Gonadotropin-Releasing Hormone Receptor Signaling: Biased and Unbiased

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Abstract: Gonadotropin-releasing hormone is a neuropeptide that acts via G_q coupled G-protein coupled receptors in the pituitary that mediate central control of reproduction. GnRH receptors (GnRHR) and GnRH ligands are also found in extra-pituitary sites including the CNS as well as reproductive tissues and cancer cells derived from such tissues. Much of the interest in the extra-pituitary receptors stems from the fact that they mediate anti-proliferative and/or pro-apoptotic effects and may therefore be directly targeted for cancer therapy. Type I mammalian GnRHR are atypical in that they do not bind to (or signal via) arrestins. In spite of this restriction on their signaling repertoire, there is good evidence for existence of multiple active GnRHR conformations and for activation of multiple upstream effectors (heterotrimeric and monomeric G-proteins). In this review GnRHR signaling is described, with emphasis on the relevance of functional selectivity for pharmacological characterization of GnRHR ligands, as well as its possible contribution to context-dependent GnRHR signaling and relevance for GnRHR-mediated effects on cell fate as well as GnRHR trafficking.

Keywords: GnRH, GPCR, signaling, trafficking, ligand bias, functional selectivity.

GONADOTROPIN-RELEASING HORMONES AND THEIR RECEPTORS

Gonadotropin-releasing hormone I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, often simply termed GnRH[1]) is a hypothalamic neuropeptide that mediates central control of reproduction. It does so via GnRH receptors (GnRHR) on pituitary gonadotropes, stimulating the synthesis and secretion of the pituitary gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thereby controlling gametogenesis and steroidogenesis in the gonads [1-6]. GnRH is secreted from the hypothalamus in pulses which are most often of a few minutes duration and at 0.5-8 hr intervals in humans. Each pulse elicits a pulse of gonadotropin secretion and effects of endogenous GnRH pulses can be blocked *in vivo* using GnRH antagonists [1-8]. Sustained exposure to GnRH (or metabolically stable GnRH agonists) causes stimulation followed by desensitization of GnRHR-mediated gonadotropin secretion. Consequently, blockade or desensitization of GnRHR-mediated gonadotropin secretion (with antagonists or agonist) both ultimately reduce circulating levels of gonadotropins and gonadal steroids, and this medical castration underlies the therapeutic use of GnRH analogs to treat sex hormone-dependent neoplasms such as those of the prostate, ovary, endometrium, or mammary [1-6,9]. In addition to expression in the pituitary, GnRHR are found (often along with GnRH) in many other tissues including the brain and tissues of the reproductive system [1-6,10,11]. Most interest in extra-pituitary GnRHR stems from the discovery that they are expressed by numerous cancers, including those of the mammary, prostate, endometrium, and ovary. In cells derived from such cancers GnRH analogs (or their cytotoxic derivatives) often exert anti-proliferative and/or pro-apoptotic effects raising the exciting possibility that such direct action may contribute to the therapeutic effects of GnRH analogs in cancer treatment [1,3,6,11-32].

GnRHR are rhodopsin-like G-protein coupled receptors (GPCRs) that were first cloned from a mouse gonadotrope-derived cell line [3,4,33,34] and subsequently from a broad range of species. The cloned GnRHR can be broadly classified into three groups with all cloned mammalian GnRHR in groups I or II [3,10]. There are also multiple forms of GnRH peptide although reptiles birds and mammals express only GnRH I and GnRH II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2). The latter is often widely distributed in the CNS [35,36] where it is thought to act as a neurotransmitter and/or neuromodulator, whereas GnRH I, acting via pituitary type I GnRHR is the essential cognate pairing for central control of mammalian reproduction. Some primates express type I and type II GnRHR [37] but in humans the type II GnRHR (pseudo)gene has a frame-shift and premature stop codon so humans have two GnRH ligands acting via a single type I GnRHR [3,6,10,38].

