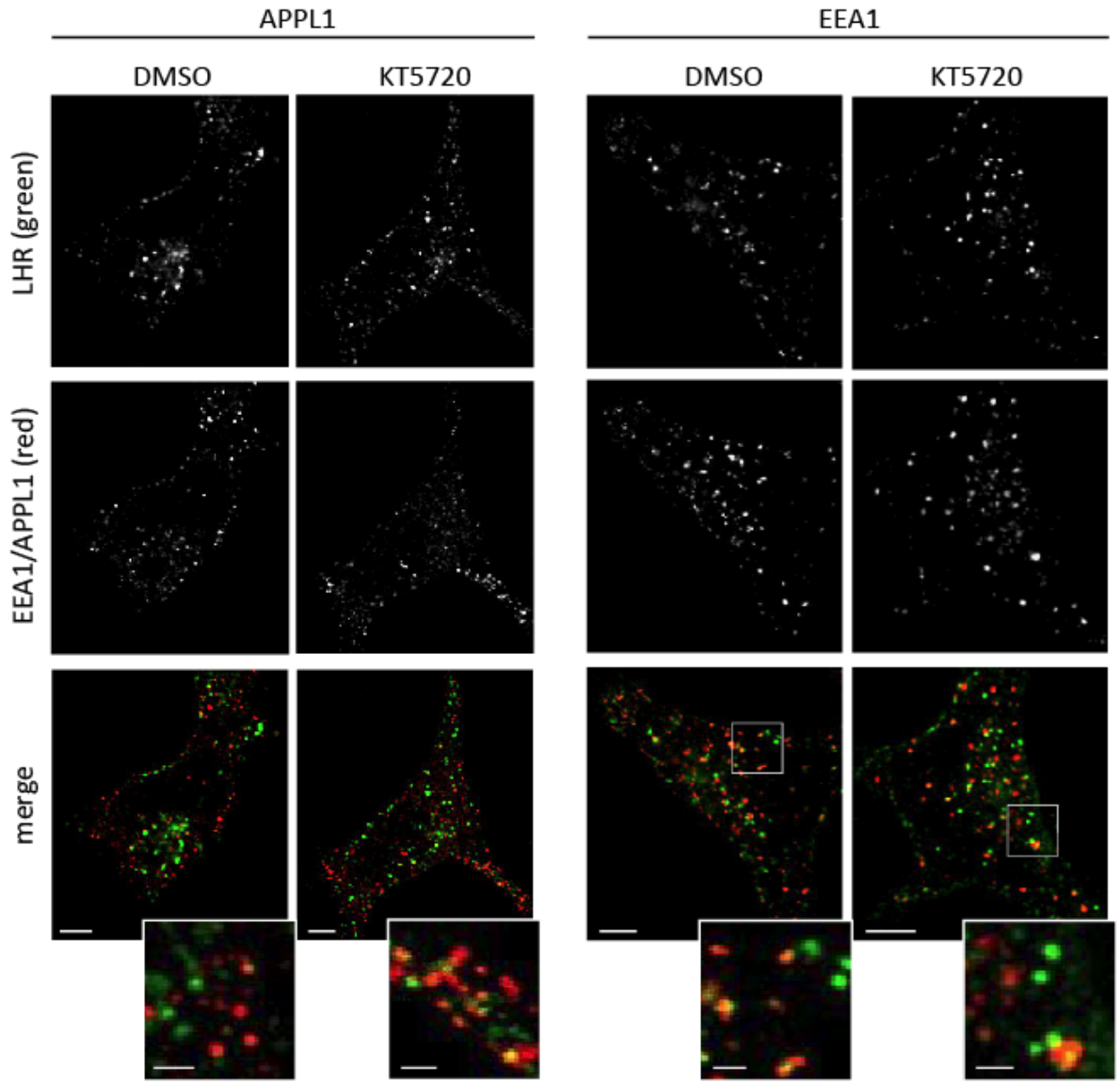


Figure 4.8 PKA inhibition induces sustained LH-induced ERK 1/2 phosphorylation. (A) HEK 293 cells stably expressing FLAG-LHR were pre-treated with either DMSO or the PKA inhibitor KT5720 (10 µM) for 15 min and phosphorylation of ERK 1/2 at stated time points after LH (10 nM) stimulation was determined by Western blotting. Total ERK was used as a loading control. A representative immunoblot is shown. (B) Densitometry analysis of ERK 1/2 phosphorylation from A. n=4; Two-way ANOVA: *p<0.05, **p<0.01.

A.



B.

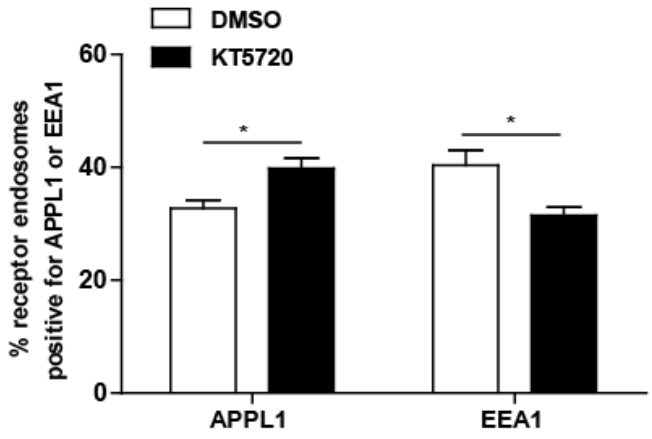
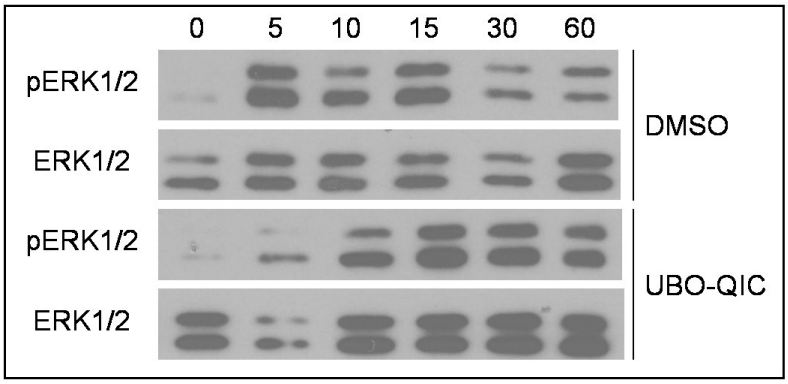


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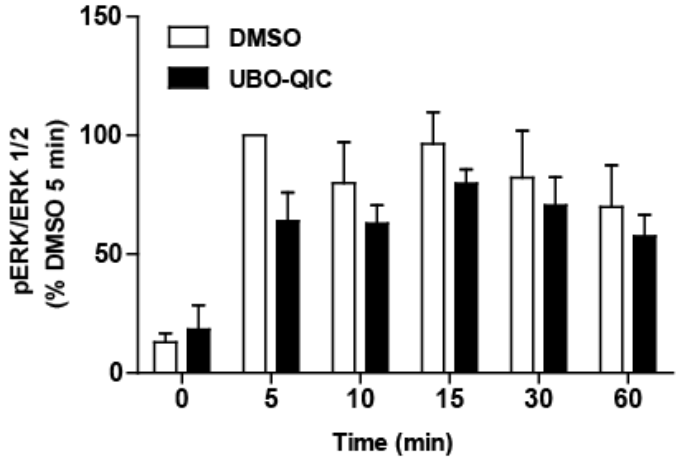
Figure 4.9 PKA inhibition increases LHR trafficking to VEE. (A) Representative confocal images showing co-localisation of LHR with either endogenous APPL1 or EEA1. HEK 293 cells stably expressing FLAG-LHR were pre-treated with either DMSO or KT5720 (10 μM) for 15 min, fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 or anti-EEA1 antibodies followed by anti-rabbit AlexaFluor555 and anti-mouse AlexaFluor488 antibodies labelling, and imaged with confocal microscopy; scale bar=5 μm, insert=1 μm. (B) Quantification of LHR endosomes positive for endogenous APPL1 or EEA1 from A; n=12-25 cells per condition, collected across at least 3 independent experiments; Two-way ANOVA: *p<0.05.

$G\alpha_{q/11}$ involvement in LH-dependent ERK 1/2 phosphorylation was assessed by blocking $G\alpha_{q/11}$ activation with the selective inhibitor UBO-QIC. UBO-QIC treatment did not affect ERK 1/2 phosphorylation profile activated by LHR (Fig 4.10 A and B). To demonstrate that UBO-QIC was effectively blocking $G\alpha_{q/11}$, I measured LH-induced calcium release in cells pre-treated with this inhibitor. Whilst cells pre-treated with DMSO generated a robust calcium response, in UBO-QIC treated cells this was completely blocked (Figure 4.10 C).

A.



B.



C.

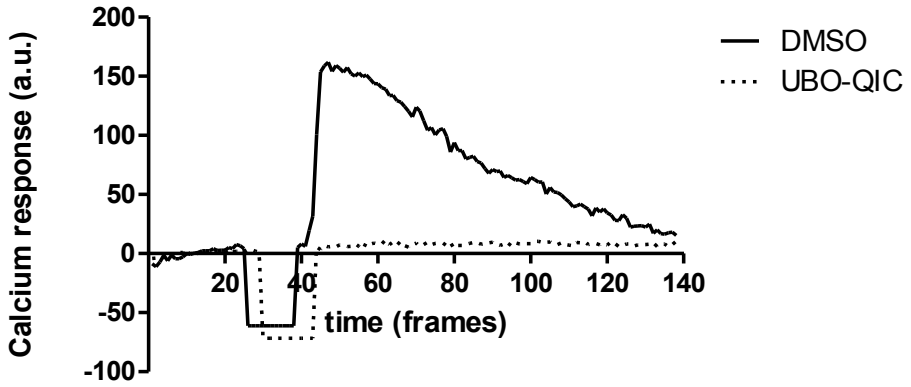


Figure 4.10 $G\alpha_{q/11}$ inhibition does not affect LH-induced ERK 1/2 phosphorylation. (A) HEK 293 cells stably expressing FLAG-LHR were pre-treated with either DMSO or the $G\alpha_{q/11}$ inhibitor UBO-QIC (1 µM) for 2 h and phosphorylation of ERK 1/2 at stated time points after LH (10 nM) stimulation was determined by Western blotting. Total ERK was used as a loading control. A representative

immunoblot is shown. (B) Densitometry analysis of ERK 1/2 phosphorylation from A; n=3. (C) Calcium release was measured in HEK 293 cells stably expressing FLAG-LHR incubated with the fluorescent calcium indicator Fluo4-AM for 1 h, pre-treated with either DMSO or the $G\alpha_{q/11}$ inhibitor UBO-QIC (1 μM) for 2 h and imaged live via confocal microscopy from 1 min before LH (100 nM) addition. A representative plot is shown.

4.3 Discussion

The data presented in this chapter demonstrate that, in addition to the MAPK pathway, the $G\alpha_s$-cAMP and $G\alpha_{q/11}$-calcium pathways also strongly rely on LHR internalisation for full activation upon stimulation with LH, supporting the recent demonstrations that GPCRs can continue, or reactivate, G protein signalling from endosomes (Ismail et al., 2016, Jean-Alphonse et al., 2014, Irannejad et al., 2015, Sposini and Hanyaloglu, 2017). Whilst for the majority of GPCRs that signal from endosomes, like B2AR and PTHR, the endosomal cAMP response refers to the persistent or 2nd phase of signalling (Irannejad et al., 2013, Ferrandon et al., 2009), which happens within tens of minutes to hours from agonist addition, my results highlight that some GPCRs could exploit endosomes as signalling stations also during the acute phase of signalling, within 5 minutes of receptor activation. It could be possible that, as the VEE is much closer to the plasma membrane compared to other endosomes, internalised GPCRs that reach this compartment can start to signal from there earlier than EE-target GPCRs. In addition to LHR, the only other receptor shown to require internalisation for acute cAMP response is the D1 dopamine receptor (Kotowski et al., 2011). However, as receptor endocytosis was only significant at high doses of agonist, Kotowski and colleagues suggest that rapid endosomal signalling may be only a feature under such concentrations where endocytosis is induced, and thus may be more pertinent under drug-induced or pathological conditions (Kotowski et al., 2011). This possibility remains to be explored for LHR, even if both low (10 nM) and high (100 nM) doses of LH are physiologically relevant. It is possible then that the high concentrations of LH registered during the “LH surge” of the menstrual cycle (described in Introduction, paragraph 1.5.1) are required not for acute cAMP signalling, which in the case of LHR happen even at low doses of ligand, but for the acute activation of the $G\alpha_{q/11}$-calcium pathway.

Interestingly, through the use of Nb37, I have been able to visualise active $G\alpha_s$ in LHR endosomes that are distinct from the EE, a finding which has never been reported before. Thanks to the higher resolution achieved by SIM, I could localise $G\alpha_s$ activation in restricted areas of the VEE, raising the possibility that, although physically smaller than EEs, active receptors within an indivudal VEE are organised in microdomains, as reported for the EE-localised B2AR (Bowman et al., 2016). The finding that Nb37 was not recruited to the plasma membrane within the first minutes of LHR stimulation (~5 minutes), nor at later time points (~30 minutes), corroborates the hypothesis that LHR signals from endosomes during the acute phase of activation. Nb37 is a potent tool which, combined with the appropriate microscopy technique, allows both visual inspection and quantitative measurement of $G\alpha_s$ signalling. Nevertheless, it has some drawbacks; it recognizes all active $G\alpha_s$ present in the cell, not only those activated by the receptor of interest, and it only binds $G\alpha_s$ subunits which are in an active, nucleotide free state, a very transient phase of the G protein cycle, thus potentially underestimating the actual proportion of active $G\alpha_s$. For these reasons, it would be more accurate to use Nb37 in conjunction with a receptor-specific Nb, even though GPCR-specific Nbs, due to their ability to bind the active receptor in a steric-occlusion manner, have been shown to physically prevent the binding of downstream effectors, hence altering cAMP levels, and lock the receptor in an active state, a feature which has been greatly convenient for the successful crystallisation of B2AR by Kobilka and co-workers. Further optimisation of Nb generation is thus needed, as well as the development of new Nbs for other GPCRs, including LHR.

I explored further the cAMP response induced by LH by focusing on the two main factors that shape cellular cAMP gradients, PDEs and ACs. PDE4 appeared to be greatly involved in cAMP degradation, as shown by the increase in cAMP values under PDE4 inhibition. It still remains to be elucidated whether other PDEs play a role in the regulation of LH-induced cAMP, and whether they have distinct roles in different subcellular locations. Unfortunately, both the use of antibodies against PDEs or transfection of tagged-PDEs are impractical, due to the fact that PDEs exist in many isoforms and that their overexpression would profoundly alter even basal cAMP levels, respectively. I also demonstrated that LH-induced cAMP is generated by both forms of AC, tmAC and sAC. Until recently, cAMP produced upon GPCR

stimulation was thought to be produced only by tmAC. Ivonnet and colleagues showed that sACs contribute to GPCR-cAMP response as well; prostaglandin EP4 receptor stimulation leads to tmAC-produced cAMP accumulation which, in turns, activates Ca^{2+} release and subsequent activation of sAC, resulting in an amplification of the cAMP response (Ivonnet et al., 2015). LHR could use a similar mechanism to fully activate its cAMP response, as inhibition of either tmAC or sAC greatly affects the amount of cAMP produced in response to LH.

This finding also strengthens the hypothesis that there could be crosstalk between cAMP and calcium pathways activated by LHR, with LH-activated $G\alpha_s$ pathway synergising with the $G\alpha_{q/11}$ to release calcium. In fact, calcium release is diminished under PKA inhibition, suggesting a strong requirement of $G\alpha_s$-cAMP-PKA activation for a full calcium response. Interestingly, the cAMP response also requires $G\alpha_{q/11}$-calcium signalling to be fully activated. This apparent link between the $G\alpha_s$-cAMP and $G\alpha_{q/11}$-calcium pathways raises a wide range of questions including at which level of the signalling cascades the crosslink takes place, whether scaffolding proteins behave as linkers between the two pathways, if it is caused by receptor homo-dimerisation/oligomerisation or LHR dimerisation with other types of receptors. The tools to address these questions might not be straightforward or available yet, but first approaches could be the use of biosensors targeting signalling pathways other than $G\alpha_s$, the knock-down of specific G proteins, and more specific and upstream inhibitors for $G\alpha_s$. The latter two could also be applied to further study of MAPK pathway elicited by LHR.

In addition to the MAPK and cAMP pathways, I showed that the $G\alpha_{q/11}$-calcium also requires internalisation for its activation, as demonstrated by the results obtained using the dynamin inhibitor. Internalisation-dependent calcium release has been observed for another $G\alpha_{q/11}$-coupled receptor, the kisspeptin receptor (Min et al., 2014), suggesting that, potentially, all G-protein dependent signalling cascades could be modulated by the intracellular location of the receptor. More evidence is needed to confirm LHR-$G\alpha_{q/11}$ activation from intracellular compartments, and also to better characterise which endosomal and/or cellular location $G\alpha_{q/11}$ is activated from. Whilst a big variety of tools is available for the study the $G\alpha_s$-cAMP pathway at subcellular level, the visualisation of where the $G\alpha_{q/11}$-calcium pathway is

activated is much more challenging, as the genetically encoded calcium indicators reveal the compartment from which calcium is released in response to GPCR activation, but not where the activated GPCR is when triggering the $G\alpha_{q/11}$-calcium response. For this purpose, it would be greatly useful the development of active-$G\alpha_{q/11}$ nanobodies.

Although the cellular and physiological role of endosomal *versus* plasma membrane signalling remains unclear, the evidence provided in this chapter highlights the importance of endosomal localisation of LHR to achieve its signalling outputs.

Chapter 5:

APPL1 integrates sorting and signalling from the VEE

5.1 Introduction

Endocytic trafficking is a critical mechanism for cells to decode complex signalling pathways, including those activated by GPCRs, by dictating the intensity and duration of specific patterns of heterotrimeric G protein signalling from the plasma membrane. The divergent sorting of GPCRs following ligand-induced endocytosis exemplifies this; in fact, receptors are either resensitised, by being re-exposed to the extracellular environment through recycling, or degraded by targeting to the lysosomes (detailed in Introduction, paragraphs 1.2.2 and 1.2.3). Therefore, membrane trafficking and signalling are viewed as an integrated system that controls diverse fundamental cellular programs and altering receptor trafficking profoundly reprograms GPCR signal transduction. Physiologically, this represents an adaptive mechanism and under pathophysiological conditions, can lead to perturbed GPCR signalling and disease. As described in Chapter 4, in the last few years it has been acknowledged that GPCRs can continue, or re-activate, signalling following receptor internalisation, highlighting a key functional role of the endocytic system in GPCR activation and changing the dogma according to which GPCR internalisation corresponds to signal inactivation. Although this concept is now well enstablished in the GPCR field, the mechanisms dictating how membrane trafficking spatially decodes complex signalling pathways remain largely unknown.

Following endocytosis cell surface receptors can be differentially sorted in the endosomal network, with certain receptors, including LHR, targeted to the VEE instead of the classic EE, where GPCRs such as B2AR internalise to (Jean-Alphonse et al., 2014). The main features of VEE-localised LHR are its ability to activate a sustained MAPK (Jean-Alphonse et al., 2014) and the $G\alpha_s$/cAMP and $G\alpha_{q/11}$-calcium pathways (Chapter 4, paragraphs 4.2.1 and 4.2.2). Further, LHR must be targeted to VEEs to recycle back to the plasma membrane in a PDZ-ligand dependent manner (Kishi et al., 2001, Galet et al., 2003). To date, the only marker of the VEE is APPL1, although its roles in LHR trafficking and signalling are unknown. In this chapter I identify the functional roles of APPL1 on LHR signalling and recycling from the VEE and the molecular mechanisms determining APPL1-mediated integration of these two processes.

5.2 Results

5.2.1 APPL1 is essential for GPCR recycling via the VEE

Previous work to study the co-localisation between LHR and APPL1 was conducted in cells overexpressing exogenous GFP-APPL1 (Jean-Alphonse et al., 2014). Overexpression could alter the cellular localisation of the protein of interest and force its interaction with other proteins that it would not come in contact with. Therefore, I measured co-localisation between LHR and either endogenous APPL1 or EEA1. After 15 minutes of LH stimulation, 34.2 ± 1.4 % and 45.7 ± 1.9% of LHR endosomes were positive for APPL1 or EEA1, respectively (Figure 5.1), confirming that APPL1 marks a subpopulation of LHR endosomes even at endogenous levels, as well as EEA1.

A.

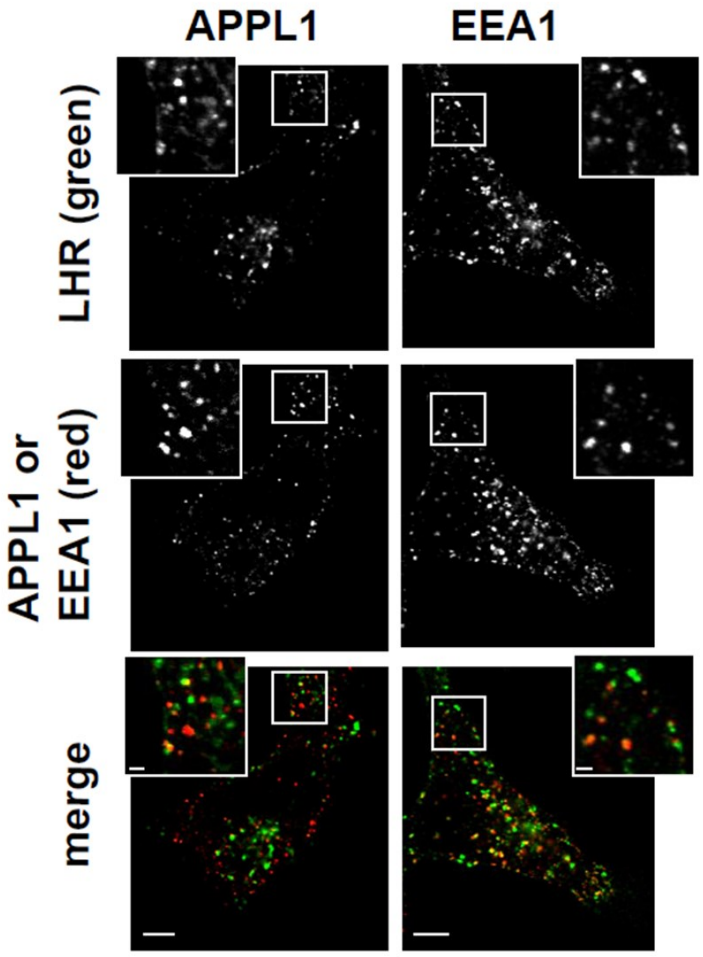


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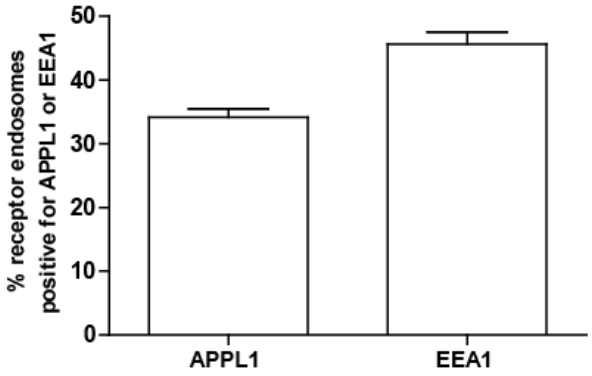


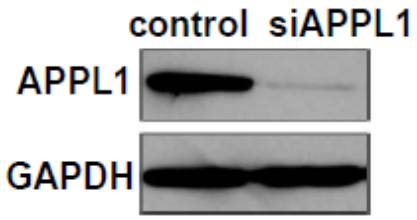
Figure 5.1 LHR is internalised into endosomes marked by APPL1 or EEA1. (A) Representative confocal images showing LHR endosomes co-localising with either endogenous APPL1 or EEA1. HEK 293 cells stably expressing FLAG-LHR were fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 or anti-EEA1 antibodies followed by anti-rabbit AlexaFluor555 and anti-mouse AlexaFluor488 antibodies labelling, and imaged with confocal microscopy. Scale bar=5 µm, insert=1 µm. (B) Quantification of LHR endosomes positive for endogenous APPL1 or EEA1, n=44 (APPL1) and 54 (EEA1) cells per condition collected across at least 3 independent experiments.

To identify the functional impact of APPL1 on LHR endosomal organisation and post-endocytic sorting, APPL1 was depleted using siRNA in HEK 293 cells stably expressing FLAG-LHR (Figure 5.2 A). Assessment of ligand-induced LHR internalisation and recycling via confocal microscopy showed a marked inhibition of LHR re-routing back to the plasma membrane and accumulation of receptor in endosomes upon ligand removal in cells depleted of APPL1 (Figure 5.2 B, top panel). Confocal microscopy confirmed that APPL1 knockdown prevented recycling of not only LHR but additional GPCRs previously shown to traffic to VEEs (Jean-Alphonse et al., 2014), the FSHR and B1AR (Figure 5.2 B, middle and bottom panels, respectively) .

I next assessed the role of APPL1 in rapid GPCR recycling via live cell TIR-FM of SEP-LHR (described in Chapter 3). Critically, recycling of SEP-LHR was strongly inhibited in APPL1-

depleted cells (Figure 5.2 C). To ascertain if APPL1 was also essential for the recycling of GPCRs that are organised to the EE, but not the VEE, and for receptors undergoing default recycling via bulk membrane flow, B2AR and Transferrin receptor (TfR) recycling were assessed, respectively. Cells expressing SEP-B2AR or SEP-TfR were transfected with non-targeting or APPL1 siRNA. siRNA-mediated APPL1 knock-down did not affect the number of recycling puffs observed in SEP-B2AR nor SEP-TfR expressing cells, confirming that APPL1 specifically modulates PDZ-ligand regulated recycling of VEE-sorted receptors (Figure 5.2 C).

A.



B.

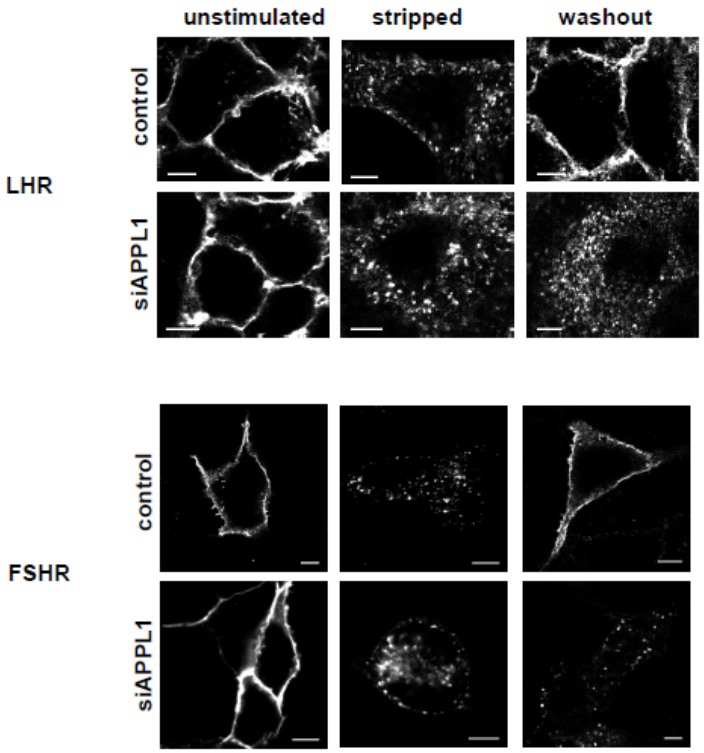
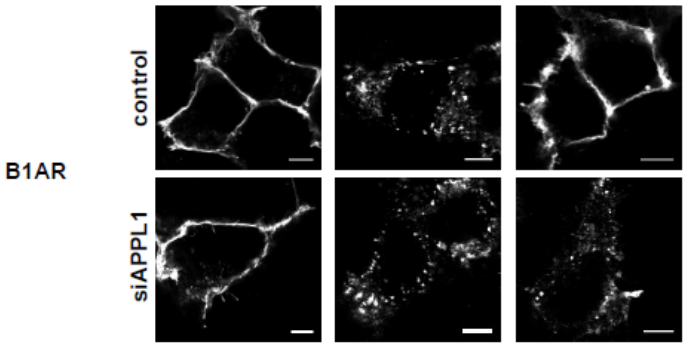


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C.

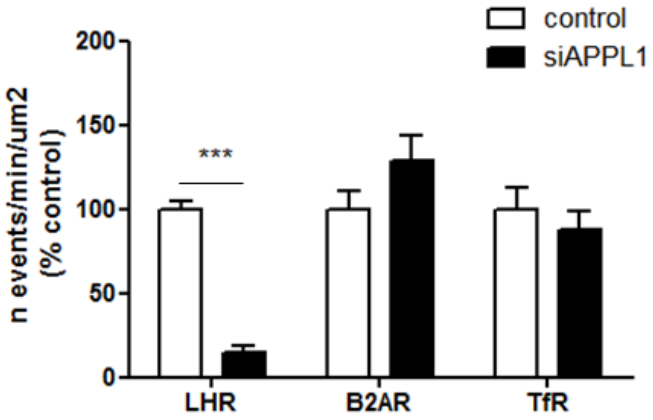
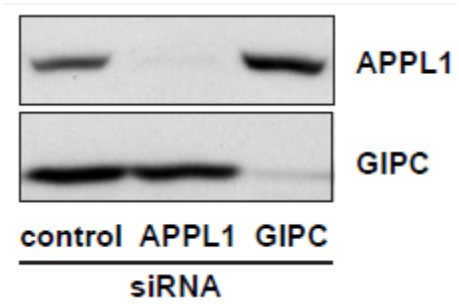


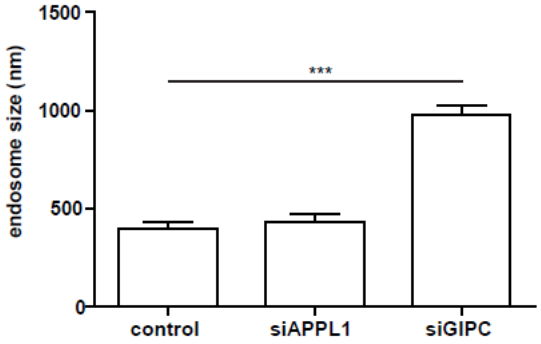
Figure 5.2 APPL1 is required for GPCR recycling from the VEE but not the EE. (A) Representative Western blot of total cellular levels of APPL1 from lysates collected from HEK 293 cells following control siRNA (control) or siRNA-mediated knockdown of APPL1 (siAPPL1). GAPDH was used as a loading control. (B) Ligand-induced internalisation and recycling following APPL1 siRNA-mediated knockdown were analyzed by confocal microscopy. HEK 293 cells expressing FLAG-LHR, -FSHR or -B1AR were treated with AlexaFluor555 conjugated-M1 FLAG antibody prior to treatment with LH (10 nM), FSH (10 nM) or ISO (10 µM) for 20 min, respectively. Surface bound M1 FLAG antibody was removed by PBS/EDTA wash and cells were incubated in ligand-free medium for 1 h to allow for receptor recycling. Scale bar= 5 µm. (C) HEK 293 cells expressing either SEP-LHR, SEP-B2AR or SEP-TfR and treated with either scrambled siRNA (control) or APPL1 siRNA (siAPPL1) were stimulated with either LH (10 nM), isoproterenol (10 µM) or PBS respectively and recycling was measured in real time, via TIR-FM, in different cells from 5 min after ligand addition. n=16, 13 or 16 cells per condition for LHR, B2AR or TfR, respectively, collected across at least 3 independent experiments; t-test: ***p<0.001.

To ensure that the inhibition of LHR recycling in APPL1-depleted cells was not a consequence of re-routing receptor to the EE, levels of GIPC, LHR endosome size and co-localisation with EEA1 (three hallmarks of the EE compared to the VEE) were determined. APPL1 knockdown did not affect cellular levels of GIPC nor the size of LHR endosomes (Figure 5.3 A-B). Co-localisation of LHR with the EE marker EEA1 demonstrated a small but significant increase following knockdown of APPL1 (<10%); however, this increase was marginal compared to the 3-fold increase in LHR-EEA1 co-localisation following GIPC knockdown (Figure 5.3 C). Taken together, these data suggest that APPL1 depletion does not abolish the VEE and does not re-route LHR to the EE.

A.



B.



C.

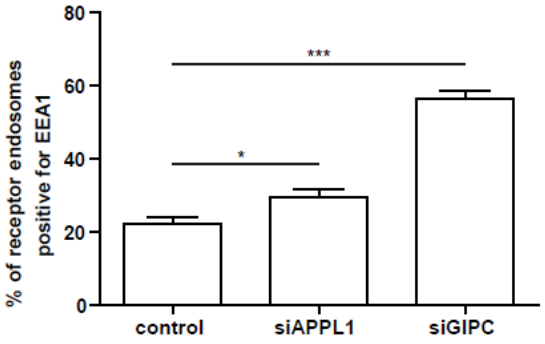


Figure 5.3 LHR is not re-routed to the EE following APPL1 depletion. (A) Representative Western blot of total cellular levels of APPL1 or GIPC from lysates collected from HEK 293 cells expressing FLAG-LHR following scramble (control), APPL1 (siAPPL1) or GIPC (siGIPC) siRNA-mediated knockdown. (B) Size of endosomes containing internalised FLAG-LHR in cells transfected with either scramble (control), APPL1 (siAPPL1) or GIPC (siGIPC) siRNA. Cells were imaged live by confocal

microscopy following 10-15 min of LH (10 nM) treatment. Endosome size was quantified using Leica LASAF software, n=6 cells per condition; t-test: ***p<0.001. (C) Quantification of LHR endosomes positive for EEA1 in HEK 293 cells stably expressing FLAG-LHR following scramble (control), APPL1 (siAPPL1) or GIPC (siGIPC) siRNA-mediated knockdown. Cells were fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 10 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-EEA1 antibody followed by anti-rabbit AlexaFluor555 and anti-mouse AlexaFluor488 antibodies labelling, and imaged with confocal microscopy. n=27 cells per condition; t-test: *p<0.05, ***p<0.001.

5.2.2 APPL1-dependent LHR recycling requires PKA activation

The next step was to uncover the molecular mechanisms of APPL1-dependent LHR recycling. Considering GPCR activation and signalling is essential for subsequent intracellular trafficking of receptors (Yudowski et al., 2009a, Vistein and Puthenveedu, 2013, Rosciglione et al., 2014) and that LHR is primarily a $G\alpha_s$-coupled receptor, I first assessed whether the cAMP/PKA activation could regulate LHR recycling. SEP-LHR expressing cells were pre-treated with a PKA inhibitor (KT5720) or activator (8-Br-cAMP) prior to stimulation with LH and live TIR-FM imaging. KT5720 potently inhibited LHR recycling compared to untreated or DMSO-treated cells (Figure 5.4). By contrast, cells pre-treated with 8-Br-cAMP exhibited a significant increase in recycling (Figure 5.4).

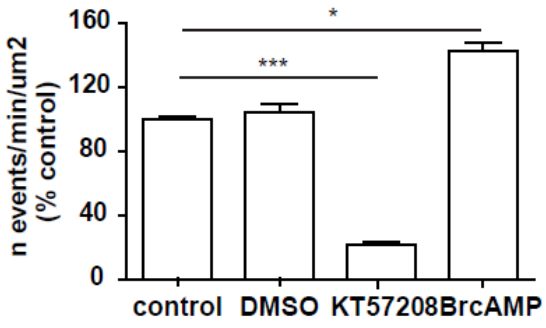


Figure 5.4 PKA activation is necessary for LHR recycling. (A) LHR recycling was measured in real time by TIR-FM in HEK 293 cells stably expressing SEP-LHR in presence of LH and pre-treated for 15 min

with either DMSO, PKA inhibitor KT5720 (10 μM) or PKA activator 8-Br-cAMP (0.5 mM). n=16 cells per condition collected across 3 independent experiments; One-way ANOVA: *p<0.05, ***p<0.001.

Given the absolute dependence on APPL1 for LHR recycling from the VEE, inhibition of PKA may impact LHR recycling by altering trafficking of internalised LHR to APPL1 endosomes or disrupting the endosomal localisation of APPL1. As previously shown in Chapter 4, the ability of APPL1 to localise to endosomes was unperturbed following treatment with KT5720, however, the number of LHR endosomes co-localising with endogenous APPL1 increased significantly (Figure 4.7 C-D). Thus, loss of recycling under inhibition of PKA is not due to impaired LHR sorting to APPL1 endosomes.

From the data presented in Figure 5.4, the number of LHR recycling events seemed to be positively correlated with cAMP levels; to further test this hypothesis I altered LH-induced cAMP levels using the PDE4 inhibitor roflumilast and the two AC inhibitors ddA (tmAC i) and 2-HE (sAC i). As expected, inhibition of both AC types determined a reduction in the number of LHR recycling events, although this was significant only for sAC i (Figure 5.5); surprisingly, even if roflumilast increases LH-induced cAMP levels (Figure 4.3 B), it decreased, instead of increased as it was expected, LHR recycling rates (Figure 5.5). This suggests that not only the levels, but also the source of cAMP, are fundamental in regulating LHR trafficking to the plasma membrane.

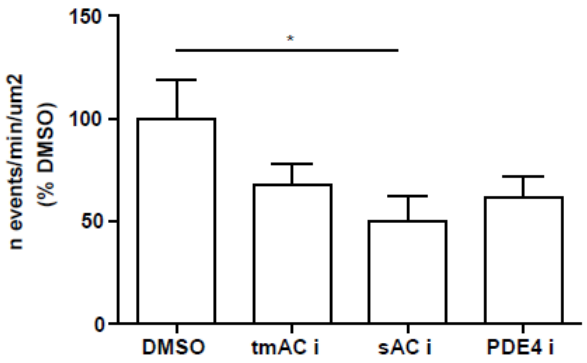


Figure 5.5 Altering cAMP production or degradation modulates LHR recycling. LHR recycling events were counted in HEK 293 cells stably expressing SEP-LHR, pre-treated with either DMSO, the transmembrane AC inhibitor (tmAC i) ddA (100 µM), the soluble AC inhibitor (sAC i) 2-HE (20 µM) or the PDE4 inhibitor roflumilast (50 nM) and stimulated with LH (10 nM). n=16 cells for DMSO and 10 cells for the other conditions; One-way ANOVA: *p<0.05.

5.2.3 LH-driven PKA phosphorylation S410 of APPL1 is essential for LHR recycling

PKA is known to negatively or positively regulate recycling of other GPCRs through phosphorylation of the receptors or associated adaptor proteins (Vistein and Puthenveedu, 2013, Nooh et al., 2014, Man et al., 2007, Gardner et al., 2006). After consultation of NetPhos 3.1 Server, no PKA consensus phosphorylation sites were found in the human LHR intracellular regions (Blom et al., 2004). However, PKA phosphorylation, specifically at Serine 410 (S410), has been shown to act as molecular switch for APPL1 interaction with other protein partners (Gant-Branum et al., 2010). To determine if phosphorylation of APPL1 on S410 mediates APPL1-dependent recycling, I generated and used both a phospho-deficient mutant (S410A) and phospho-mimetic mutant (S410D) of mCherry-APPL1 to assess if VEE-directed recycling of LHR could be restored in APPL1 depleted cells. To exclude the possibility that the mutations could disrupt the endosomal co-localisation between LHR and APPL1, I first measured the percentage of LHR-containing endosomes co-localising with either endogenous APPL1 or mCherry-tagged wild-type (WT), S410A (S/A) or S410D (S/D) APPL1 mutants 15 min after LH stimulation. The level of co-localisation of mCherry-tagged WT, S/A, or S/D APPL1 with LHR was comparable to the level observed with endogenous APPL1 (Figure 5.6 A-B).

A.