An intriguing feature of these receptors is that type I mammalian GnRHR lack the intracellular COOH-terminal tail region (C-tail) found in all other GPCRs, and in all non-mammalian GnRHR [1-6,33,34]. For many GPCRs, the C-tail plays a key role in desensitization and trafficking [39,40]. Thus, GPCR activation often causes homologous receptor desensitization, a process in which the activated receptors are phosphorylated by GPCR kinases (GRKs) on Ser and Thr residues most often within their C-tails. This

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1875-5607/12 $58.00+.00

facilitates the binding of non-visual arrestins (arrestins 2 and 3) that prevent G protein activation. They also act as adapters targeting the desensitized receptors for internalization, a process that can lead to receptor down-regulation, or recycling and resensitization [39,44]. The functional correlate is that type I mammalian GnRHR do not undergo agonist-induced phosphorylation, do not bind arrestins, internalize relatively slowly and do not rapidly desensitize [41-50]. In contrast, where characterized type II GnRHR do bind arrestins and show rapid homologous desensitization as well as rapid internalization [41-50]. For many GPCRs, arrestins also mediate signaling by recruiting effectors to the GRK phosphorylated receptors. Indeed, arrestins can bind a range of enzymes including cRaf1 and the ERK (extracellular signal-regulated kinase) mitogen-activated protein kinase (MAPK), both of which can bind to MAPK/ERK kinase (MEK) so that the 3 component Raf/MEK/ERK cassette can be assembled on the receptor-associated arrestin scaffold. This provides an additional mechanism for GPCR-mediated ERK activation that is not dependent upon heterotrimeric G-protein activation [51-55]. Comparison of signaling in cells expressing a mouse type I or a *Xenopus laevis* GnRHR revealed that they both cause G-protein-dependent ERK activation but arrestin-mediated ERK activation was only seen with the C-tail expressing *Xenopus* GnRHR [56,57]. Thus modulation and mediation of signaling by arrestins may be characteristic of non-mammalian GnRHR. The absence of such effects for type I mammalian GnRHR may enable them to faithfully transduce the pulsatile GnRH I signal into an intracellular G-protein activation response. This could have adaptive advantage in situations such as the pre-ovulatory gonadotropin surge where there is an increase in GnRH I pulse frequency and amplitude and where consequent G-protein activation might otherwise be prevented by receptor desensitization.

Another important feature of GnRHR related to the C-terminal tail is that a large proportion of these receptors are actually intracellular [58-65]. This was revealed by the study of human GnRHR mutants that cause infertility and were found to be non-functional because of impaired trafficking rather than impaired signaling [58,59,63-65]. It subsequently became clear that even wild-type human GnRHR are relatively poorly expressed at the cell surface. The presence of a primate specific Lys^{191}, the absence of a second N-terminal glycosylation site and the absence of a C-tail are all implicated in poor cell surface expression of human GnRHR [58-66]. Indeed, when we used a high content imaging approach to quantify HA-tagged GnRHR at the cell surface and within cells [60-62,64], we found that at least 95% of GnRHR were intracellular after heterologous expression in hormone-dependent cancer derived cell lines (MCF7, T47D, PC3, LnCAP). In MCF7 cells <1% of human GnRHR were at the cell surface and this remarkably small proportion was increased as much as 10-50 fold for GnRHR with C-tails (*X. laevis* GnRHR or chimeric type I mammalian GnRHR with added *X. laevis* C-tails). The proportional cell surface expression of human GnRHR was also approx. 5 fold higher in gonadotrope-derived cell lines (LβT2 and αT4) than in the hormone-dependent cancer cells, demonstrating that GnRHR trafficking is cell context-dependent [60,64]. Cell permeant GnRHR ligands are currently being developed as potential orally active GnRHR antagonists [67] and we found that the proportion of human GnRHR at the cell surface was also increased (10-20 fold) by a non-peptide indole antagonists (IN3). This compound had previously been shown to rescue signaling by the trafficking impaired human GnRHR mutants noted above [58,59,63-65] and is thought to act as a pharmacological chaperone, aiding the folding of endoplasmic reticulum (ER) resident GnRHR into a conformation needed to meet ER exit quality control criteria, and thereby aiding GnRHR trafficking to the cell surface [60-62,64]. In the absence of reliable antibodies to human GnRHR, these studies have been performed primarily with tagged receptors. It is therefore important to recognizes that the chaperone effects on GnRHR trafficking are paralleled by effects on receptor function (i.e. rescue of signaling via trafficking deficient mutants and enhanced signaling via wild-type human GnRHR), effects that can also be seen with tag-free receptor constructs [58-65]. The caveat remains, however, that the number and proportion of native receptors at the cell surface has not been defined for human GnRHR in pituitary or extra-pituitary sites.