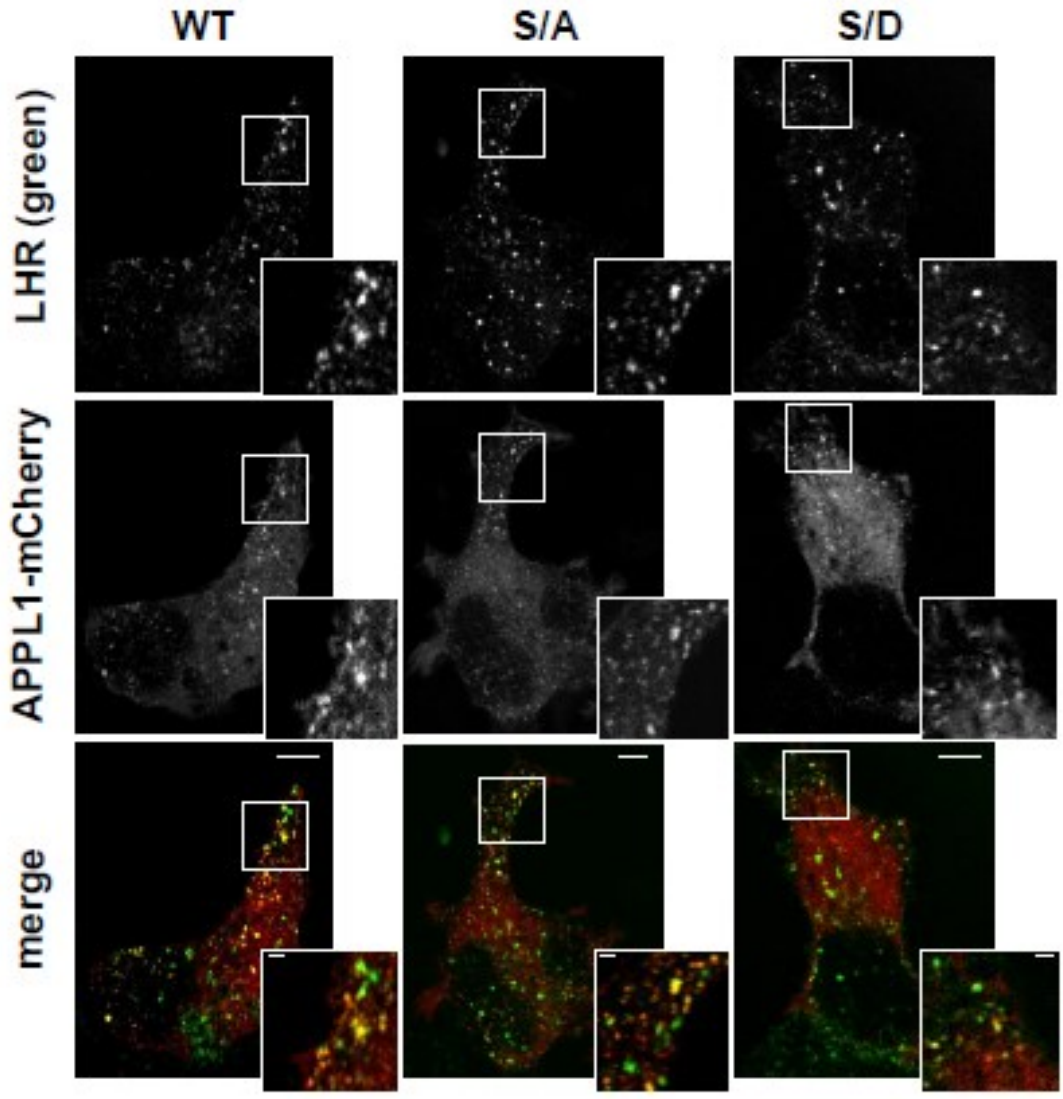


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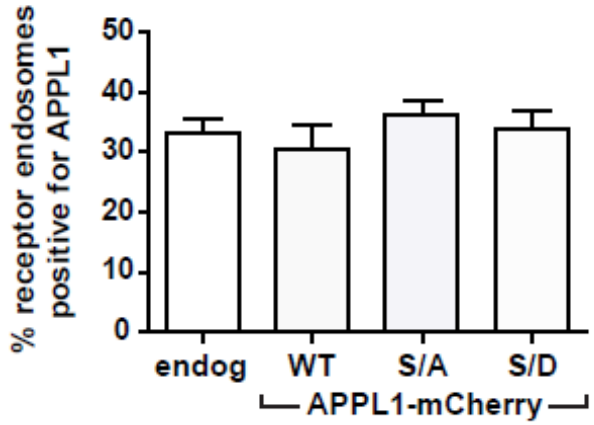
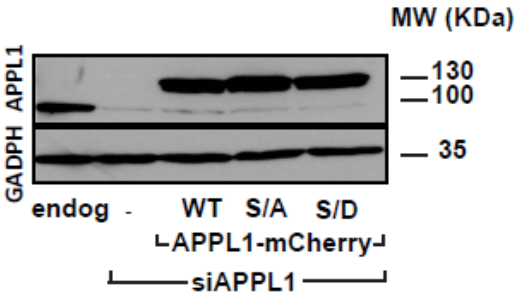


Figure 5.6 APPL1 phospho-mutants retain the ability to co-localise with LHR. (A) Representative confocal images of HEK 293 cells stably expressing FLAG-LHR and transiently transfected with either mCherry-WT, -S410A (S/A) or S410D (S/D) APPL1. Cells were fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised, stained with anti-mouse AlexaFluor488 antibody and imaged. Scale bar=5 µm, inset=1 µm. (B) Quantification of Figure 5.6 A, n=15 cells per condition, collected across 3 independent experiments across 3 independent experiments.

The expression of the three mCherry-constructs following siRNA-mediated depletion of endogenous APPL1, monitored by Western blot, was equivalent and similar to levels of endogenous APPL1 prior to siRNA treatment (Figure 5.7 A). Therefore, I measured LHR recycling in cells where APPL1 expression was rescued by transfection with each of the mCherry-constructs after depletion of endogenous APPL1. LHR recycling was only restored upon expression of either WT or the phospho-mimetic mutant S/D (Figure 5.7 B). Notably, expression of the phospho-deficient APPL1 did not rescue LHR recycling (Figure 5.7 B).

A.



B.

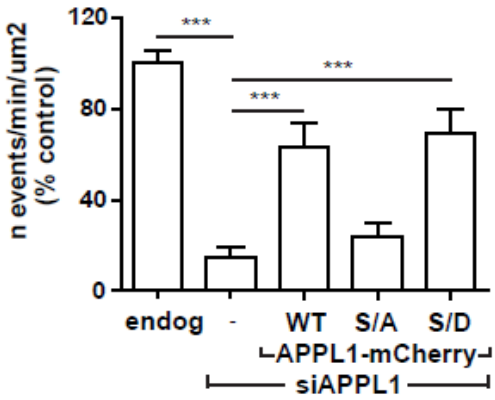
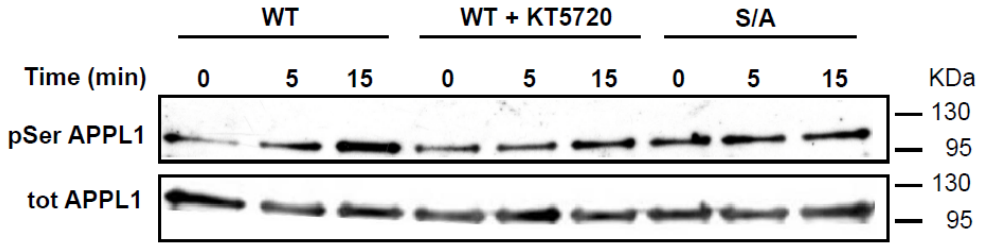


Figure 5.7 APPL1 phospho-mimetic mutant S410D rescues LHR recycling in endogenous APPL1-depleted cells. (A) Representative Western blot analysis of total cellular levels of APPL1 from lysates collected from HEK 293 cells stably expressing SEP-LHR and transiently transfected with mock (endog), siAPPL1 (-), siAPPL1 + mCherry-WT (WT) or -S410A (S/A) or -S410D (S/D) APPL1. GAPDH was used as loading control. (B) LHR recycling measured by TIR-FM in HEK 293 cells stably expressing SEP-LHR and transfected as in A. n≥16 cells per condition were imaged across at least 3 independent experiments; One-way ANOVA: ***p<0.001.

The above data indicate that APPL1-dependent recycling of LHR is driven by PKA-dependent phosphorylation of APPL1 at S410. To ascertain that LH stimulation induces phosphorylation of APPL1 in a PKA-dependent manner and specifically on S410, cells expressing WT APPL1-GFP were treated with LH and immunoprecipitated using a GFP nanobody and eluates analysed by Western blot using a phospho-serine antibody and APPL1 antibody. The phospho-serine antibody detected a single band of ~110 kDa corresponding to APPL1-GFP which increased following LH treatment (Figure 5.8 A). Strikingly, this increase in phospho-serine levels of APPL1 was significantly inhibited by pre-treatment with KT5720 at both time points analysed (Figure 5.8 A-B). Although there was a significant increase in phosphorylation of S/A APPL1 following 15 min of ligand stimulation (Fig 5.8 A-B), comparison of LH-induced S/A APPL1 phosphorylation levels to those of WT APPL1, revealed a significant reduction at this time point (Figure 5.8 A-B).

Overall, these data suggest that LHR activation of the cAMP/PKA pathway drives its own recycling from VEEs via a mechanism that depends on ligand-dependent phosphorylation of APPL1 at S410.

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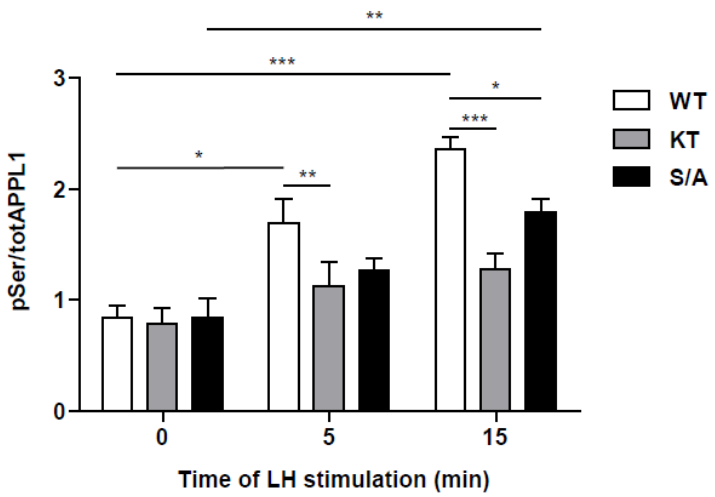
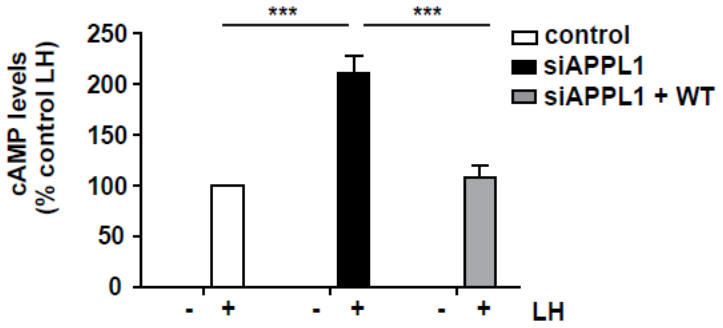


Figure 5.8 LHR stimulation induces PKA-dependent phosphorylation of APPL1, predominantly on S410. (A) HEK 293 cells stably expressing FLAG-LHR following transfection with either GFP-WT APPL1 or GFP-S/A APPL1 were ± stimulated with LH (10 nM, 5 and 15 min), ± pre-treatment with KT5720 (10 µM, 15 min). After collection of lysates, GFP-APPL1 was immunoprecipitated and APPL1 phosphoserine levels were determined by Western blotting. Total APPL1 was used as a loading control. A representative immunoblot is shown. (B) Densitometric analysis of APPL1 serine phosphorylation from A. n=3; *p<0.05, **p<0.01, ***p<0.001.

5.2.4 APPL1 negatively regulates cAMP signalling of VEE-targeted GPCRs

Given that APPL1-dependent LHR recycling requires cAMP/PKA activation, I then determined whether APPL1 depletion impacts LHR-mediated cAMP signalling. Agonist-induced cAMP production was measured in cells stably expressing FLAG-LHR and transfected with either non-targeting or APPL1 siRNA. There was no effect of APPL1 knock-down on the basal levels of cAMP; however, there was an unexpected 2-fold increase in LH-induced cAMP levels in cells lacking APPL1 (Figure 5.9 A). This increase in cAMP levels was reversed upon transfection of WT APPL1 (Figure 5.9 A). Further, APPL1 depletion had a similar effect on the VEE-targeted receptors FSHR and B1AR, but did not impact ligand-induced cAMP signalling from the B2AR (Figure 5.9 B). Collectively, these results demonstrate that APPL1 has a specific role in negatively regulating cAMP production by VEE-targeted GPCRs.

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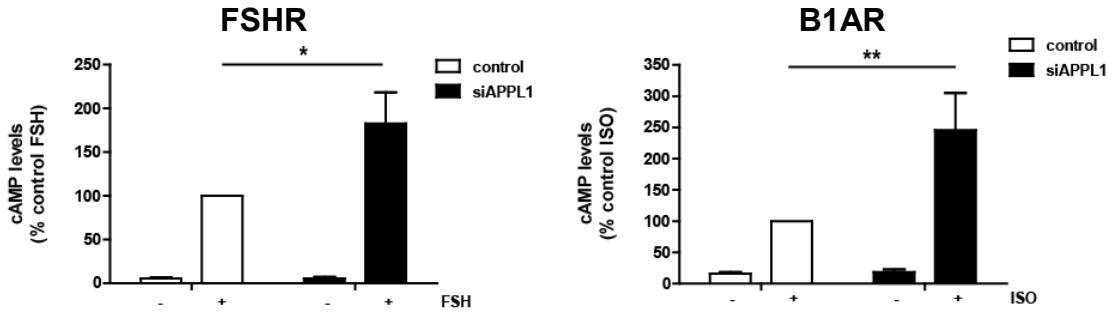


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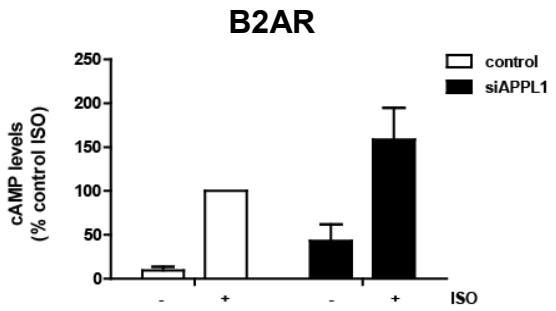
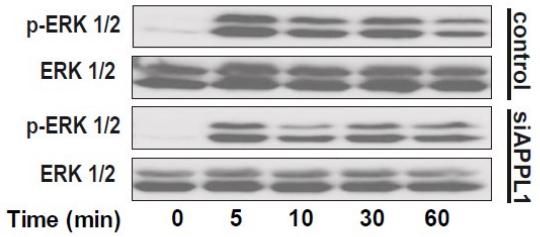


Figure 5.9 APPL1 depletion increases cAMP levels generated from VEE-, but not EE-, targeted GPCRs. (A) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR following transfection with either scramble siRNA (control), APPL1 siRNA (siAPPL1) or APPL1 siRNA and mCherry-WT APPL1 (siAPPL1 + WT). Cells were ± stimulated with LH (10 nM) for 5 min. n=3; Two-way ANOVA: ***p<0.001. (B) Intracellular levels of cAMP were measured in HEK 293 cells expressing either FLAG-FSHR, -B1AR or -B2AR following transfection with either scramble siRNA (control), APPL1 siRNA (siAPPL1). Cells were ± stimulated with FSH (10 nM, 5 min) or isoproterenol (ISO, 10 µM, 5 min). n=3, 4 or 5 independent experiments for FSHR, B1AR or B2AR, respectively; Two-way ANOVA: *p<0.05, **p<0.01.

To test whether APPL1 was involved in the regulation of another LHR pathway known to be activated also from the VEE (Jean-Alphonse et al., 2014), ERK 1/2 phosphorylation was measured in cells depleted of APPL1. ERK 1/2 was not influenced by APPL1 knock down (Figure 5.10), suggesting APPL1 role in regulating LHR signalling is specific for the $G\alpha_s$-cAMP pathway.

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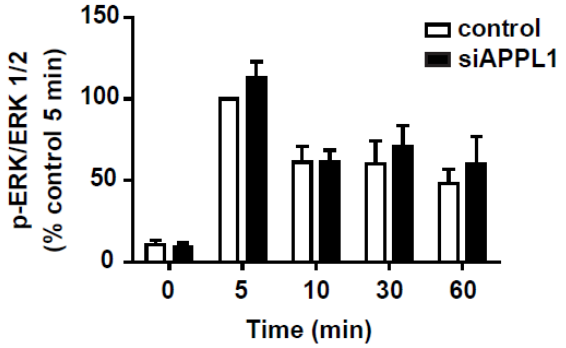
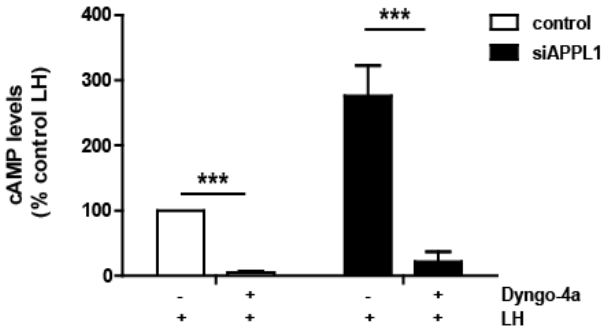


Figure 5.10 APPL1 depletion does not affect LH-induced ERK 1/2 phosphorylation. HEK 293 cells stably expressing FLAG-LHR were treated with either scramble (control) or APPL1 siRNA (siAPPL1) and phosphorylation of ERK 1/2 at stated time points after LH (10 nM) stimulation was determined by Western blotting. Total ERK was used as a loading control. A representative immunoblot is shown. (B) Densitometry analysis of ERK 1/2 phosphorylation from A was normalised to 5 min control. n=4.

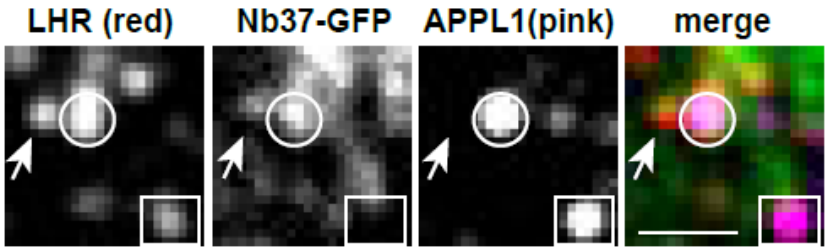
I also tested if the elevated cAMP produced in cells depleted of APPL1 was still generated from intracellular compartments. Dyngo-4a pre-treatment resulted in an almost total loss of cAMP response induced by LH, also in cells lacking APPL1 (Figure 5.11 A). This result may suggest that LHR/APPL1 endosomes may be distinct from the LHR-signalling VEEs as observed in Chapter 4, in order to exert its negative action on cAMP. To test this hypothesis, I imaged FLAG-LHR cells also expressing Nb37, which recognizes active $G\alpha_s$, with endogenous APPL1. TIR-FM was employed as LHR-Nb37 endosomes were more prevalent in the peripheral region of cells, consistent with the peripheral distribution of APPL1 (Miaczynska et al., 2004, Erdmann et al., 2007, Gan et al., 2013). TIRF-FM analysis revealed that LHR signalling endosomes, as marked by Nb37, were heterogeneous and characterised by LHR-Nb37 endosomes with and without APPL1, however, LHR-APPL1 endosomes with no Nb37 were also observed (Figure 5.11 B). Analysis of LHR-Nb37 endosomes after LH stimulation (5 and 15 minutes) revealed that ~40% of LHR-Nb37 endosomes were marked by APPL1 after 5 minutes of LH stimulation and that this number decreased to 26% after 10 minutes (Figure 5.11 C i). Interestingly, although the number of LHR-Nb37 endosomes significantly decreased with time, the total number of LHR-APPL1 endosomes remained

constant (Figure 5.11 C ii). Consequently, the proportion of LHR-APPL1 endosomes with Nb37 significantly decreased over time (Figure 5.11 C iii). These data confirm that APPL1 is also a marker of a subpopulation of LHR-Gα_s-signalling endosomes highly dynamic in the duration of their signalling activity and APPL1 residency time.

A.



B.



C.

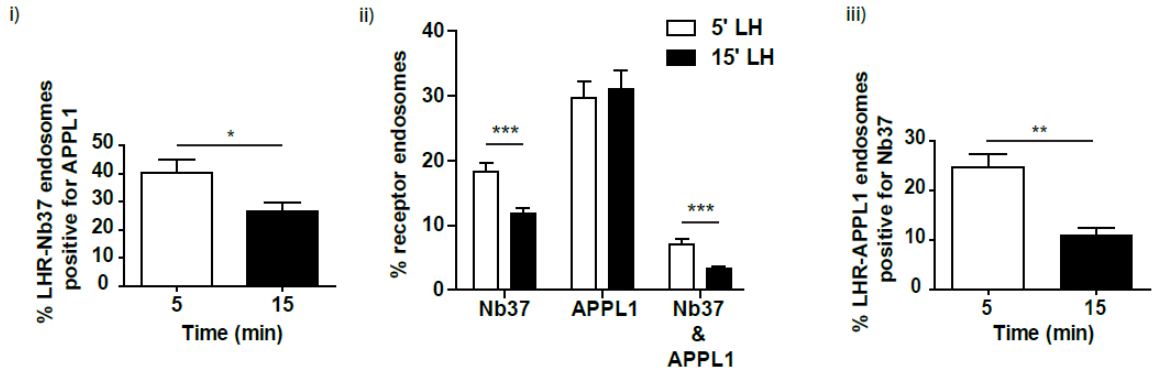


Figure 5.11 APPL1 negatively affects cAMP produced from a subpopulation of VEEs. (A) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR following transfection with either scramble siRNA (control) or APPL1 siRNA (siAPPL1). Cells were stimulated with LH (10 nM, 5 min) ± pre-treatment with Dyngo-4a (30 µM, 45 min). n=3; Two-way ANOVA:

***p<0.001. (B) Representative TIR-FM images of HEK 293 cells stably expressing FLAG-LHR, and co-expressing Nb37-GFP, after stimulation with LH. Cells were fed live with anti-FLAG M1 antibody, stimulated with LH (10 nM) for 5 or 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 antibody followed by anti-rabbit AlexaFluor647 (pink) and anti-mouse AlexaFluor568 (red) antibodies labelling. Arrow indicates LHR endosome positive for Nb37 only, circle indicates LHR endosome positive for Nb37 and APPL1, and square indicates LHR endosome positive for APPL1 only. Scale bar=1 µm. (C) Quantification of LHR-Nb37 endosomes positive for APPL1 (i), LHR endosomes positive for either Nb37, APPL1 or Nb37 and APPL1 (ii) and LHR-APPL1 endosomes positive for Nb37 (iii) and n=15 cells per condition from B were quantitated across 3 independent experiments; t-test: *p<0.05, **p<0.01, ***p<0.001.

5.2.5 The role of G proteins in APPL1 negative regulation of LH-stimulated cAMP

APPL1 could exert its negative action on LH-stimulated cAMP at different levels. I first assessed if the number of cell surface receptors was increased in cells depleted of APPL1, but there was no significant change in surface levels of LHR (Figure 5.12).

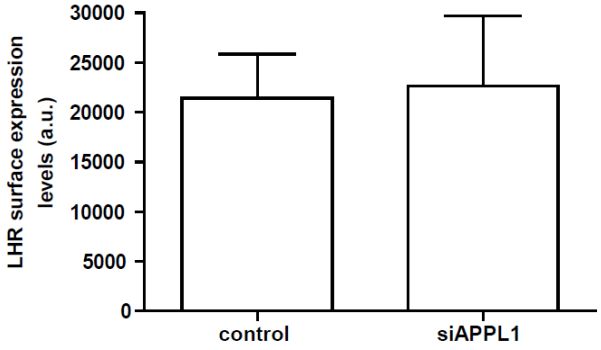


Figure 5.12 APPL1 depletion does not affect LHR surface expression levels. HEK 293 cells expressing FLAG-LHR treated with either scrambled siRNA (control) or APPL1 siRNA (siAPPL1) were incubated with FLAG-antibody to label the surface receptors and analyzed by flow cytometry. Data is expressed in arbitrary unit (a.u.) values calculated from the % cells gated x mean fluorescence. Mean ± SEM, n=4.

I then sought if APPL1 was regulating cAMP production at the level of G protein. I reasoned that APPL1 could either impact on $G\alpha_s$ activation, possibly by altering Gβγ release, and/or interact with G protein subunits, preventing their interaction with downstream effectors (e.g. ACs). To test these hypotheses, I utilised Bioluminescence Resonance Energy Transfer (BRET), a biophysical technique which allows the study of protein-protein interaction in live intact cells (described in Methods, paragraph 2.2.8). If the two proteins of interest interact, a signal emitted by the energy acceptor is registered, as the BRET donor and acceptor (the proteins of interest have been previously tagged with) are close enough for energy transfer to take place. I first looked at the effect of altering cellular levels of APPL1 on $G\alpha_s$-Gγ association, in both unstimulated and LH-treated FLAG-LHR cells. As expected, a specific BRET signal was observed (indicated by the saturation at increasing levels of acceptor protein) when cells transfected with $G\alpha_s$-Rluc8 and Gγ-Venus were analysed (Figure 5.13). The maximal BRET signal increased when APPL1 was overexpressed yet decreased when APPL1 was knocked-down (Figure 5.13). Due to the high variability of the BRET values across different experiments and that LH stimulation did not correspond to a change in BRET signal, $BRET_{max}$ and $BRET_{50}$, which indicate the distance and the affinity between energy donor and acceptor molecules, respectively (Mercier et al., 2002), were not calculated.

Considering the possible implication of APPL1 in regulating G protein activation, I tested the hypothesis that APPL1 could directly interact with either $G\alpha_s$ or Gγ subunit. Also in this case, both the BRET values and the BRET profiles were not consistent across experiments in both net BRET values and the ability to produce a saturation curve indicative of a specific interaction, thus unsuitable for quantitative analysis (Figure 5.14).

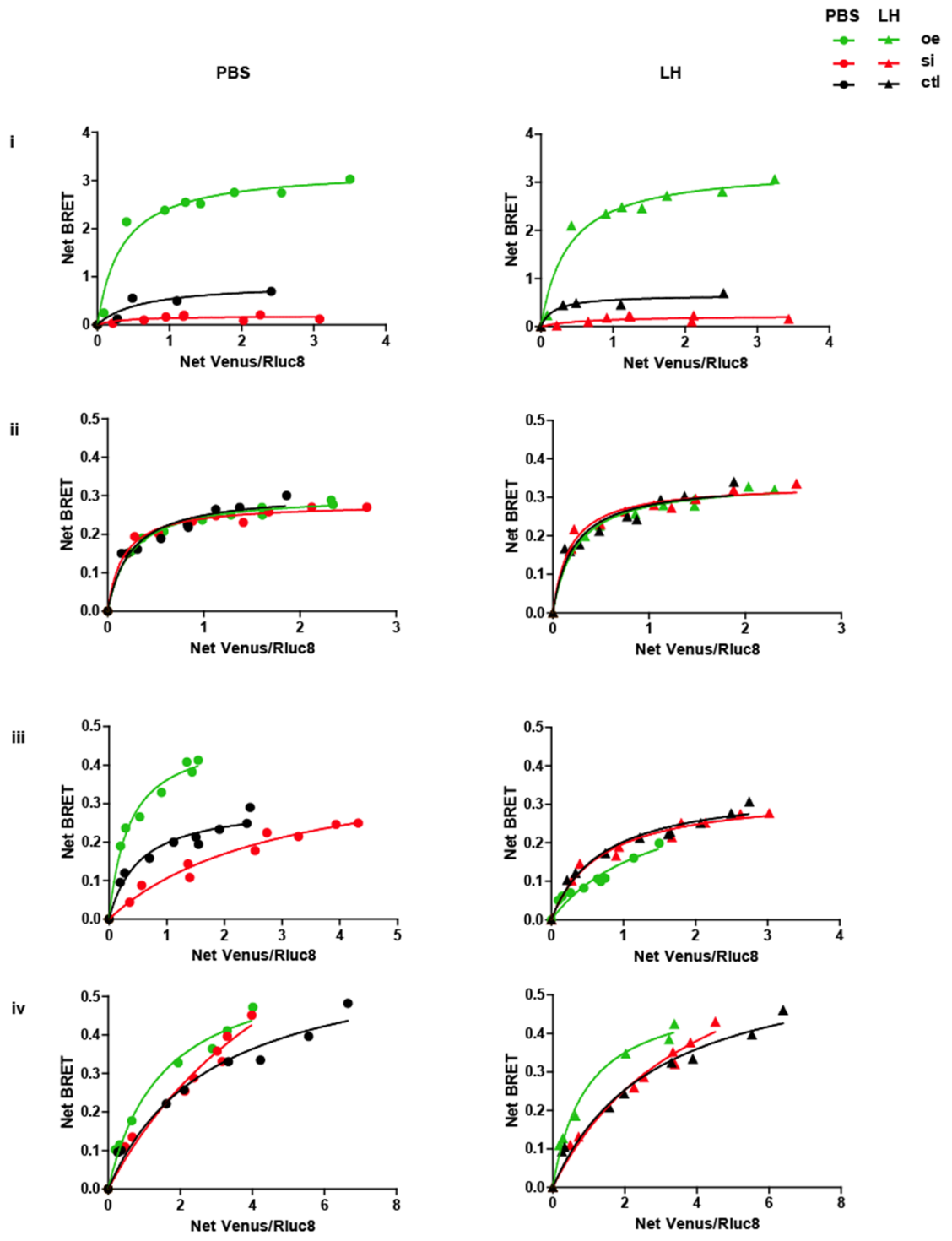


Figure 5.13 APPL1 levels may affect Gα$_s$-Gγ association. HEK 293 cells expressing FLAG-LHR were transfected with the BRET donor Gα$_s$-Rluc8, the BRET acceptor Gγ-Venus and untagged Gβ to keep the amount of G protein subunits in the cell constant, and either with mock (ctl), APPL1 siRNA (si) or mCherry-WT APPL1 (OE). A basal BRET measurement was taken right after coelenterazine-h addition (5 µM), then PBS (black) or LH (10 nM) (red) were added and measurements were taken immediately. Four different experiments are shown (i-iv).

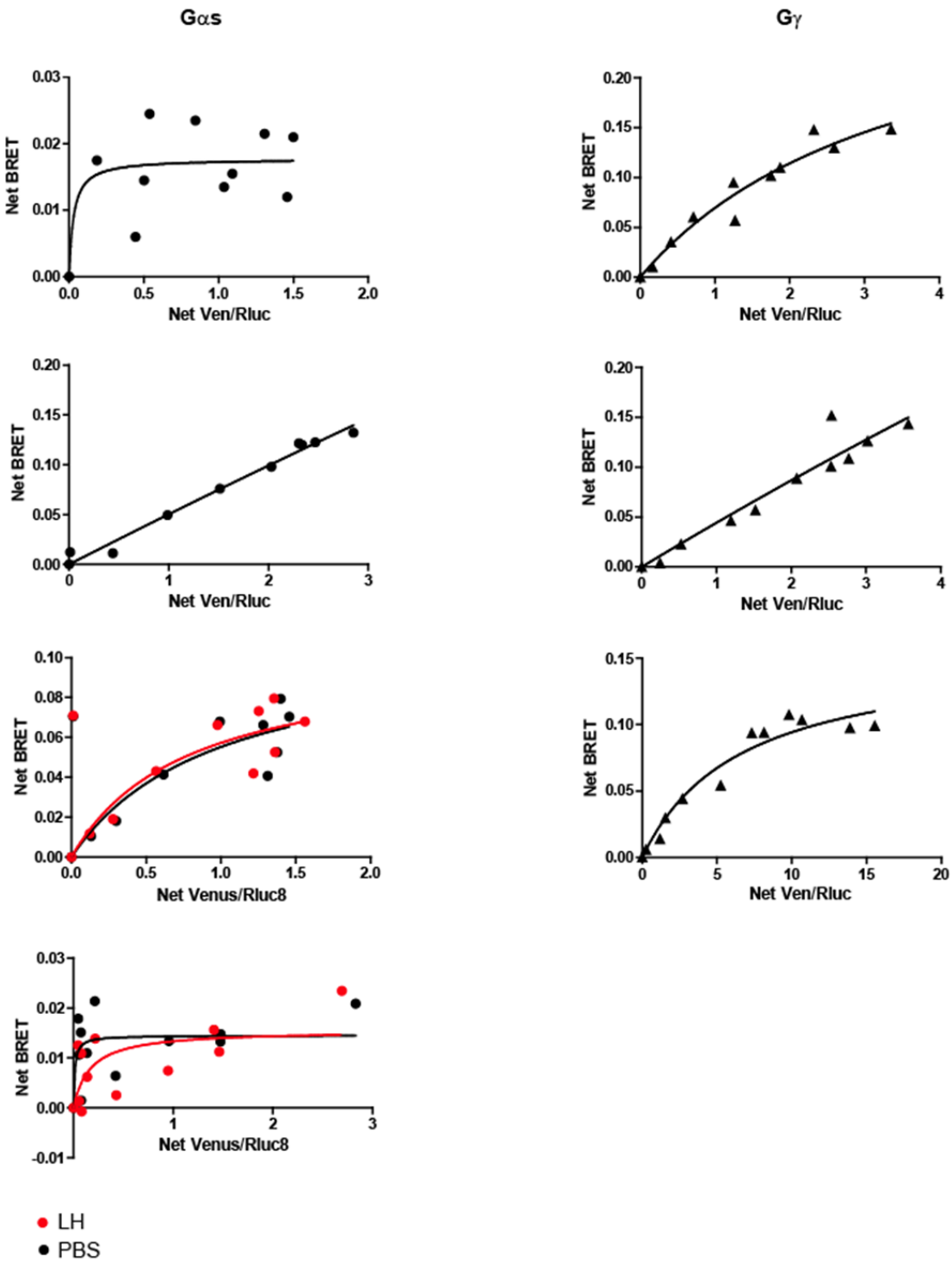


Figure 5.14 APPL1 may not directly interact with Gγ or Gα$_s$ subunit. HEK 293 cells expressing FLAG-LHR and transfected with the BRET donor APPL1-Rluc8, the BRET acceptor Gαs- (left column, circles) or Gγ-Venus (right column, triangles), untagged Gγ or Gαs and untagged Gβ. A basal BRET measurement was taken right after coelenterazine-h addition (5 µM), then PBS (black) or LH (10 nM) (red) were added and measurements were taken immediately. Four and three different experiments are shown for Gα$_s$ and Gγ, respectively.

5.2.6 The phospho-status of APPL1 regulates LH-dependent cAMP signalling

APPL1 is essential for LHR recycling from the VEE via a mechanism that involves LHR-dependent cAMP/PKA activation and phosphorylation of APPL1 on S410. In turn, APPL1 negatively regulates LHR endosomal signalling. Consequentely, I examined if PKA activation and APPL1 phosphorylation attenuated endosomal cAMP signalling. Intracellular cAMP levels were measured in FLAG-LHR cells pre-treated with the PKA inhibitor KT5720. In contrast to the increase in agonist-induced cAMP signalling induced by loss of APPL1, cells pre-treated with KT5720 showed a partial but significant reduction in the levels of LH-stimulated cAMP production when compared to DMSO-treated cells (Figure 5.15 A), despite retention of LHR in APPL1-positive VEEs (Figure 4.9). This suggests that PKA activity may be a key determinant in the ability of APPL1 to negatively regulate LH-induced cAMP signalling. Therefore, I determined whether the phosphorylation status of APPL1 regulates LHR-dependent cAMP signalling expressing either mCherry-WT, phospho-mimetic (S/D) or phospho-deficient (S/A) APPL1 in FLAG-LHR cells. LH-induced increase in cAMP was not significantly different between cells transfected with or without WT APPL1 (WT APPL1 110.50 $\pm$ 6.65% compared to untransfected cells, p=0.165) (Figure 5.15 B). A small but significant decrease in cAMP was observed in cells expressing the phospho-deficient S/A APPL1 compared to cells expressing WT APPL1, similarly to the effect on LH-induced cAMP following PKA inhibition with KT5720 (Figure 5.15 A-B). On the contrary, in cells expressing phospho-mimetic S/D APPL1, LH-induced cAMP was increased by ~30% (Figure 5.15 B). These data support a mechanism whereby phosphorylation of APPL1 at S410 negates repression of LH-induced cAMP signalling.

A. B.

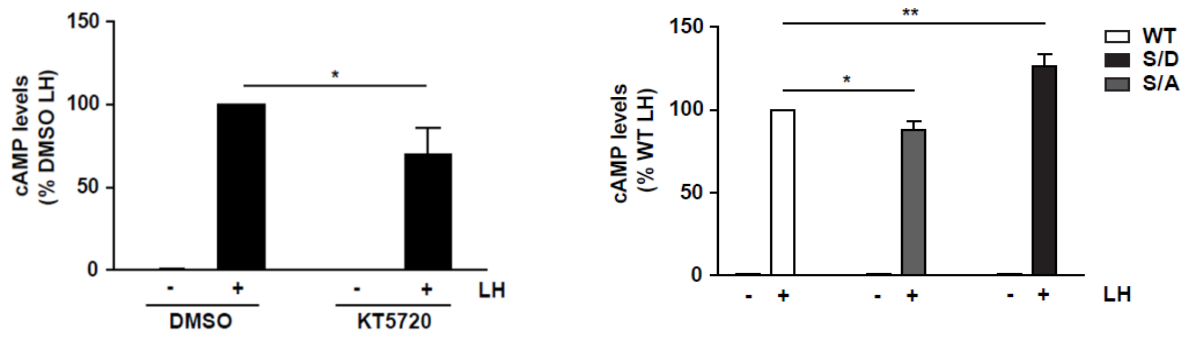


Figure 5.15 LH-induced cAMP is affected by PKA inhibition and phosphorylation status of S410 of APPL1. (A) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR following stimulation with LH (10 nM, 5 min) following pre-treatment with either DMSO or KT5720 (15 min), n=3; Two-way ANOVA: *p<0.05. (B) Intracellular levels of cAMP were measured in HEK 293 cells stably expressing FLAG-LHR following transfection with mCherry-WT (WT), -S410A (S/A) or -S410D (S/D) APPL1. Cells were ± stimulated with LH (10 nM, 5 min). n=4; Two-way ANOVA: *p<0.05, **p<0.005.

5.2.7 OCRL as possible protein partner of APPL1 in the regulation of LHR functions

The finding that the phosphorylation of APPL1 on S410 acts as a molecular switch for the regulation of both LHR recycling and cAMP signalling, combined with prior reports showing that phospho status of S410 of APPL1 dictactes its interaction with the phosphatase OCRL (Erdmann et al., 2007), led to a preliminary investigation of a possible role for OCRL in regulating LHR sorting and/or signalling fates.

OCRL is the most efficient of the ten PstIns-5 phosphatases present in eukaryotes in removing the phosphate in position 5 of PstIns-4,5-P2, Ins-1,4,5-P3 and, to a less extent, PstIns-3,4,5-P3 (De Matteis et al., 2017). In addition to its phosphatase catalytic domain, it is composed by various protein- and membrane-interacting domains; these dictate its cellular localisation (plasma membrane, clathrin-coated pits and vesicles, early endosomes, lysosomes, TGN and the primary cilium) and its protein partners (including components of the cytoskeleton, AP-2, clathrin, Rabs, SNX and APPL1) (De Matteis et al., 2017). For these

reasons, OCRL is one of the major regulators of PstIns balance along the endosomal pathway and, due to its ubiquitous expression, the physiological functions it mediates vary according to the cell type and proteins and cellular compartments it associates with (Mao et al., 2009, Choudhury et al., 2005). Mutations in OCRL have been identified in patients suffering from Lowe syndrome and Dent disease 2, both characterised by renal Fanconi syndrome and, for the more severe Lowe syndrome, by congenital cataracts, central hypotonia and cognitive disability (Richards et al., 1965, Attree et al., 1992, Hoopes et al., 2005). All mutations reported so far have detrimental effects on OCRL catalytic activity, leading to accumulation of its major substrate PstIns-4,5-P2 (Hichri et al., 2011).