OVERVIEW OF GnRHR SIGNALING

The native GnRHR of pituitary gonadotropes couple primarily to G_q [1-6]. Consequently they stimulate phospholipase C β (PLCβ), elevating cytoplasmic [Ca^{2+}] and activating isozymes of protein kinase C (PKC), both of which are important for GnRHR-mediated effects on gonadotropin synthesis and secretion [1-10,68,69]. However, GnRHR also have the potential to activate other G-proteins as indicated by early experiments in which GnRHR mediate elevation of cyclic adenosine 3', 5' monophosphate (cAMP) in pituitary tissue [70]. This does not necessarily reflect activation of G_s in gonadotropes, and in various models GnRHR have been shown not to increase cAMP or to do so by mechanisms other than G_s activation [71-73]. However, Arora *et al*. (1998) showed that GnRHR expressed in COS cells mediate cAMP elevation and identified amino acids in the 1st intracellular loop required for the cAMP response but not for stimulation of [^{3}H]inositol phosphate (IP) accumulation, uncoupling that argues against a causal relationship between the IP and cAMP responses [74]. GnRH can also increase cAMP accumulation in LβT2 gonadotropes [72,75], an effect that has been attributed to sequential activation of G_q and adenylyl cyclase (i.e. PKC-mediated activation of adenylyl cyclases 5 and 7 [72]) or to direct activation of G_s [75]. In the latter case, GTP loading provided direct evidence for G_s activation. Probably the most compelling evidence for activation of multiple G-proteins by endogenous GnRH comes from work with GnRH neurons [76,77]. In these cells GnRH causes activation of G_s, G_i and G_q as evidenced by agonist-induced release of activated G-protein subunits from membranes and associated functional responses. Temporal differences were observed in G-protein activation, and a molecular switch was described (G_s being activated at lower GnRH concentrations than G_i and G_q) and implicated in control of pulsatile GnRH secretion [76-77]. There is also considerable evidence for GnRHR-mediated activation of G_i in cancer cell lines as, for example, in JEG-3 human choriocarcinoma cells, and BPH-1 human benign prostate hyperplasia cells [29], where GnRH inhibits

forskolin-stimulated cAMP accumulation, and this effect is blocked by pertussis toxin (PTX). Moreover, in several models the anti-proliferative effects of GnRHR ligands have been inhibited by PTX suggesting mediation by G_i [29]. In general, it appears that G_i is an important mediator of the anti-proliferative effects of extra-pituitary GnRHR, whereas G_q is the primary mediator of pituitary GnRHR effects on gonadotropin synthesis and secretion [6]. Together, these studies demonstrate the potential for GnRH to activate distinct heterotrimeric G-proteins and to do so in a manner that is dependent upon cell context and stimulation paradigm.

Further downstream GnRHR activate a large number of important signaling pathways [1-6,57,68,69, 78-89]. Notably, they mediate activation of phospholipases A2 and D as well as PLC [68] and also activate the ERK, JNK and p38 MAPK pathways [57,68,69,81,83-88]. Although mechanisms vary according to cell context, PKC isozymes are important mediators of GnRH effects on MAPKs [57,68,81,83]. GnRHR also mediate activation of calmodulin, calmodulin-dependent kinases and nuclear factors of activated T-cells (NFATs), as well as activation of diacylglycerol kinase, proline rich tyrosine kinase-2 and Wnt signaling, and inhibition of glycogen synthase kinase [6,12,68, 78-80]. In some models, GnRHR cause a PKC-mediated liberation of membrane bound epidermal growth factor (EGF) receptor ligands, thereby activating EGF receptors and engaging their effectors [12,68], whereas in others GnRHR mediate induction of protein phosphatases that apparently inhibit trophic effects of EGF [19]. Moreover, the transfected rat GnRHR in SCL60 cells mediate cytoskeletal remodeling and tyrosine phosphorylation of cytoskeleton associated proteins including focal adhesion kinase (FAK), c-Src and ERKs [29]. In this model ERK activation is dependent upon assembly of a protein-protein complex with FAK and c-Src at focal adhesion complexes and the cell remodeling event is mediated by activation of the monomeric G-protein Rac. It is not dependent upon PKC, PLC or Ca^{2+} (as judged by experiments with chemical inhibitors) revealing a novel monomeric G-protein-mediated pathway for GnRHR signaling [29].