To assess the role of OCRL on LHR function, I attempted to knock down endogenous OCRL or increase its cellular levels by overexpressing mCherry-OCRL. I first optimized the knock down of OCRL in HEK 293 cells (Figure 5.16). Due to technical issues in detecting OCRL via Western blot and optimisation of siRNA-mediated OCRL knock down, I was unable to test its effects on LHR function.

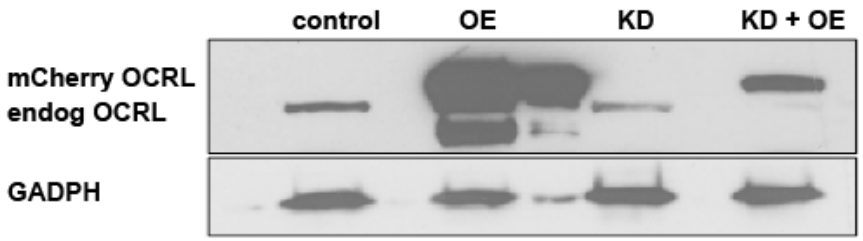


Figure 5.16 siRNA-mediated depletion of OCRL in HEK 293 cells. Representative Western blot of total cellular levels of OCRL in lysates collected from HEK 293 cells following mock (control), mCherry-OCRL (OE), OCRL-directed siRNA (KD) or mCherry-OCRL and OCRL-directed siRNA (KD+OE) transfection. GAPDH was used as a loading control.

The effect of mCherry-OCRL expression on SEP-LHR recycling via TIR-FM was assessed. There were no significant differences in the number of LHR recycling events between cells with endogenous or elevated levels of OCRL, similarly to the effect of APPL1 overpression (Figure 5.17). However, preliminary data indicate that a population of LHR endosomes were also

positive for mCherry-OCRL (Figure 5.18), suggesting OCRL might play a role in LHR functions at the VEE.

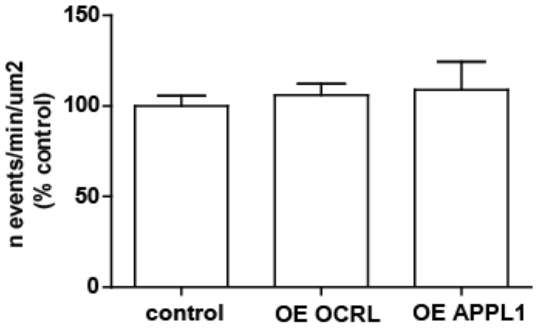


Figure 5.17 Overexpression of either OCRL or APPL1 does not impact LHR recycling. HEK 293 cells expressing either SEP-LHR and transiently transfected with either mCherry-OCRL (OE OCRL) or mCherry-APPL1 (OE APPL1) were stimulated with LH (10 nM) and recycling was measured in real time, via TIR-FM, in different cells from 5 min after ligand addition. n=16 cells per condition for control and OE APPL1, 6 for OE OCRL.

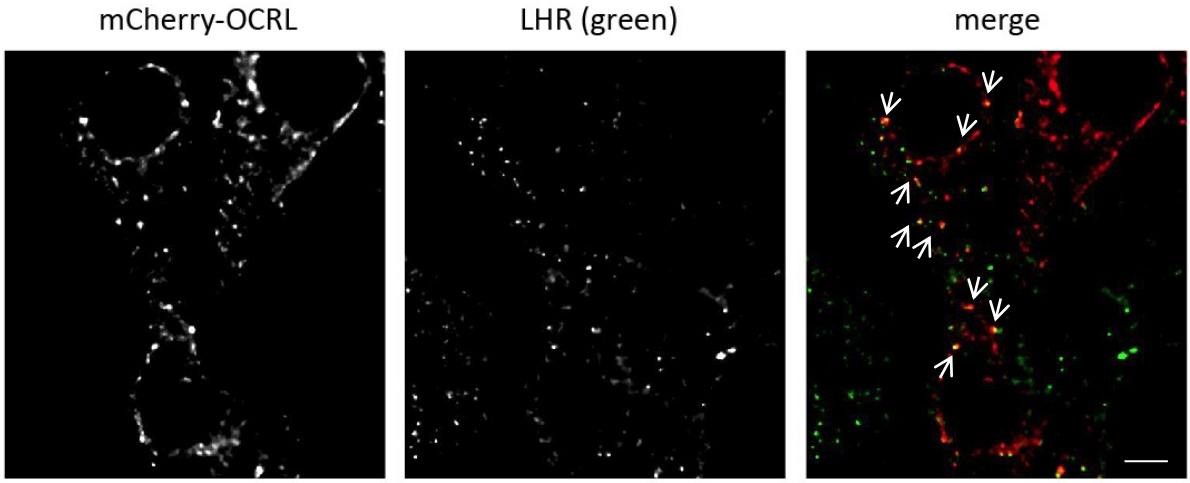


Figure 5.18 LHR endosomes are partially marked by mCherry-OCRL. Representative wide-field images of fixed HEK 293 cells stably expressing FLAG-LHR and transiently expressing mCherry-OCRL, after stimulation with LH for 15 min. LHR was stained using anti-FLAG M1 antibody followed by AlexaFluor488 labelling. Arrows indicate structures which are positive for both LHR and OCRL. Scale bar= 5 µm.

5.2.8 Searching for additional markers of the VEE

In the attempt to identify the molecular and biochemical composition of LHR endosomes, extensive research has been conducted by previous members of our laboratory aimed to identify endosomal proteins and membrane phospholipids markers of the VEE.

In regard to the phospholipids, most of the available phosphoinositide-specific biosensors were tested, including 3xFYVE domain for PI3P and PH domain of AKT and PLC for PIP2, without achieving satisfactory results. Given the importance of phosphoinositides in shaping membrane identity, in addition to signal transduction and cytoskeleton regulation, the increased demand for more biosensors led to the generation of new compounds in the recent years. One of those, P4M, a new PI4P probe, has been shown to recognize pools of this phosphoinositide other than the Golgi pool (Hammond et al., 2014). I took advantage of this biosensor to assess if PI4P could reside in the VE endomembrane. Following transfection with iRFP-P4M, cells were labelled for LHR, then stimulated with LH and immediately imaged live via confocal microscopy. When LHR endosomes started to generate, I noticed they did not co-localise with P4M even after several minutes after LH addition and, although P4M was marking some intracellular vesicles, they did not correspond to LHR endosomes (Figure 5.19).

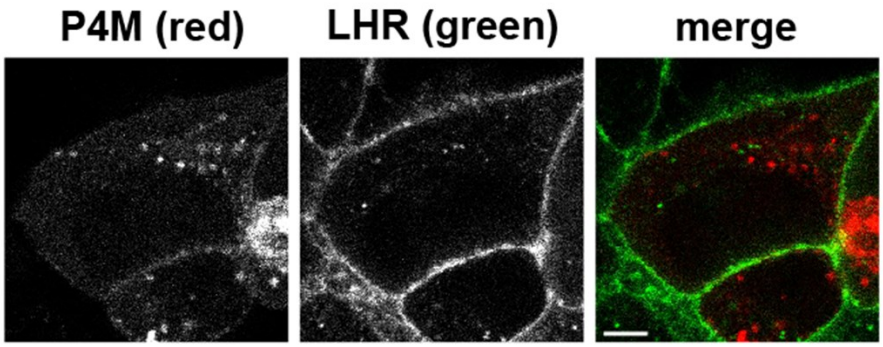


Figure 5.19 LHR endosomes are not composed by PI4P. Representative confocal images of live HEK 293 cells stably expressing FLAG-LHR and transiently expressing the PI4P biosensor P4M, after stimulation with LH.

In addition to phosphoinositides, another class of molecules which greatly dictate the characteristic structure, biochemical composition and function of endosomal vesicles are the Rab members of the Ras GTPase superfamily (detailed in Introduction, paragraph 1.3.3). These proteins are recruited to endomembranes through hydrophobic moieties and, once bound to the organelle, can bind a variety of effectors in a GTP-dependent manner (Rink et al., 2005). Effectors include cellular motors, enzymes and adaptor proteins which constitute function-specific microdomains within an endosome and determine the intracellular fate of endosomally localised cargos through budding, tethering and fusion of vesicles (Grosshans et al., 2006). In regard to GPCRs, a number of Rabs have been identified for their presence on the membrane of endosomes designated for specific trafficking routes; the most known are Rab5 marker of EE, Rab7 which replaces Rab5 during the maturation to late endosomes and Rab11 and Rab4 signatures of slow and fast recycling pathways, respectively (Esseltine and Ferguson, 2013). In our hands, none of these co-localised with LHR at VEEs ((Jean-Alphonse et al., 2014) and unpublished observations from Dr. Jean-Alphonse). Considering that the VEE serves as sorting station for LHR to recycle back to the plasma membrane and the presence of APPL1 at this compartment, I sought other Rabs that were known to have a similar role to Rab11 or Rab4 and/or to interact with APPL1, that were not investigated before in our laboratory. I focused my attention on two members of the Rab family: Rab35, which has been found on endosomes at the cell periphery and has been proposed as mediator of fast recycling (Cauvin et al., 2016, Kobayashi et al., 2014), and Rab31, which has been shown to interact with the APPL1 isoform APPL2 (Rodriguez-Gabin et al., 2010). FLAG-LHR cells transiently expressing Rab35- or Rab31-GFP were stained for LHR, stimulated with LH and fixed. Visual inspection of TIR-FM images of these cells revealed that some LHR endosomes were marked by Rab35 or Rab31, with Rab31-LHR co-localisation being qualitatively higher than Rab35-LHR (Figure 5.20). Although these are just representative images of n=1 cell per condition, preliminary results might suggest Rab31 as a VEE marker to pursue for future and further analysis.

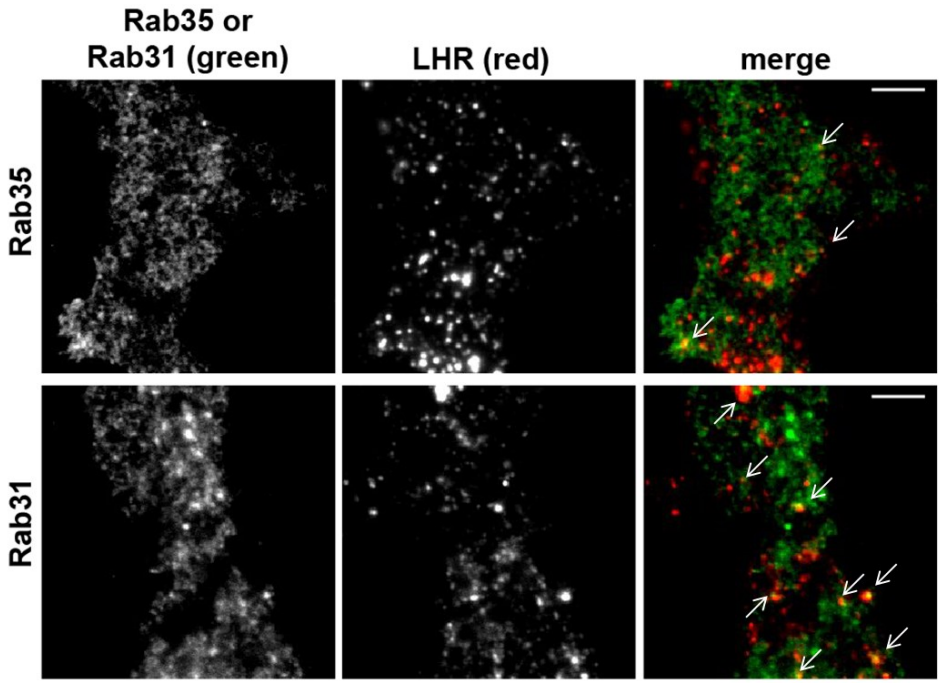


Figure 5.20 LHR endosomes are partially marked by Rab31 but not Rab35. Representative TIR-FM images of fixed HEK 293 cells stably expressing FLAG-LHR and transiently expressing Rab35- or Rab31-GFP, after stimulation with LH. LHR was stained using anti-FLAG M1 antibody followed by AlexaFluor555 labelling. Scale bar= 5 µm.

5.3 Discussion

In this chapter, I propose a central role for APPL1 in directing the sorting and G protein signalling of LHR from the VEE. Even though APPL1 has been implicated in the trafficking and signalling of a variety of membrane receptors including FSHR (Nechamen et al., 2007, Nechamen et al., 2004), here I present two unprecedented functions for APPL1. First, APPL1 is essential for rapid GPCR recycling from VEEs to the plasma membrane. Second, APPL1 functions as a negative regulator of GPCR-mediated cAMP production from VEEs. The fact that APPL1 lacks enzymatic activity and that it binds to a variety of proteins including Rabs, GPCRs, kinases and phosphatases, suggests that APPL1 could serve as a scaffold protein which, in response to LH stimulation, could constitute 'megaplexes' that mediate rapid LHR trafficking and signalling. This assembly of GPCR, regulatory protein and signalling effectors has been already shown to be mediated by β-arrestin (Thomsen et al., 2016), but it is highly probable that different GPCRs, which do not utilize arrestins for their intracellular

prolongation of signalling, and traffick to diverse endosomal compartments, could exploit other adaptor proteins, like APPL1.

Despite the differential endosomal fate of VEE- and EE-targeted GPCRs and their opposite requirement for APPL1, recycling of both LHR and B2AR is regulated via the activation of their $G\alpha_s$-cAMP-PKA pathway. Importantly, it is not just the increase of cAMP that dictates GPCR recycling, but also its spatial confinement in microdomains (Calebiro and Maiellaro, 2014), as proven by the fact that LH-induced cAMP increase due to PDE4 inhibition does not elevate LHR recycling rates. The wide range of PDEs available could be used by different GPCRs at distinct cellular locations, to generate a spectrum of cAMP gradients. cAMP/PKA activation further diversifies the mechanisms that regulate receptor activity by exerting opposite functions, for example with LHR and B2AR, by phosphorylating distinct targets; sequence-dependent recycling of B2AR is negatively regulated via PKA phosphorylation of the receptor C-tail (Yudowski et al., 2009a, Vistein and Puthenveedu, 2013), whilst the data presented in this chapter show that LHR recycling requires PKA phosphorylation of APPL1. Although APPL1 can be phosphorylated at distinct sites by different kinases (Gant-Branum et al., 2010), prior studies have demonstrated that S410 is phosphorylated specifically by PKA (Erdmann et al., 2007) and here I show that PKA phosphorylation S410 is essential to stimulate LHR recycling, as evidenced by the phospho-deficient and phospho-mimetic mutants. Furthermore, I provide evidence that LH stimulation directly determines PKA-dependent APPL1 phosphorylation, predominantly on S410, proving that LHR signalling promotes its own sorting. This is not a unidirectional relationship, as LHR trafficking to the VEE determines its signalling output, which, in turn, is negatively influenced by the unphosphorylated form of APPL1, as revealed by both PKA inhibition and employment of the APPL1 phospho-mutants. Furthermore, this is the first report that APPL1 has a role in regulating GPCR endosomal signalling, when considering that APPL1 was known as adaptor of signalling molecules contributing to the propagation of the signal (Miaczynska et al., 2004, Ryu et al., 2014, Xin et al., 2011, Zhou et al., 2009), rather than inhibition, as in this study. This negative regulation is consistent with the decreased presence over time of active LHR/$G\alpha_s$ signalling endosomes that contain APPL1 with an increased proportion of LHR/APPL1 endosomes without active $G\alpha_s$.

It appears evident that the phosphorylation status of S410 of APPL1 regulates both LHR recycling and signalling, but in an opposing manner, suggests that there could be a range of phosphorylated forms of APPL1 across different VEEs, or perhaps even across microdomains of an individual VEE, to control distinct LHR functions as the receptor traverses the VEE compartment. Indeed, microdomains of active G protein within an individual LHR endosome were captured via super-resolution imaging (Figure 4.2 C). The inactivation of endosomal signalling, prior to receptor sorting to a recycling pathway, has been shown to be a necessary step for other GPCRs (McGarvey et al., 2016, Feinstein et al., 2011), thus the role of APPL1 in negative regulation of VEE signalling is consistent with this. An alternative model is also reasonable, where both cAMP signalling and recycling take place within the same microdomain, possibly a tubule, marked by phosphorylated APPL1; the rest of the VEE is marked by unphosphorylated APPL1 and, thus, where quiescent LHR resides. Additional research is needed to determine whether different phosphorylated forms of APPL1 are present within the same endosome. Due to the restrictive size of the VEE, techniques which allow the detection of single molecule localisation, such as photo-activable localisation microscopy (PALM) or stochastic optical reconstruction microscopy (STORM), would be required.

The evidence reported in this study that S410 of APPL1 is a key residue for the coordination of LHR functions, combined with the fact that APPL1 modulates its interaction with OCRL through the phosphorylation of the same residue (Erdmann et al., 2007), leads to the speculation that OCRL might be involved in the regulation of LHR activity where S410 act as molecular switch depending on its phosphorylation status; interestingly, OCRL was observed at endosomes where LHR traffics to. To address this possibility, I generated an mCherry-tagged OCRL mutant where the tryptophan in position 739 was substituted with an alanine (W739A). This mutation has been previously shown to abolish OCRL-APPL1 interaction, akin to the effect of PKA phosphorylation on S410 of APPL1 (Pirruccello et al., 2011). I hypothesised that APPL1 mediates LHR recycling by dissociating from OCRL, via PKA phosphorylation of S410. However, due to lack of time these hypotheses remain to be tested, but delineate a possible mechanism that could explain how an adaptor protein is able to modulate GPCR activity.

Alternatively, APPL1 could exert its regulatory function directly at the level of G protein. This was investigated via BRET (Figure 5.13 and 5.14); even though the results I obtained were not consistent, it is tempting to speculate that APPL1 levels might affect $G\alpha_s$-Gγ interaction even at basal levels, since the BRET signal seemed to increase when APPL1 was overexpressed and decrease when APPL1 was knocked-down (Figure 5.13). This could be due to a physical interaction between APPL1 and Gγ; in fact, while BRET values obtained for APPL1-$G\alpha_s$ were too low to be accounted for a real interaction (Figure 5.14 left column), BRET values recorded for APPL1-Gγ were within a reasonable range and reached saturation in two experiments out of three (Figure 5.14 right column).

Apart from the elucidation of the molecular mechanisms used by APPL1 to mediate LHR recycling and signalling, a better characterisation of the VEE compartment is needed. Although we have identified ~50% of non-EEA1 endosomes LHR internalises to as APPL1-positive VEEs, we still have not been able to identify any marker for the remaining 50%. They may represent VEEs that have lost or will acquire APPL1 or, alternatively, a completely different population of LHR endosomes offering a new environment for LHR to elicit diverse functions. Attempts to determine whether LHR endosomes are recruiting APPL1, or if LHR endosomes fuse with pre-existing APPL1 vesicles via live dual colour TIR-FM movies, however, have been technically challenging, due to the highly rapid dynamics of these vesicles and the lack of appropriate tools to track and analyse these compartments.

Identifying the phospholipid composition of the VEE would also be very informative. This will not only help in defining which Rab/Rabs could tag the VEE but also allow the use of different approaches to study this compartment; for example, by selectively isolating the VEE from the rest of the cellular membranes, it may be possible to co-immunoprecipitate those proteins specifically interacting with LHR at the VEE.

Overall, in this chapter I demonstrate a strong bidirectional relationship between GPCR signalling and endocytic trafficking, as well as unprecedented roles for APPL1 in orchestrating GPCR function. I also started to elucidate the molecular mechanisms that finely tune these processes; further study aimed at the identification of APPL1 interacting

proteins and alternative markers of the VEE are necessary to better understand how LHR action is modulated.

Chapter 6:

The role of APPL1 in spatial regulation of LHR activity

in human endometrial stromal cells

6.1 Introduction

Understanding spatial control of GPCR signalling has opened novel therapeutic avenues to create compounds that target specific signalling pathways, and has provided new insight in to diseases with known perturbed receptor activity. With a growing demand to design drugs with pathway selectivity, the ability to pharmacologically reprogram signalling at a spatial level is a fruitful area to identify compounds with improved specificity and fewer side effects.

To address this for LHR, it was critical to demonstrate the role of APPL1 in trafficking and signalling in a cell type that endogenously expresses this receptor. Given rodent and human LHRs differ in their post-endocytic sorting fate (Kishi et al., 2001, Galet et al., 2004), primary human cells were important. LHR is classically expressed in testicular Leydig cells and ovarian theca and granulosa cells, however, it is not possible to obtain the former two and, although accessible and routinely cultured, granulosa cells are not readily manipulated by transfection (personal communication Dr. Lisa Owens and Prof. Steve Franks). As TIR-FM imaging requires use of SEP-tagged receptors, I opted for primary human endometrial cells (hESCs). In addition to the gonads, LHR is also expressed in extra-gonadal, but reproduction-associated, tissues, such as breast, cervix, oviduct and uterus (Ziecik et al., 2005). In the uterus, LHR is expressed in both myometrium of pigs and humans, where it stimulates cell growth and relaxation of the musculature, and endometrium of baboons, mice and humans, where it induces morphological and functional changes of stromal cells (decidualisation) and increases the receptivity of this tissue to embryo implantation (Ziecik et al., 1986, Reshef et al., 1990, Ziecik et al., 1992, Ziecik et al., 2005).

Prior studies from our group conducted in hESCs recapitulated the involvement of GIPC and the LHR-C-tail in LHR trafficking and signalling previously demonstrated in HEK 293 cells (Jean-Alphonse et al., 2014, West, 2016). Therefore, I used hESCs to determine the role of APPL1 on LHR recycling from the VEE and cAMP signalling in a physiologically relevant context.

Both LHR and APPL1 have been previously, though independently, implicated in PCOS (detailed in Introduction, paragraph 1.5.4). Increased levels of LHR mRNA and higher

responsiveness to LH have been indentified in PCOS patients (Jakimiuk et al., 2001; Willis et al, 1998) as well as insulin and adiponectin signalling abnormalities have been reported in endometrial stroma of PCOS patients (Pasquali et al., 2002, Dehghan et al., 2016). Furthermore, APPL1 has been proven to be the primary regulator in determining adiponectin-driven insulin sensitivity with conflicting reports on altered APPL1 levels in the endometrium of PCOS individuals (Garcia et al., 2015).

I tested the hypothesis that LHR recycling and/or LH-induced cAMP production could be altered in the endometrium from women with PCOS compared to non-PCOS, due to incorrect LHR trafficking to the VEE or to reduced APPL1 levels.

6.2 Results

6.2.1 APPL1 role is recapitulated in hESCs

First, to verify that LHR internalised into APPL1-positive endosomes in hESCs, I measured the level of co-localisation between endogenous APPL1 and FLAG-LHR. After stimulation with LH, 32.33 ± 1.04% of LHR endosomes were also positive for APPL1 (Figure 6.1), similarly to what I observed in HEK 293 cells (Figure 5.1).

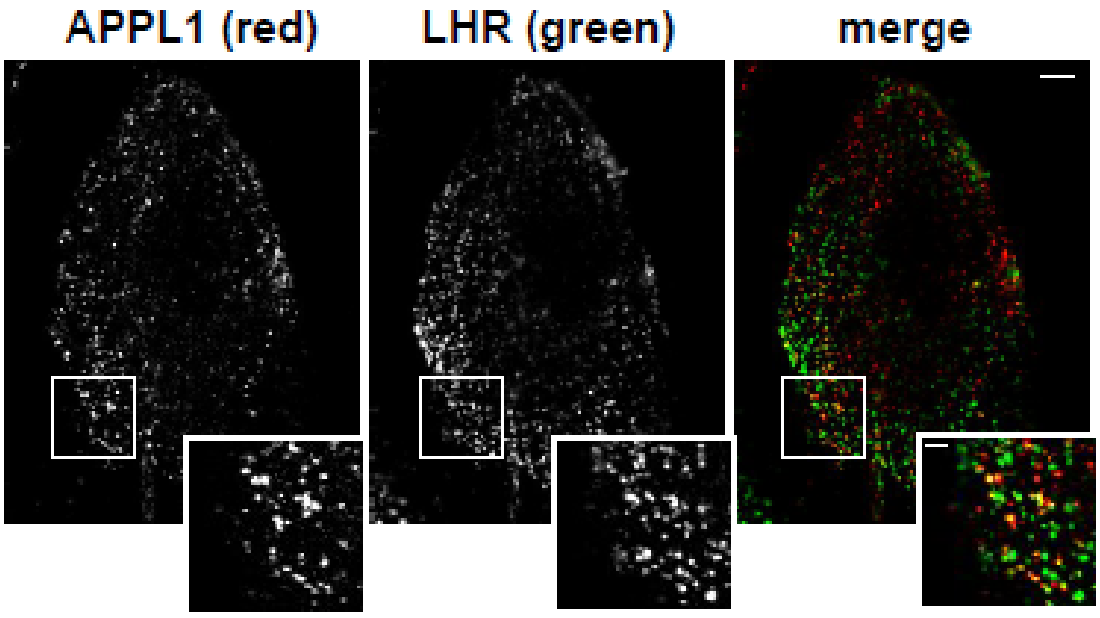
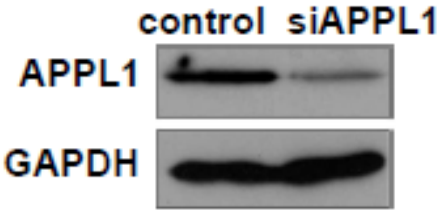


Figure 6.1 LHR internalises to APPL1-positive endosomes in hESCs. Representative confocal images showing LHR endosomes co-localising with endogenous APPL1 in primary hESCs. Cells transfected with FLAG-LHR were fed live anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 antibody followed by anti-rabbit AlexaFluor555 and anti-mouse AlexaFluor488 antibodies labelling, and imaged with confocal microscopy. Scale bar=5 µm, inset=1 µm.

With the same approach used to study the effects of APPL1 on LHR trafficking and signalling in HEK 293 cells, hESCs were depleted of endogenous APPL1 via transfection with siRNA targeting APPL1 (siAPPL1), and levels of APPL1 protein were measured by Western blot. Although not as efficient as in HEK 293 cells, APPL1 levels were reduced in hESCs treated with siAPPL1 compared to cells treated with non-targeting siRNA (control) (Figure 6.2 A). I then measured the number of LHR puffs in hESCs expressing SEP-LHR, via TIR-FM, to assess the impact of APPL1 knock down on LHR recycling as in HEK 293 cells. Strikingly, the recycling of LHR was reduced by 70% in APPL1 depleted cells (Figure 6.2 B).

A.



B.

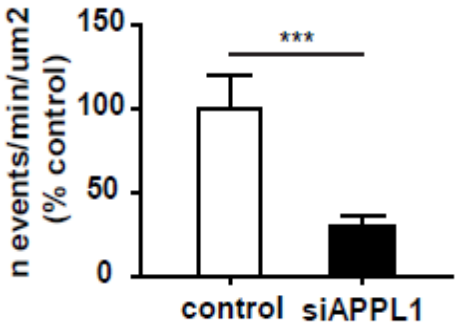


Figure 6.2 Depletion of APPL1 impairs SEP-LHR recycling in hESCs. (A) Representative Western blot of total cellular levels of APPL1 from hESC lysates following transfection with scramble (control) or APPL1 siRNA (siAPPL1). GAPDH was used as loading control. (B) SEP-LHR recycling in hESCs following siRNA-mediated knock-down of APPL1 was analysed in real time by TIR-FM after LH (10 nM) addition. n=29 cells per condition collected across 3 independent experiments; t-test: ***p<0.001.

Together with its requirement for LHR recycling from the VEE, I have demonstrated that APPL1 negatively regulates LH-induced cAMP production (described in Chapter 5). To verify if this additional role of APPL1 was recapitulated in hESCs, I measured cAMP levels in hESCs with endogenous or decreased levels of APPL1. Stimulation of hESCs with LH did not significantly increase cAMP levels compared to basal in both control or siAPPL1 treated cells (Figure 6.3 A). To exclude the possibility that LH was failing to induce a $G\alpha_s$/cAMP response, or potentially activating a $G\alpha_i$ pathway, I combined LH with Forskolin, which elevates cAMP by direct activation of AC. When cells were treated with LH in conjunction with Forskolin, cAMP levels were higher than those registered for Forskolin treatment alone, but not significantly. Moreover, APPL1 depletion did not cause any significant change in any of these treatments (Figure 6.3 A). Although expression of LHR in the endometrium has been previously reported (Kundu et al., 2012, Viswanath et al., 2007, Srisuparp et al., 2003), I hypothesised that the levels of LHR at the plasma membrane could be too low, and/or LHR expressing cells represent a subpopulation of stromal cells, to generate a detectable LH response. Therefore, hESCs were transfected with FLAG-LHR and cAMP levels were measured. The elevated LHR levels resulted in detectable levels of cAMP following both LH and Forskolin plus LH treatment. Interestingly, the LH-induced cAMP response was significantly higher in cells depleted of APPL1 (Figure 6.3 B), recapitulating the finding in HEK 293 cells (Figure 5.9 A).

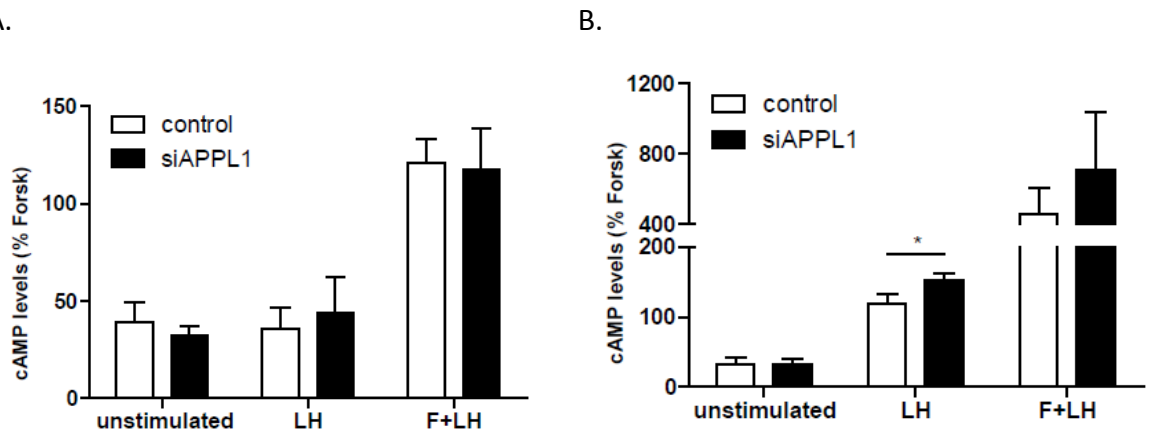


Figure 6.3 APPL1 depletion determines an increase in LH-stimulated cAMP response in hESCs expressing FLAG-LHR. Intracellular cAMP levels were measured in hESCs following tranfection with either scramble (control) or APPL1 (siAPPL1) siRNA. Cells were transfected without (A) or with (B)

FLAG-LHR, pre-treated with the PDEs inhibitor IBMX (0.5 mM, 5 min) and ± stimulated with LH (10 nM), Forskolin (F, 3 μM) or Forskolin plus LH (F+LH) for 5 min. n=4 independent experiments. Two-way ANOVA: *p<0.05.

6.2.2 Assessment of APPL1 protein levels and co-localisation with LHR in hESCs from PCOS patients

To test the hypothesis that APPL1 levels could be altered in PCOS samples I conducted a Western blot analysis of lysates from hESCs from PCOS, non-PCOS and non-PCOS depleted of APPL1, with the latter serving as negative control for APPL1 expression levels. Preliminary data indicated there may be no difference in APPL1 levels between the PCOS and non-PCOS groups (Figure 6.4).

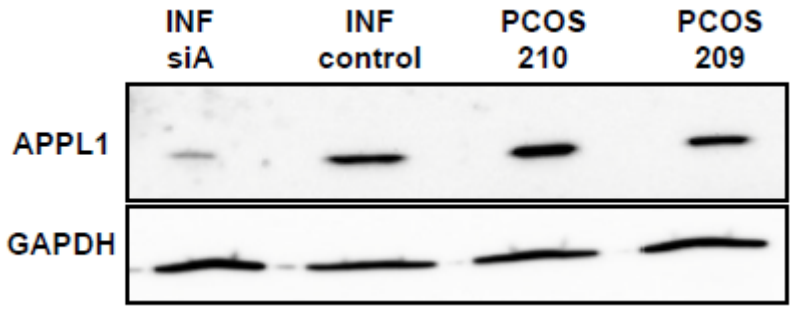
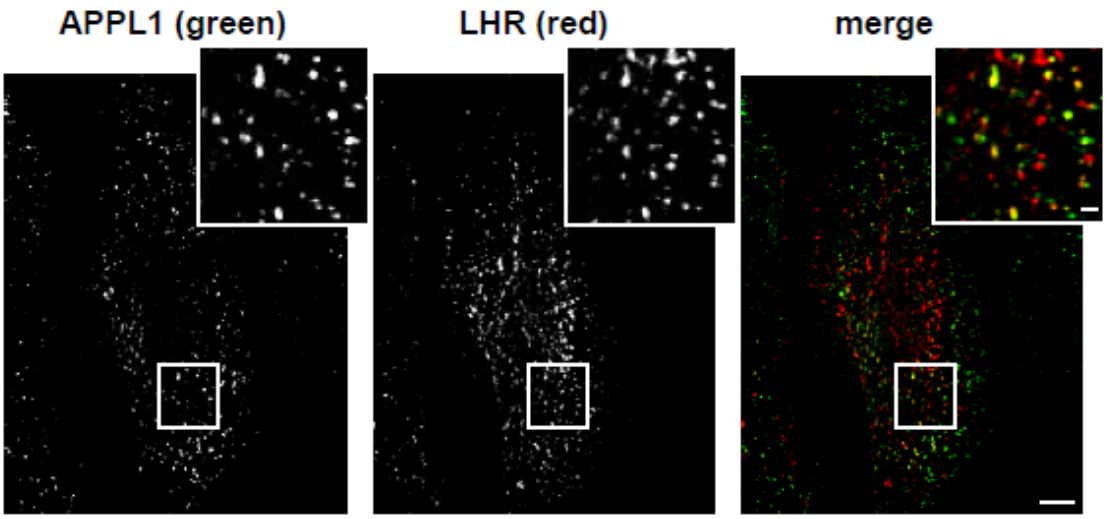


Figure 6.4 hESCs from PCOS and non-PCOS samples exhibit similar levels of APPL1. Representative Western blot of total cellular levels of APPL1 from hESC lysates from non-PCOS (INF) and PCOS samples. APPL1 levels in non-PCOS hESCs treated with APPL1 siRNA (INF siA) are shown as reference for reduced levels of APPL1. GAPDH was used as loading control.

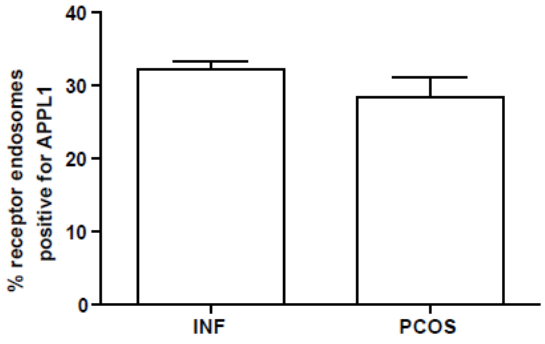
The fact that APPL1 expression levels were unchanged could not exclude the possibility that the amount of receptor trafficking to APPL1-positive VEE was unperturbed. Thus, I measured the levels of co-localisation between LHR and APPL1 in three different PCOS samples and compared to three non-PCOS ones. Qualitative analysis indicated that APPL1 in PCOS samples retained its endosomal localisation as in non-PCOS cells (Figure 6.5 A). The number of LHR endosomes co-localising with APPL1 was then counted; the pooled values did not show any significant difference between the two groups (32.33 ± 1.04% INF vs 28.39

± 2.71% PCOS, p-value=0.186) (Figure 6.5 B), suggesting similar LHR-APPL1 co-localisation levels between the two groups. However, analysis of individual PCOS patients indicated more heterogeneity in co-localisation levels than the control group (Fig 6.5 C), highlighting the need of more samples to be analysed to draw firmer conclusions.

A.



B.



C.

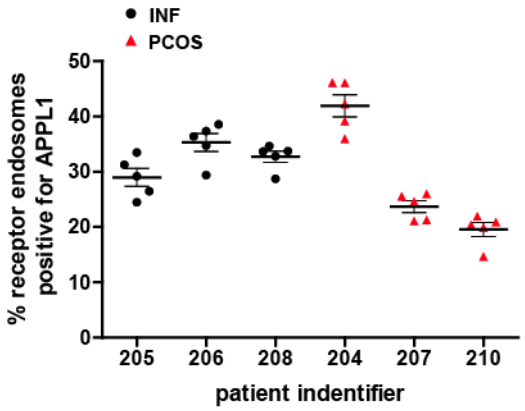


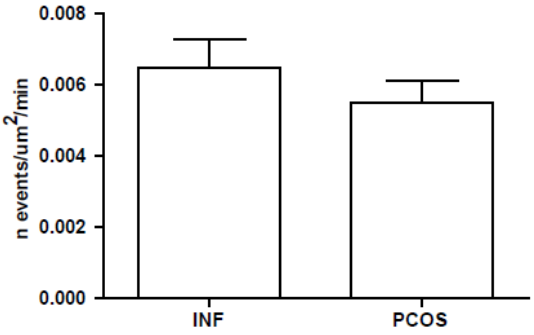
Figure 6.5 LHR internalises to APPL1-positive endosomes in hESCs from PCOS samples. (A) Representative confocal images showing LHR endosomes co-localising with endogenous APPL1 in primary hESCs from PCOS patients. Cells transfected with FLAG-LHR were fed live anti-FLAG M1 antibody, stimulated with LH (10 nM) for 15 min, stripped to remove surface bound antibody, fixed, permeabilised and stained with anti-APPL1 antibody followed by anti-rabbit AlexaFluor555 and anti-mouse AlexaFluor488 antibodies labelling, and imaged with confocal microscopy. Scale bar=5 µm,

inset=1 µm. (B) Quantification of LHR endosomes positive for endogenous APPL1. Mean ± SEM of n=15 cells per condition collected across 3 different experiments. (C) Quantification of LHR endosomes positive for endogenous APPL1. Values from individual patients are shown. n=5 cells per patient.

6.2.3 Assessment of LHR recycling and cAMP signalling in hESCs from PCOS patients

The work previously presented in Chapter 5 shows that APPL1-mediated LHR recycling is not only dependent on LHR trafficking to APPL1-positive VEEs, but requires PKA phosphorylation of S410 of APPL1. Furthermore, I also demonstrated that although LHR-APPL1 endosomal co-localisation was not affected by mutation of S410 of APPL1 in HEK 293 cells (Figure 5.6), S410 phosphorylation levels still impact on LHR recycling (Figure 5.7). Thus, LHR recycling could still be perturbed in hESCs. I assessed LHR recycling by expressing SEP-LHR in hESCs from PCOS patients and subsequent TIR-FM imaging. LH-induced SEP-LHR recycling was not significantly different between PCOS and non-PCOS groups (p-value=0.340) (Figure 6.6).

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B.