The vast majority of work on GnRHR signaling has involved sustained stimulation whereas GnRH I is actually secreted from the hypothalamus in pulses of only a few minutes duration. However, brief or pulsatile GnRH I stimulation is known to activate a number of key effectors including G_q, G_s and G_i [68,69,83]. Downstream of G_q, the Ca^{2+}/calmodulin/calcineurin/NFAT and Raf/MEK/ERK signaling modules are both activated by pulsatile GnRH I [88,89], and gonadotrope ERK has been shown to be essential for reproduction [90] consistent with its role as an effector of pulsatile GnRHR activation *in vivo*. The mechanisms by which gonadotropes decode pulse frequency are largely unknown [2,8,88-93], as is the relevance of such mechanisms for GnRHR signaling in extra-pituitary sites (where patterns of GnRH exposure are unknown).

As noted previously, arrestin scaffolded effectors can mediate G-protein independent GPCR signaling. In many cases there is a transient wave of G-protein-mediated signaling from the plasma membrane, followed by a more sustained phase of arrestin-mediated signaling from endosomal receptor-arrestin signalsomes [51-55]. This model is not applicable to type I mammalian GnRHR for which neither arrestin-mediated nor endosomal signaling have been reported. Nevertheless, a G_q-independent mechanism for GnRHR-mediated ERK activation has been described [56,57] and cellular compartmentalization of GnRHR is certainly important for GnRHR signaling. Indeed, differential centrifugation has revealed that GnRHR are enriched in low buoyant membrane fractions suggesting their preferential localization in membrane rafts [83,94,95] along with several proteins implicated in GnRH signaling (i.e. G_q, calmodulin, Raf-1, MEK and ERK). Moreover, chemical treatments known to disrupt membrane rafts can also prevent GnRHR-mediated activation of PLC and ERK [83,95]. A more recent study revealed the presence of type I mammalian GnRHR in the nuclear membrane [96]. This is perhaps unsurprising given the large proportion of GnRHR thought to be retained in the ER and the fact that the nuclear and ER membranes are contiguous. However, it was also found that these receptors mediated effects of GnRH on histone phosphorylation and acetylation, demonstrating that GnRHR signaling is not necessarily restricted to the plasma membrane [96].

FUNCTIONAL SELECTIVITY AT GnRHR: CELL FATE

Functional selectivity of GPCR ligands (also known as ligand bias, biased receptor signaling, pluridimensional efficacy, biased agonism or ligand-induced selective signaling) requires the existence of at least two distinct active receptor conformations that are preferentially stabilized by different agonists and preferentially coupled to different effectors [29,83,97-99]. In this scenario the ligands are functionally selective or "biased" in that they selectively activate only a subset of the receptor's signaling repertoire. In reality the system is much more complex as molecular dynamic studies predict the occurrence of numerous different tertiary structures predominantly found in related groups of preferred conformations known as receptor ensembles [98]. Here, ligands alter the thermodynamics of receptor ensemble formations, and the distinct conformations within them differentially activate different effectors. In this way ligand efficacy reflects stabilization of ensembles of receptor conformations; it is thought unlikely that any two ligands would produce identical receptor ensembles or that different receptor ensembles would activate effectors identically [98] so ligand bias emerges as a likely (possibly universal) feature of GPCR function. In the case of type I mammalian GnRHR the absence of agonist-induced receptor phosphorylation and arrestin-mediated signaling may limit the number of GnRHR conformations and the effector repertoire, but even with these receptors there is clear evidence for multiple active receptor conformations and ligand bias for upstream signaling pathways (i.e. G_q versus G_s), global downstream readouts (i.e. inhibition of proliferation) as well as receptor trafficking (to and from the plasma membrane). Functional selectivity has a number of important implications [97-99] including the fact that conventional descriptors such as agonist, antagonist and

partial agonist must be qualified because efficacy is dependent upon the response measured. It also provides a possible explanation for qualitative differences in receptor function in different cell types (as the repertoire of active conformations adopted and effectors activated may be cell context-dependent). Indeed, the earliest evidence for ligand bias at GnRHR comes from experiments showing functional differences between endogenous GnRHR in different cell types.

To a large extent, cell context-dependent GnRH effects may be the inevitable consequence of differences in effector abundance and signal architecture. Accordingly, the fact that GnRH activates G_s in LβT2 cells [75] and activated G_i in numerous hormone-dependent cancer cells [29], but apparently has neither effect in αT3-1 cells [71,73], does not necessarily reflect stabilization of different active GnRHR conformations. Similarly, the fact that GnRHR mediate ERK activation by distinct mechanisms in different cell types [100] does not necessarily require activation of different receptor conformations. The alternative explanation is that identical receptor conformations signal differently because of differences in access to effectors, either because whole cell expression levels differ or because the proteins are differentially insulated from one-another physically or temporally. However, other differences are not easily explained without invoking multiple receptors or multiple conformations of a given receptor. Thus, for example it has long been known that the peptide "antagonist" cetrorelix is a pure antagonist of GnRH effects on IP accumulation and gonadotropin secretion in pituitary cells, but actually mimics anti-proliferative effects of GnRH in some models (20, 24,29). Similarly, GnRH II is more potent than GnRH I at inhibition of proliferation in some models [6,15,20,24-29,31,32], which is the reverse of the situation for stimulation of IP accumulation by type I GnRHR in pituitary cells. Reversal of potency or efficacy could potentially be explained by activation of distinct receptors [15,20] but as noted previously, humans express only one GnRHR [3,6,10,38]. This has been somewhat controversial but two key observations are that GnRHR transcripts are identical in pituitary and hormone-dependent cancer cells [23], and that effects of GnRH I and GnRH II are both prevented by depletion of type I GnRHR in epithelial and prostate cancer cell lines, confirming action via a single GnRHR [14,31]. Accordingly, ligand bias emerges as the most likely explanation for much data showing differences in ligand specificity when effects mediated by endogenous GnRHR have been compared in different cell types.

Nevertheless, caution is needed when interpreting such studies because, although GnRHR-mediated anti-proliferative and/or pro-apoptotic effects have been reported in many hormone-dependent cancer cell lines, it has proven difficult to unambiguously demonstrate the presence of endogenous GnRHR by radioligand binding, and some groups have found it necessary to heterologously express GnRHR in order to observe effects of GnRHR ligands [16-18, 26]. This could simply reflect existence of GnRHR positive and negative sub-clones of these cell lines (i.e. MCF-7, T47D, PC3 and LnCAP cells) but it is noteworthy that in the latter case, the functional characteristics (affinity, ligand specificity, $G_{q/11}$ coupling) of heterologously expressed receptors can mirror those of the endogenous GnRHR in gonadotropes, and where explored anti-proliferative effects appear to be mediated by G_q rather than G_i [16-18]. Such experiments with heterologous systems are consistent with a role for GnRHR in mediation of direct effects on cell fate and also revealed the importance of GnRHR number and function (i.e. the avoidance of desensitization) as determinants of efficacy. It remains unclear, however, why the endogenous type I GnRHR of GnRHR positive breast and prostate cancer cells should mediate proliferation inhibition by distinct mechanism to type I GnRHR expressed heterologously in GnRHR negative versions of these lines [16-18]. With this in mind, the most compelling data for GnRHR ligand bias comes from work with different ligands and heterologously expressed receptors. For example, we found as expected that *X. laevis* GnRHR expressed in HeLa cells have higher affinity for GnRH II than for a type I mammalian GnRHR-selective ligand (buserelin) and that PKC activation (with phorbol 12, 13 dibutyrate (PDBu)) increased the affinity of the *X. laevis* GnRHR for GnRH II but not for buserelin [101]. This was paralleled by an increase in Ca^{2+} mobilization by GnRH II (but not buserelin) and, interestingly, the C-tail was shown to be crucial for this effect as chimeric receptors (type I sheep or human GnRHR with an added *X. laevis* C-tail) showed the same PDBu induced increase in binding to GnRH II but not buserelin. These data demonstrate a novel form of "inside-out-signaling" and the existence of distinct conformations of C-tailed GnRHR that differ in relative specificity for GnRH II and buserelin [101]. They do not, however, demonstrate biased signaling and this mechanism is unlikely to be relevant to type I mammalian GnRHR. Much more direct evidence for ligand bias at such receptors has come from experiments comparing effects of series of GnRH analogues on different type I mammalian GnRHR-mediated responses.

Maudsley *et al*. (2004) demonstrated that a series of GnRHR ligands all inhibited proliferation (as indicated by a reduction in cell number) in JEG-3 cells and BPH-1 (both of which have endogenous human GnRHR) and in SCL60 cells (which have exogenous rat GnRHR). They all apparently activated G_i and caused PTX toxin sensitive inhibition of proliferation [29]. Marked ligand bias was observed because GnRH I stimulated IP accumulation, activated ERK, p38 and JNK and inhibited proliferation, whereas a GnRH analogue (135-25) mimicked all other GnRH I effects but did not stimulate IP accumulation [29]. In the SCL60 cells GnRH I has greater potency for stimulation of IP accumulation (EC_{50}<1 nM) than for inhibition of proliferation (EC_{50} approx. 100nM), whereas GnRH II has comparable potency for both measures (EC_{50}s 10-100 nM). There are only three amino acid differences between GnRH I and II, and subsequent work with peptide analogues revealed that Tyr^8 of GnRH II is the main determinant of its bias toward proliferation inhibition. Here, the idea is that interaction of Arg^8 of GnRH I interacts with Asp^{302} of the GnRHR receptor to stabilize an active GnRHR that preferentially activates G_q, whereas no such interaction occurs with ligands containing Tyr^8. Instead the Tyr^8 of GnRH II faces away from Asp^{302} (toward ECL2) and the ligand stabilizes a distinct receptor conformation that preferentially activates G_i [102]. More