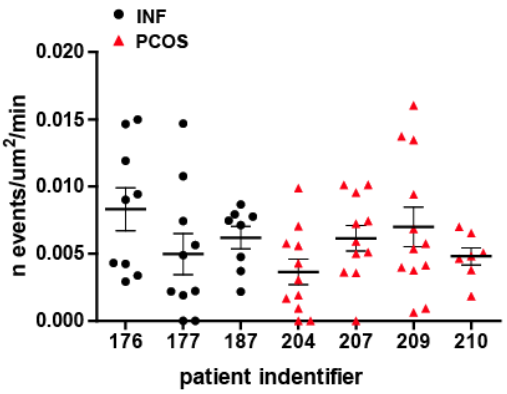
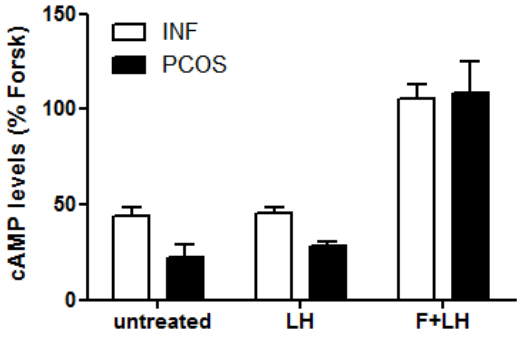


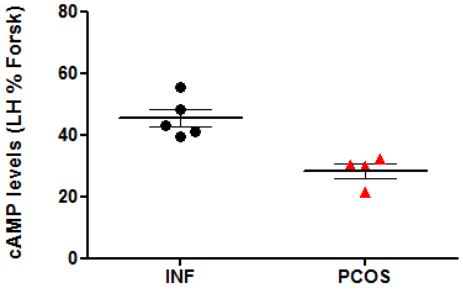
Figure 6.6 SEP-LHR expressed in hESCs from PCOS samples exhibits similar recycling rates to those observed in non-PCOS samples. (A) SEP-LHR recycling in hESCs from PCOS samples was analysed in real time by TIR-FM after LH (10 nM) addition and compared to non-PCOS samples (INF). n=27 and 41 cells per condition for INF and PCOS, respectively, collected across at least 3 independent experiments. Values from individual patients are shown in (B).

In addition to recycling, I also determined whether there were any differences in cAMP production between PCOS and non-PCOS samples. Measurement of cAMP levels produced by LH activation of both endogenous LHR in PCOS hESCs highly resembled the pattern observed in non-PCOS samples, with no increase in cAMP levels over basal upon LH stimulation (Figure 6.7 A). Although cAMP levels in response to LH appeared lower in the PCOS group compared to the non-PCOS (28.40 $\pm$ 2.42% PCOS versus 45.46 $\pm$ 2.91% INF, t-test: *p<0.05), this seemed to reflect the difference in basal levels of cAMP between these two groups (22.18 $\pm$ 7.10% PCOS *versus* 43.18 $\pm$ 4.99% INF, t-test: *p<0.05) (Figure 6.7 A). Within each group, different patients showed similar cAMP levels in response to LH (Figure 6.7 B). Transfection of FLAG-LHR in hESCs from both PCOS and non-PCOS patients eliminated the difference in the basal levels of cAMP between the two groups and, in presence of LH, cAMP levels appeared higher in the PCOS group, although this difference was not statistically significant (Figure 6.7 C). Plotting cAMP values from individual patients highlighted that some patients responded to LH producing more cAMP then others (Figure 6.7 D). This could be due to different transfection efficiency among patients. I tested this hypothesis calculating the fluorescence intensity of both PCOS and non-PCOS cells transfected with SEP-LHR and prior to stimulation with LH. In the non-PCOS group, similar intensity levels were observed across patients, suggesting LHR is expressed at similar levels in these cells (~10000 a.u.) (Figure 6.8 A). On the contrary, the mean fluorescence values for the four PCOS samples analyzed did not follow a common trend and seemed to be divided in two sub-groups: high (>10000 a.u.) and low (<10000 a.u.) intensity, both different from the intensity levels characteristic of the non-PCOS group (Figure 6.8 A). As LHR expression levels could be associated with cAMP production, I conducted a correlation analysis which showed a strong negative correlation (r^2=0.943) between these two variables (Figure 6.8 B). Similarly, LHR expression levels negatively correlate (r^2=0.945) with LHR recycling rates as well (Figure 6.8 C). In agreement with the data presented in Chapter 5 providing evidence that cAMP levels seem to dictate LHR recycling frequency in HEK 293 (Figure 5.4), LHR recycling rates positively correlate (r^2=0.942) with cAMP levels in hESCs (Figure 6.8 D).

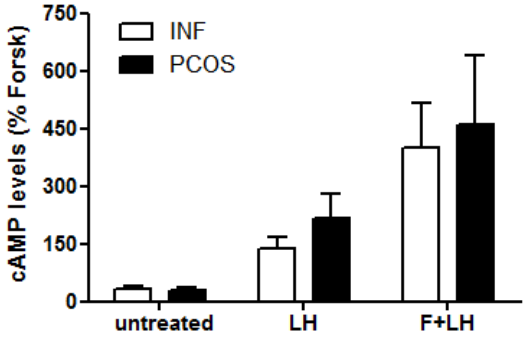
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C.



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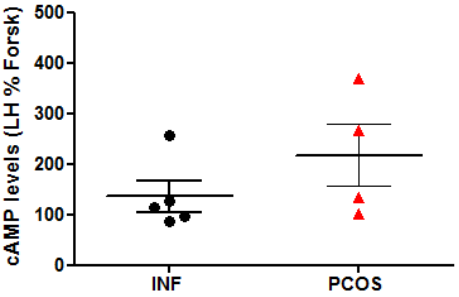
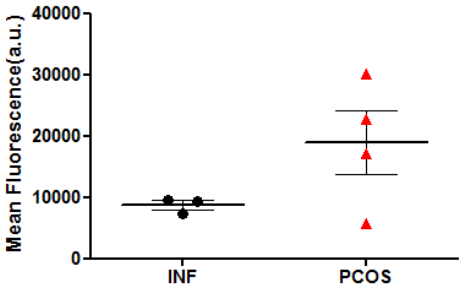
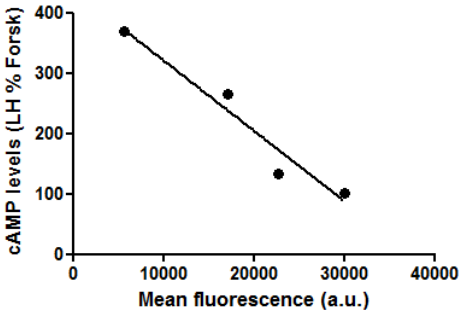


Figure 6.7 LH-stimulated cAMP response is similar in hESCs from both PCOS and non-PCOS samples. Intracellular cAMP levels were measured in hESCs from non-PCOS (INF) or PCOS samples, transfected without (A-B) or with (C-D) FLAG-LHR, pre-treated with the PDEs inhibitor IBMX (0.5 mM, 5 min) and ± treated with LH (10 nM), Forskolin (F, 3 µM) or LH an Forskolin (F+LH) for 5 min; n=5 for INF and 4 for PCOS. Individual patients for LH condition are shown in (B) and (D).

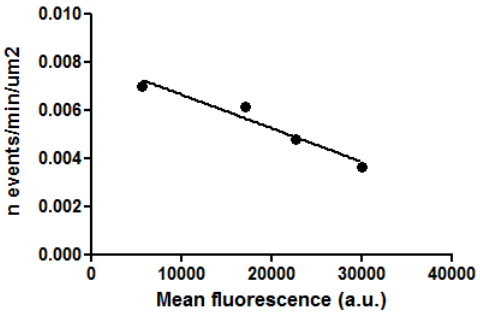
A.



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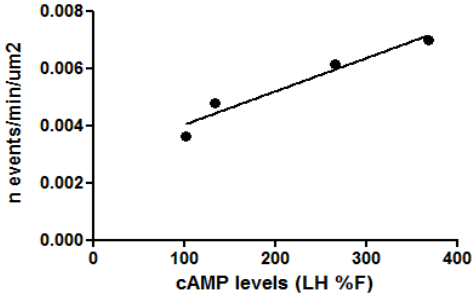


Figure 6.8 LHR expression levels negatively correlate with LH-induced cAMP production and LHR recycling. (A) Mean fluorescence intensity values were calculated from the first frame of TIR-FM movies by selecting the whole surface of each unstimulated cell and using the average tool of Time Series Analyzer plugin of ImageJ. Each point on the graph represents a different patient, n=3 cells per patient. (B) cAMP values from figure 6.7 D were plotted against fluorescence intensity values from figure 6.8 A. (C) Recycling values from figure 6.5 C were plotted against fluorescence intensity values from figure 6.8 A. (D) Recycling values from figure 6.5 C were plotted against cAMP values from figure 6.7 D.

6.3 Discussion

The results presented in this chapter represent, to my knowledge, the first studies on LHR signalling and trafficking in response to LH in human non-pregnant endometrium. Although the importance of hCG-LHR interaction for a successful pregnancy is undoubted, it is equivalently important to investigate the effects of LH binding to LHR. In females LH undergoes a sharp increase in the middle of the menstrual cycle, and LHR is implicated in regulating decidualisation, a process not only important for early pregnancy and embryo implantation, but also for normal menstruation (Gellersen and Brosens, 2014). Unveiling the effects of LH on its receptor in this tissue could help understand the reasons underlying problems associated with a variety of reproductive health issues, such as PCOS and endometriosis.

So far, the very few studies focusing on endometrial LHR have looked at human LHR responses induced by the placental hormone hCG (Srisuparp et al., 2003, Viswanath et al., 2007). The recent findings reporting hCG-LHR biased agonism (Riccetti et al., 2017, Ayoub et al., 2016, Casarini et al., 2012) confirm that results obtained from experiments conducted using either one of the two hormones should not be considered as universal mechanisms of LHR action. Similarly, because rodent and human LHR are known to behave differently, especially in their trafficking pathways (Kishi et al., 2001), results from studies focused on one species should not be expanded to LHR from a different origin.

Therefore, I propose that the cAMP pathway is not activated by LHR in the human endometrium by LH nor by hCG (this study and (Srisuparp et al., 2003, West, 2016)), whilst this has been observed in mice (Kundu et al., 2012, Lyga et al., 2016), making the cAMP response a species-specific feature of LHR. The lack of cAMP production in human samples could be related to low receptor levels, as increased LHR expression lead to LH-induced cAMP activation (Figure 6.3). This hypothesis is corroborated by the finding that FSHR preferentially signals through β-arrestin at low receptor density, and via $G\alpha_s$-cAMP upon increase of receptor expression (Tranchant et al., 2011). Importantly, increased LHR levels also revealed that LH-induced cAMP was sensitive to APPL1 depletion, in a similar manner to what has been found in HEK 293 cells. In addition to APPL1's negative regulatory role on

LHR signalling, I showed that its positive regulation of LHR recycling is also recapitulated in hESCs.

According to my results, APPL1 levels appeared to be similar between non-PCOS and PCOS samples. As demonstrated in Chaper 5, the regulatory role of APPL1 on both LHR signalling and trafficking is mediated by the phospho-status of APPL1 S410. Thus, while the overall protein levels may not be different between these patient groups, it is still possible that the phosphorylation status may be altered in PCOS, leading to impaired recruitment of effectors by APPL1, as it has been proposed before that phosphorylation at S410 of APPL1 represents a molecular switch for the interaction with proteins recruited by APPL1 (Erdmann et al., 2007). It is also reasonable to consider that both the PCOS and non-PCOS groups are charaterised by high variability across the individual samples; the patients recruited in this study suffer from infertility, a highly heterogeneous condition dictated by a variety of factors which, in most cases, have not been clearly identified. Patients grouped as non-PCOS in this study had idiopathic infertility or due to the combination of more than one factor (see table 10), and, although the PCOS group is charaterised by anovulation, polycystic ovaries and hyperandrogenism, the metabolic effects of PCOS can greatly vary (severity or appearance of diabetes, obesity, etc.). Such heterogeneity stresses the need for a larger number of samples in order to reliably analyse and speculate on this preliminary study.

Overall, the data presented in this chapter underline the importance of APPL1 in the regulation of both LHR signalling and trafficking in a cell type that requires LHR functioning to fulfil its physiological tasks.

Chapter 7:

General discussion and future perspectives

This thesis has investigated the molecular mechanisms underlying LHR post-endocytic sorting from the VEE and the signalling pathways activated in response to LH, with primary focus on the $G\alpha_s$-cAMP-PKA cascade. The main findings of my thesis are: i) LHR recycling from the VEE is dependent on both cytoskeletal components (actin and microtubules) and on the adaptor protein APPL1; ii) APPL1-dependent LHR recycling requires LH-driven PKA phosphorylation of Ser410 of APPL1; iii) LHR activates both $G\alpha_s$-cAMP and $G\alpha_{q/11}$-calcium pathways also from intracellular compartments; iv) APPL1 plays another distinct role on LHR negatively regulating LH-induced cAMP production. These findings lead to two major conclusions which both add to and corroborate the current knowledge on GPCR regulation: there is a strong bidirectional relationship between LHR trafficking and signalling, which are linked by the endosomal adaptor protein APPL1, and the requirement for this receptor, to reach the VEE compartment to successfully fulfil its signalling activities.

The evidence that LHR co-localises with endogenous APPL1 at the VEE led to the investigation of possible functional roles on LHR intracellular localisation and trafficking, similarly to the approach previously used to study the role of GIPC on LHR activity (Jean-Alphonse et al., 2014). Depletion of APPL1 revealed its fundamental implication in LHR recycling back to the plasma membrane after endocytosis; importantly, this is the first time this role has been ascribed to APPL1 for any membrane cargo (Figure 5.2). APPL1 triggers LHR recycling via a mechanism involving LHR $G\alpha_s$-cAMP-PKA pathway (Figure 5.4 and 5.8). The involvement of signalling in the regulation of GPCR recycling is not a new concept, as other GPCRs have been shown to use their signalling effectors, specifically PKA, to regulate their post-endocytic sorting. In the case of LHR, though, the target of PKA is APPL1 rather than the receptor itself. This doesn't exclude that other adaptor proteins could be phosphorylated by PKA, in order to regulate LHR function by either mediating its trafficking, for example the VEE-EE transition which has not been investigated in this study, or its signalling. Indeed, LHR activates other signalling pathways in addition to $G\alpha_s$-cAMP-PKA, and, for the MAPK pathway, it has been demonstrated that APPL1 is not involved (Figure 5.10). It still remains to be elucidated whether APPL1 influences the $G\alpha_{q/11}$-calcium pathway; this could be highly possible, as LH-induced calcium release has been shown to depend on LHR internalisation. In the hypothesis $G\alpha_{q/11}$ is also activated at the VEE, APPL1 could explain the crosstalk between cAMP and calcium i provided evidence for. Moreover, LH-activated $G\alpha_{q/11}$

can result in activation of PKC, which has already been shown to phosphorylate APPL1 on Ser430 (Liu et al., 2012), making this a mechanism that LHR could exploit too to differentiate between the different roles of APPL1 on LHR function. Different serines of APPL1 could then serve as independent molecular switches, which get activated/inactivated via phosphorylation by distinct kinases. In fact, the phospho-serine analysis conduncted in FLAG-LHR cells stimulated with LH at different time points does not exclude the phosphorylation of other serines of APPL1, as inhibition of PKA through KT5720 or Ser410A mutation do not completely abolish the LH-induced phospho-serine levels of APPL1 (Figure 5.8). To discriminate between the differential involvement of Ser410 and Ser430, phospho-specific antibodies could be used. This will help understanding which serine is phosphorylated under specific conditions and in which pathways it is involved, in addition to provide spatial information on the cellular distribution of these forms of APPL1.

The search for other endosomal proteins involved in the regulation of LHR signalling is thus needed and could be addressed by selective co-immunoprecipitation of VEE-localised LHR partners combined with mass spectrometry. Indeed, a similar approach has been recently developed to spatiotemporally resolve protein interaction networks in living cells. This approach relies on the engineered ascorbic acid peroxidise (APEX) for its excellent ability to bind, in a highly rapid manner (sub-minute), biotinylated proteins of interest and on quantitative proteomics (Lobingier et al., 2017, Paek et al., 2017). Briefly, this method uses APEX to tag the GPCR of interest, to obtain information about the interaction protein network in a time specific manner, and targets APEX to specific cellular locations (e.g. plasma membrane, EE and cytosol), to obtain spatial resolution of the previously identified proteome (Lobingier et al., 2017, Paek et al., 2017). This will lead to the identification of GPCR interaction profiles which are both time- and organelle-specific. In order to apply this methodology to LHR, more information about the VEE is required, most importantly its PI content.

LH-induced PKA phosphorylation of APPL1 on Ser410 and its pivotal, yet opposite, functions on LHR recycling and cAMP signalling have been clearly demonstrated by the use of both of Ser410 phosphorylation mutants and the PKA inhibitor (Chapter 5). What remains unclarified is the source of the PKA-activating cAMP, whether it is from the plasma

membrane or from endosomes. Considering the very small proportion of cAMP produced at the plasma membrane (Chapter 4), and that tmAC activation triggers subsequent sACs activation, I hypothesize that cAMP starts to be produced at the plasma membrane, followed by a much more robust $G\alpha_s$-mediated cAMP production at the endosome (as revealed by Nb37), which is amplified by the synergistic effect of both endosomal tmACs and sACs, as sAC have been demonstrated to participate to the LH-induced cAMP response (Chapter 4). One approach to visualise cellular loci of PKA action is by localising AKAPs. These scaffolding proteins bind PKA and other signalling and regulatory proteins, including PKA targets and PDEs, and physically tether these signalling complexes to specific cellular locations, further refining the spatial control of cAMP dynamics. hCG signalling in the placenta appears to be mediated by AKAPs (Weedon-Fekjaer and Tasken, 2012), suggesting a role for these proteins in LHR functions. Indeed, a proteomic screening conducted in our laboratory has identified AKAP-Lbc as an LHR interacting protein, paving the way for further research on LHR-cAMP pathway. Furthermore, previous studies conducted on B2AR describe a role for AKAPs in differentially regulating the signalling and recycling of this receptor (Tao and Malbon, 2008); taking into account that both cAMP production and recycling of LHR take place at the VEE (Chapter 4 and 5), a deeper investigation into the involvement of AKAPs in LHR signalling and trafficking could shed a light the relationship between these two aspects.

The identification and localisation of LHR signalling loci would greatly benefit from the optimisation of the super-resolution techniques I carried out throughout my PhD. It is now evident that classic optical methods, such as confocal microscopy, do not provide the resolution needed to identify the microdomain structure of the VEE. In fact, other groups have used super-resolution microscopy techniques, namely SIM and PALM, to further characterise the spatial organisation and co-localisation at vesicles smaller than the classic EE, including APPL1-positive endosomes (Eichel et al., 2016, Masters et al., 2017). As suggested for GPCR sorting from the EE, different microdomains with different capabilities exist at the endomembrane (Varandas et al., 2016, Bowman et al., 2016), raising the intriguing hypothesis that more than one type of signalling microdomain perhaps co-exist at the same endosome to allow for parallel activation of different signalling pathways. This could be verified by the use of nanobodies specific for distinct G proteins (e.g. $G\alpha_s$ and

$G\alpha_{q/11}$), in combination with the aforementioned super-resolution microscopy techniques. If this hypothesis is confirmed, spatial regulation of GPCR signalling will not only be interpreted as plasma membrane *versus* intracellular compartments or, for example, EE *versus* VEE; instead, a new layer of complexity will be added by the microdomain structure of each individual endosome, where multiple signalling pathways could be activated at the same time, or in response to different stimuli (e.g. type of ligand, duration of exposure to the ligand, etc), and with different intensities. One way to measure the intensity of the activation of each signalling pathway at the G protein level is to conduct PALM imaging to count the number of G protein molecules recruited and/or activated at a specific location, and compare it across different G protein types. Another hypothesis emerging from the model of endosomes as multi-tasking signalling stations, is that APPL1 could behave as a crosstalk coordinator between LHR signalling pathways (e.g. $G\alpha_s$-cAMPand $G\alpha_{q/11}$-calcium, as mentioned above) activated at the VEE, thanks to its ability to interact with multiple proteins. Interestingly, I found that APPL1 acts as an integrator of signalling and trafficking of LHR at the VEE: although LH-induced cAMP is necessary for APPL1-mediated LHR recycling, APPL1 also negatively regulates cAMP (Chapter 5). In fact, its depletion determines a robust increase in cAMP levels, which I demonstrated not to result from trapping LHR at the VEE (Figure 5.3, 5.9 and 5.15). Consequently, the repression of cAMP response by APPL1 at the VEE could be a mechanism to confine the $G\alpha_s$-cAMP pathway and avoid the activation of other signalling pathways in close proximity. The detailed description of APPL1-mediated cAMP constraint is still unclear. It could happen at G protein levels, with APPL1 acting as a brake on Gβγ release from $G\alpha_s$; indeed, APPL1 could physically interact with Gβγ through its PH domain (Wang et al., 1994), as hinted in my BRET experiments (Figure 5.14).