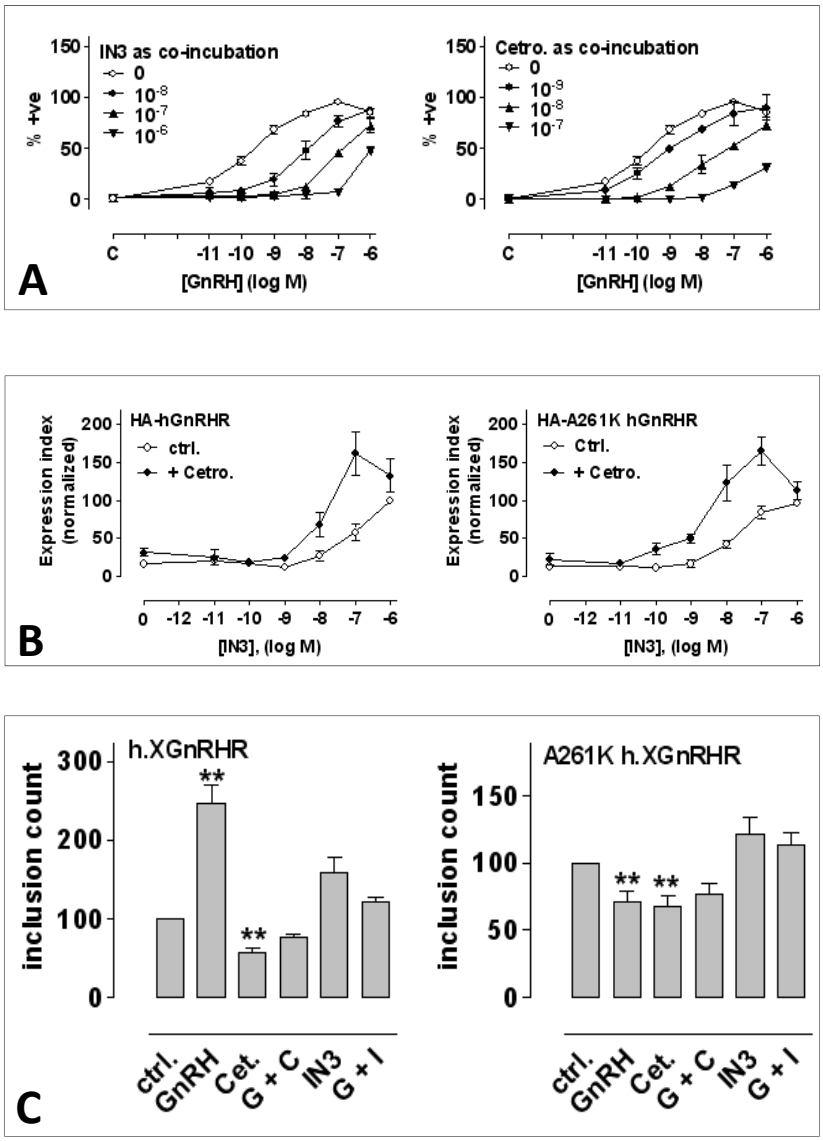

Fig. (1). Pluridimensional efficacy and qualification of pharmacological descriptors. Panel A: HeLa cells transduced with human GnRHR and an NFAT-EFP reporter were stimulated for 45 min with the indicated concentrations of GnRH I in the presence of the non-peptide IN3 and the peptide cetrorelix. Here, the translocation of NFAT-EFP from the cytoplasm to the nucleus is used as a readout for GnRH/Gq/PLC-mediated elevation of cytoplasmic [Ca^{2+}] and short treatment period ensures that IN3 has insufficient time to affect GnRHR trafficking. Under these conditions IN3 and cetrorelix both behave as competitive antagonists. **Panel B:** Cell surface GnRHR expression was assessed in HeLa cells transduced with HA-tagged human GnRH or A261K human GnRHR and incubated for 24 hr with varied concentrations of IN3 with or without cetrorelix as indicated. Under these conditions, IN3 causes the expected increase in cell surface HA-human GnRHR expression. Cetrorelix has little or no effect alone but clearly synergizes with IN3. Similar data are seen with the A261K "signal dead" mutant demonstrating that these trafficking effects are not dependent upon heterotrimeric G-protein activation. **Panel C:** An antibody loading protocol was used to monitor trafficking of GnRHR from the cell surface in HeLa cells transduced with HA-tagged version of a chimeric human GnRHR with an added *X.laevis* GnRH C-terminal tail, or with the signal dead A261K version of this construct. Acute stimulation with GnRH caused the expected increase in receptor internalization measured by counting labeled inclusions (presumptive endocytic vesicles) an effect that was dependent upon signaling as it was not only blocked by cetrorelix or IN3 but was also not seen with the A261K mutant receptors. Importantly, cetrorelix alone also reduced internalization (of both receptor constructs) suggesting that its ability to slow internalization (C) underlies its synergistic effect on cell surface GnRHR expression (B). Together these data illustrate that IN3 is a competitive antagonist in terms of cell surface GnRHR-mediate G_q activation but an agonist in terms of GnRHR trafficking to the cell surface. These effects are likely mediated by binding to distinct GnRHR conformations (intracellular conformations that have failed to meet quality control for ER exit, and cell surface GnRHR conformations that have met ER exit quality control criteria). Cetrorelix is also a competitive antagonist in terms of cell surface GnRHR-mediated G_q activation and apparently has no effect on GnRHR trafficking to the cell surface (it presumably does not access ER resident GnRHR) but is revealed as an inverse agonist for GnRHR internalization. See refs. 43 and 61 for further details.

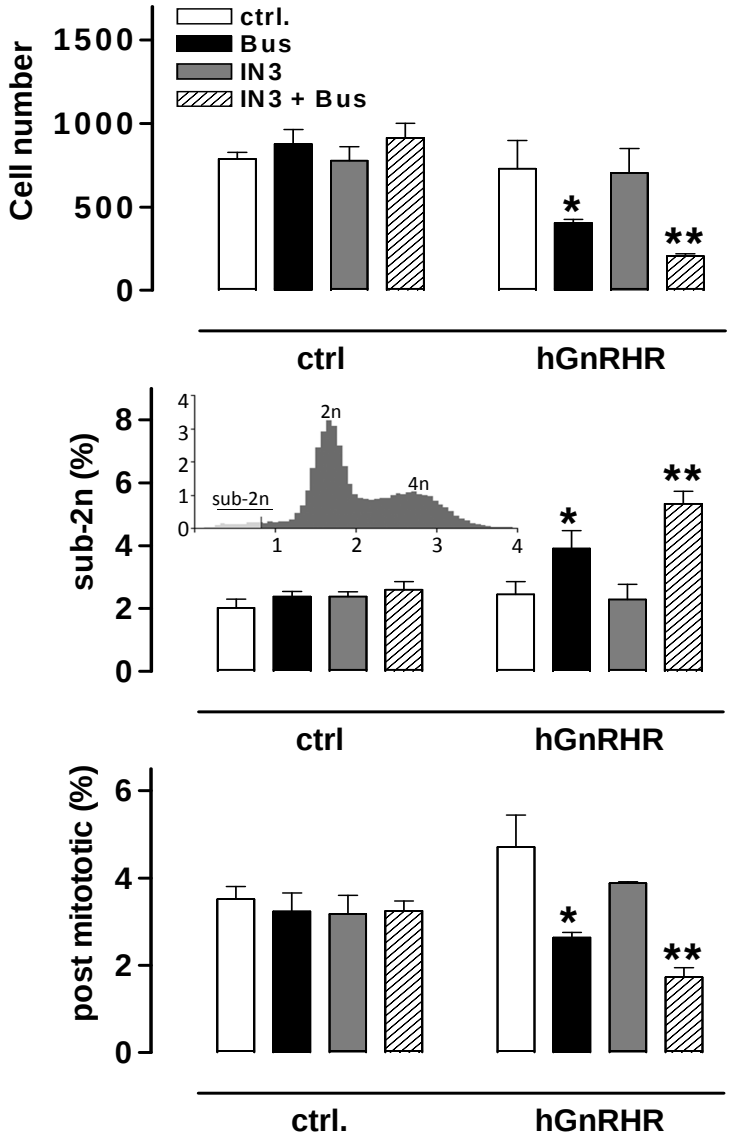

Fig. (2). GnRHR-mediated regulation of MCF7 cell fate. MCF7 breast cancer cells were infected with recombinant adenovirus expressing human GnRHR (hGnRHR) or with control virus (ctrl) and then cultured in with or without the GnRH agonist buserelin (Bus) and the non-peptide GnRHR ligand IN3 before using an automated imaging system to monitor cell number as determined by counting nuclei (upper panel), apoptosis, as determined by quantifying sub-2n cells from frequency distributions of nuclear stain intensity (middle panel) and cell proliferation, as determined by quantification of post-mitotic cells from nuclear stains (lower panel). In this model we find no evidence for endogenous GnRHR (so it is necessary to express human GnRHR in order to explore function), and in terms of acute cell surface GnRHR-mediated G_q activation, buserelin is a full agonist and IN3 behaves as a competitive antagonist. On chronic exposure IN3 greatly increases cell surface human GnRHR expression in these cells, just as it does in HeLa cells (Fig. **1**). As shown, buserelin has no effect on cell fate in control cells but in cells with heterologous human GnRH, it causes a pronounced reduction in cell number that is associated with an increase in apoptosis and a reduction in proliferation. IN3 also does not influence any of these parameters alone, but in combination it enhanced the effects of buserelin. This illustrates the complex pharmacology due to pluridimensional efficacy. Thus, IN3 is an antagonist in terms of cell surface GnRHR signaling but also increases cell surface GnRHR so its overall effect reflects relative potency and efficacy for these two responses. In the data shown, the non-peptide "antagonist" actually increases the antiproliferative and apoptotic effects of the agonist. See ref. 60 for further details.

recently Pfleger *et al.* used a domain swopping approach (human-catfish and human-chicken GnRHR chimeras) to demonstrate the importance of extracellular domains as determinants of ligand specificity, and provided further evidence that GnRH I and GnRH II stabilize distinct active GnRHR conformations [103].

FUNCTIONAL SELECTIVITY AT GnRHR: RECEPTOR TRAFFICKING

As noted previously, non-peptide pharmacological chaperones can increase GnRHR trafficking to the cell surface. Although the molecular mechanisms are poorly