In addition to how APPL1 regulates LHR signalling and sorting, another unanswered question is how APPL1 itself is regulated. Although it is still under debate whether APPL1 is a resident or transient marker of the VEE, the mechanisms that determine the effectiveness of APPL1 action in specific cellular functions is unclear. Two of the most exploited ways to modulate protein activity is via dimerisation between monomers and post-translational modification. APPL1-APPL2 dimerisation has already been identified and could be used by these adaptor proteins to mask some of their interacting modules, thus preventing

scaffolding of other molecules. Additionally, one example of a PTM that has been demonstrated to impact on GPCR sorting without physically targeting the GPCR itself, is ubiquitination of adaptor proteins. PAR1 activation induces ubiquitination of its adaptor protein ALIX via the α-arrestin ARRDC3, which is fundamental for PAR1 lysosomal sorting (Dores et al., 2015). Thus, a similar mechanism could be involved in the regulation of APPL1 and, at the same time, dictate LHR post-endocytic sorting. In addition to PAR1, also B2AR utilizes ARRDC3, in this case to regulate its residency time in endosomes before recycling (Tian et al., 2016a). A preliminary approach to study α-arrestin involvement in APPL1 regulation could be to conduct BRET experiments to test whether ARRDC3 and APPL1 interact in LHR expressing cells, and if this interaction is ligand-induced and/or dependent on the phospho-status of APPL1.

It is interesting to point out that, although increasing evidence has highlighted the role of β-arrestins in endosomal G protein signalling via its simultaneous association with receptor and G protein, including both Gα and Gβγ subunits (Jean-Alphonse et al., 2016), this remains pertinent for those GPCRs exhibiting sustained β-arrestin associations via receptor C-tail Ser/Thr clusters, and co-trafficing with arrestin to endosomes (Thomsen et al., 2016, Kumari et al., 2016, Wehbi et al., 2013). GPCRs such as the B2AR and LHR do not contain these C-tail clusters and associate with β-arrestins uniquely at clathrin-coated pits, yet both exhibit endosomal G protein activation (Jean-Alphonse et al., 2014, Irannejad et al., 2013, Kang et al., 2009, Bhaskaran et al., 2003a). Accordingly, previous studies in our laboratory have found β-arrestins associated with LHR only at the plasma membrane and I observed no recruitment of β-arrestin 1 nor 2 to VEEs when imaging LHR-expressing cells with either confocal microscopy or TIR-FM (personal communication with Dr. Jean-Alphonse and data not shown). As, to my knowledge, the involvement of α-arrestins in endosomal singalling or post-endocytic sorting of LHR has not been explored.

While the importance of endosomal localisation of GPCRs is undeniable in the achievement of controlled and diversified signalling outputs, it is still unclear how cells decipher membrane *versus* endosomally activated pathways and how this translates into distinct cellular functions. One proposed mechanism for cells to differentiate between different sources of signals is by physically confining the elements of a certain pathway in a discrete

cellular location. The importance of compartmentalised signalling is highlighted by studies conducted in cardiomyocytes from failing hearts, where disruption of PKA anchoring to AKAPs or reorganisation of B2AR cellular localisation cause loss of cAMP compartmentalisation (Zakhary et al., 2000, Nikolaev et al., 2010, Zaccolo, 2011).

For some GPCRs the biological meaning of endosomal signalling has started to be explored; these include TSHR, PTHR, V2R, B2AR, GLP1R and pituitary adenylate cyclase activating polypeptide type 1 receptor, where sustained or 2nd phase cAMP production has been linked to actin re-organisation, bone calcium homeostasis, renal water and sodium transport, differential gene expression, insulin secretion and neuronal excitability, respectively (Calebiro et al., 2009, Okazaki et al., 2008, Feinstein et al., 2013, Tsvetanova and von Zastrow, 2014, Kuna et al., 2013, Merriam et al., 2013). For LHR specifically, the downstream role of VEE-targeting and endosomal signalling remains to be determined. However, LH does induce a sustained ERK signalling profile in human ovarian granulosa cells that negatively regulates expression of aromatase (Casarini et al., 2012), a signalling profile that may result from VEE-localised LHR. Furthermore, transgenic expression of FRET-based cAMP biosensors, indicated that the mouse LHR exhibits also a sustained cAMP profile in ovarian follicles (Lyga et al., 2016). Use of chemical inhibitors to block internalisation demonstrated a loss of LH-mediated resumption in meiosis (Lyga et al., 2016), although whether the VEE is involved is unclear as rodent and human LHRs differ in their ability to internalise, associate with GIPC and undergo regulated recycling (Galet et al., 2004, Galet et al., 2003, Hirakawa et al., 2003).

One of the newest and most powerful tools to discriminate the physiological significance between either plasma membrane- *versus* endosomal, G protein *versus* non-G protein signalling, or even among G protein subtypes, is optogenetics (Spangler and Bruchas, 2017, Siuda et al., 2015, Nakamura et al., 2000). With this approach it is possible to finely tune GPCR localisation or bias signalling just by using light; in the first case the GPCR of interest is fused to a light sensing domain which, in response to light, dimerises with its counterpart, which has previously immobilised at the plasma membrane through a signal peptide, thus forcing the receptor to localise only in that precise cellular compartment; in the second instance, cells are transfected with G protein-coupled opsins, which are selective for specific G protein subtypes or biased to β-arrestin, hence allowing optical control of distinct

signalling pathways (Karunarathne et al., 2015). It has to be pointed out thought, that in the latter case ligand or receptor specificity is lost as the ligand/receptor activation step is by-passed. So far the application of these methods has resulted in a better understanding of the signalling pathways beneath cell migration, neurite growth and circadian rhythms (Karunarathne et al., 2013, Bailes et al., 2017); a broader use is expected in the interrogation of the mechanistic basis of many other GPCR-modulated cellular functions, including those mediated by LHR.

The application of these methods would be much more informative if conducted in physiologically relevant cell types implicated in LHR physiology. For this purpose, my findings employing primary hESCs are highly useful for future studies on the physiological significance of human LHR endosomal signalling, as they demonstrate LHR internalisation to APPL1-positive VEEs, APPL1-dependent LHR recycling and APPL1 negative regulation of LH-induced cAMP are conserved in this cell type where LHR is physiologically expressed (Bernardini et al., 2013) (Figure 6.1, 6.2 and 6.3). Both APPL1 functions have been validated only in specific conditions, such as decidualised non-pregnant endometrium, as my experiments were conducted in decidualised hESCs stimulated with LH. It would be highly informative to conduct the same experiments in non-decidualised cells and/or stimulated with hCG, in order to understand if the requirement of APPL1 is restricted to a specific time during the menstrual cycle or it represents a general mechanism implied in the regulation of endometrial LHR. As the use of LH or hCG results in different recycling kinetics in HEK 293, it would be useful to measure LHR recycling in hESCs to investigate ligand bias on receptor sorting in this, or other LHR-expressing cell types. Furthermore, assessing the downstream impact of APPL1 knock down in hESCs by testing the expression of key candidate genes implicated in reproductive functions of the uterus, metabolism and inflammation, such as COX2, AdipoR, GLUT4 and the cytokines IL6, IL8 and CXCL1. According to the results of this analysis, further investigation of hESCs transcriptome and secretome could be carried out.

Additional clinical and pharmaceutical applications of this work could be in the area of ovarian epithelial cancer. The development of epithelial ovarian cancer has been related, although not unanimously, to gonadotrophic action. The incidence of ovarian cancer increases around the perimenopausal period, when the serum levels of gonadotrophins are

elevated; cases of ovarian cancer development during or after ovulation induction therapy have been reported; moreover, there is higher risk of developing ovarian cancer for PCOS patients, where LH levels are increased (Longo and Young, 1981, Schildkraut et al., 1996, Mandai et al., 1997, Mandai et al., 2007). Interestingly increased APPL1 levels are associated with greater invasiveness of ovarian cancer (Zhao et al., 2010). Together, these may suggest that "hyper-VEE" signalling from LHR is detrimental; for this purpose, small allosteric molecules that affect not only the signalling output of the receptor, but also its trafficking and post-endocytic sorting, could be exploited (described in Introduction, paragraph 1.5.5). As only a few allosteric modulators are available for LHR, one avenue is to use NAMs or PAMs originally developed for FSHR, but that could also work for the closely related LHR (Ayoub et al., 2016). An alternative to the use of small molecules to control LHR activity is to reduce APPL1, although its effects on LHR cAMP generation should be assessed. Delivery of siRNA with tissue specificity is still a challenging task in biomedicine, but in a not too far future RNA therapeutics could become a breakthrough technology (Bobbin and Rossi, 2016).

In this study I had the opportunity to compare hESCs between non-PCOS and PCOS samples in respect to LH-driven LHR recycling and cAMP production (Figure 6.6 and 6.7). However, these preliminary data have not identified any difference. Accordingly, APPL1 levels do not appear different between the two groups (Figure 6.4). Although the importance of Ser410 of APPL1 has not been confirmed yet in hESCs, the strong correlation between cAMP levels and LHR recycling rates implies that PKA activation is required for LHR to be targeted back to the plasma membrane after internalization (Figure 6.8). Assuming PKA is still targeting Ser410 of APPL1, one could argue that there might be differences in its phosphorylation status that could dictate the PCOS phenotype. This is not surprising as a very similar mechanism has been reported to link APPL1 to insulin resistance in hepatocytes (Liu et al., 2012). In this case PKC phosphorylates Ser430 APPL1 under obesity and ER-stress conditions (Liu et al., 2012). As previously mentioned, an anti-phospho Ser410 antibody should be produced to better evaluate APPL1 phosphorylation status in different samples and possibly relate it to the severity of PCOS. Moreover, as PKC is one of the LHR downstream effectors, the involvement of Ser430 phosphorylation should be tested as well. Furthermore, although this work has reported no differences between the PCOS and non-PCOS groups in terms of LHR recycling and cAMP signalling, it is still possible that LHR activity might be perturbed in

another cell type where LHR is endogenously expressed, namely granulosa cells. Similar experiments to those conducted in hESCs could be repeated in primary human granulosa cells acquired following ovarian stimulation as part of IVF. However, transfection of such cells needs to be optimised, or, alternatively, an immortalised cell line, like KGN, could be employed (Nishi et al., 2001, Wu et al., 2008, Wolf-Ringwall et al., 2011). Interestingly, in addition to LHR these granulosa cells express also FSHR, which has been shown to utilise APPL1 for the regulation of its trafficking and signalling, similarly to LHR. Further investigation on how APPL1 affects gonadotrophic signalling could be conducted by quantifying steroid production in this cell type. Thus, granulosa cells could be a useful model to study the effect of trafficking and signalling on the physiology of two closely related, yet different, GPCRs.

In conclusion, my work has characterised the molecular mechanisms dictating LHR recycling and signalling from the VEE, which are summarised in the following model (Figure 7.1). After binding to LH, LHR is internalised to the VEE, a newly identified compartement which is composed by a variety of forms, some of which are APPL1-positive. From the VEE, LHR actives $G\alpha_s$ leading to the production of the majority of cAMP induced by LH, as well as partially activates the $G\alpha_{q/11}$-calcium pathway. cAMP production is achieved by the action of both tmAC and sAC and is restricted to microdomains. This cAMP triggers the activation of PKA which, in turn, phosphorylates APPL1. Phosphorylation of Ser410 of APPL1 is fundamental for LHR recycling back to the plasma membrane, which is mediated by both actin and microtubules. Possibly in another VEE, or even at the same VEE, but in distinct microdomains, unphosphorylated APPL1 negatively regulates cAMP production.

Our view of endocytic trafficking in modulating GPCR signalling has evolved from a system that regulates cellular and tissue sensitivity of a ligand via plasma membrane signalling, to a model where trafficking and signalling are highly integrated at multiple levels, across distinct endomembrane compartments and microdomains, to permit a higher degree of diversification. The discovery of the VEE as both signalling and sorting hub for a group of GPCRs has opened a path for the search of additional endosomal compartments used by other GPCRs. Given GPCRs represent an established and successful therapeutic target, GPCR endosomal signalling and the existence of a variety of distinct endosomes have the potential

to impact on not only how compounds are assessed but also provide novel therapeutic avenues. With the information provided by the incredible advances in the development of tools (e.g. nanobodies), imaging technologies (super resolution microscopy) and methods (e.g. APEX-based interrogation of time- and space-specific protein interaction networks), the same GPCR signalling pathway will be selectively targeted not only between plasma membrane and endomembrane, but even among distinct endosomal compartments or microdomains.

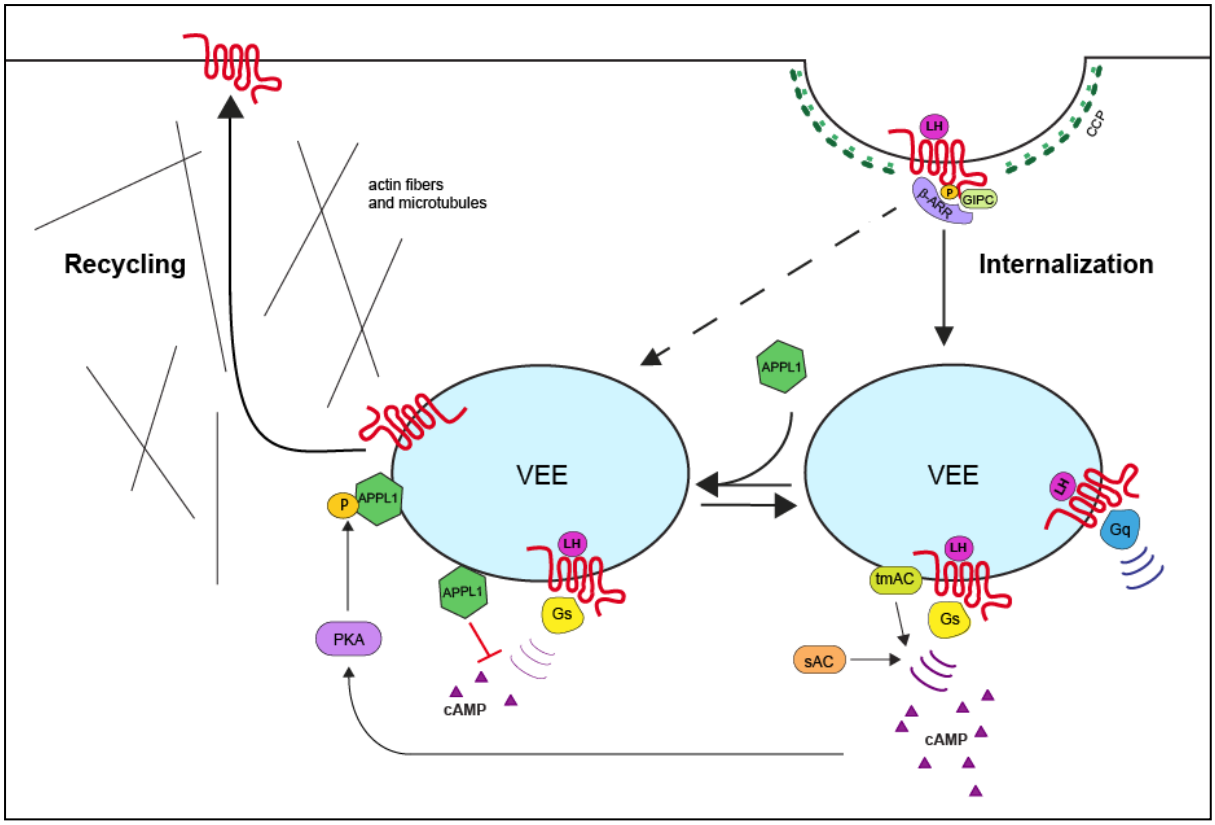


Figure 7.1 Model for APPL1-dependent regulation of LHR recycling and endosomal signalling via a heterogeneous VEE compartment. Ligand-induced LHR internalisation to VEEs is dependent on association with GIPC at clathrin-coated pits (Jean-Alphonse *et al.*, 2014). It is possible that LHR may traffic directly to APPL1-positive VEEs (dotted arrow) or through APPL1-negative VEEs. LHR-mediated activation of endosomal $G\alpha_s$-cAMP occurs from distinct subset of VEEs both devoid of, and with, APPL1, and implies the action of both tmAC and sAC. LHR-mediated activation of endosomal $G\alpha_{q/11}$-calcium occurs from endosomal compartments and, just for simplicity, has been depicted at APPL1-negative VEEs. Trafficking of LHR to APPL1 endosomes negatively regulates the endosomal cAMP. For simplicity, this negative regulation is depicted here at the same VEE that LHR recycling via phosphorylation of APPL1 on Ser410, however, these could also be distinct endosomes, given the presence of LHR/APPL1 endosomes without active $G\alpha_s$. Furthermore, cAMP-PKA activation leads to phosphorylation of APPL1 on Ser410 necessary for LHR recycling back to the plasma membrane, suggesting inter-endosomal communication. LHR recycling exploits both cytoskeletal components, actin fibres and microtubules.

Chapter 8:

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List of Publications

Sposini S, Jean-Alphonse FG, Ayoub MA, Lavery S, Brosens JJ, Reiter E, Hanyaloglu AC. "APPL1 integrates GPCR sorting and signalling from very early endosomes". (2017). *Submitted to Cell Reports (in revision).*

Sposini S and Hanyaloglu AC. (2017). "Spatial encryption of G protein-coupled receptor signalling in endosomes; mechanisms and applications". *Biochem. Pharmacol.* doi: 10.1016/j.bcp.2017.04.028.

Sposini S and Hanyaloglu AC. (2017). "Endocytosis and Signalling- Chapter 9: Evolving view of membrane trafficking and signalling systems for G protein-coupled receptors". *Submitted to Springer Nature.*

Sposini S, Caltabiano G, Hanyaloglu AC, Miele R. (2015). **"**Identification of transmembrane domains that regulate spatial arrangements and activity of prokineticin receptor 2 dimers**"**. *Mol Cell Endocrinol*. doi: 10.1016/j.mce.2014.10.024.

Conferences and Worskshops

Gordon Research Seminar and Conference - Molecular Pharmacology "Understanding GPCR Function from Structural Biology to In Vivo Models", 11st-17 th Mar 2017, Lucca, IT *(oral presentation + poster).*

Young Life Scientists symposium "Advanced Imaging with Light Microscopy", 28th Oct 2016, London, UK *(organiser).*

6th BPS Focused Meeting on Cell Signalling, 18th-19th April 2016, Leicester, UK *(poster).*

Society for Endocrinology BES 2016, 2nd-4th November 2016, Edinburgh, UK *(poster).*

Young Life Scientists symposium "Modelling Aproaches in Molecular Signalling", 13th Nov 2015, London, UK *(poster + oral presentation). Best poster award.*

Inserm workshop "New trends in super-resolution optical microscopy", Oct-Nov 2015, Bordeaux and Paris, FR.

Gordon Research Conference - Molecular Pharmacology "Connecting G Protein-Coupled Receptor Mechanisms to Physiological Functions", 1st-6 th Feb 2015, Ventura, CA, USA *(poster)*.

Biochemical Society workshop "Advancing Applications of Super-Resolution Imaging", 10 th Nov 2014, London, UK *(poster)*.

ICGRIII - International conference on gonadotrophins and receptors, 7th-10th Sep 2014, Tours, FR *(poster)*.

Keystone Symposium "G Protein-Coupled Receptors: Structural Dynamics and Functional Implications", 30 March – 4 April 2014, Snowbird, UT, USA *(poster + oral presentation)*.

Society for Endocrinology BES 2014, 24th-27th March 2014, Liverpool, UK *(poster)*. *Highly commended basic science poster award.*

Society for Endocrinology BES 2013, 18th-21st March 2013, Harrogate, UK *(poster)*.