understood, this effect provides an additional end-point for consideration of ligand bias. Indeed, the work with pharmacological chaperones provides a marked example of pluridimensional efficacy as non-peptide ligands can act as competitive or non-competitive antagonists at cell surface GnRHR, but as agonists in terms of anterograde trafficking. An intriguing possibility is that ligands could be developed that are selective for these effects. Indeed, comparing 3 non-peptide antagonists (IN3, NBI42902 and NBI54048) we found marked differences in potency for inhibition of cell surface GnRHR signaling but not for effects on cell surface expression (unpublished data). This not only implies that the effects may be selectively manipulated, but also that the cell surface and intracellular GnRHR have different conformations, which is not unexpected as most GnRHR within the cell have apparently failed quality control criteria for ER exit whereas those at the cell surface evidently have not.

In assessing the effects of pharmacological chaperones on cell surface GnRHR expression, control experiments were performed with two peptide antagonists (antide and cetrorelix). These are membrane impermeant GnRH ligands that would not be expected to access intracellular GnRHR and, as expected, had no measurable effect on the proportion of human GnRHR at the cell surface in MCF7 cells [60]. However, when the *X. laevis* C-tail was added to the human GnRHR in order to increase cell surface expression, it was noted that the peptide antagonists also increased cell surface GnRHR expression. Although the effect was modest (2 fold increase, as opposed to the 8 fold increase caused by IN3) it raised the possibility that the peptides might act at the surface to increase GnRHR number by slowing internalization. Subsequent experiments revealed that this is indeed the case [61], and that a marked synergism can occur when a non-peptide pharmacological chaperone is used to increase GnRHR trafficking to the cell surface and a peptide antagonists (cetrorelix) is used to slow internalization from the cell surface (Fig. **1**). This effect was seen with human and mouse GnRHR and in gonadotrope-lineage LβT2 cells, as well as in HeLa cells. Cetrorelix is not known to stimulate signaling in these models and similar data were obtained using the signal-dead A261K mutant of the human GnRHR [61]. Thus, although the molecular mechanisms remain unknown, this work clearly demonstrates that the cetrorelix-occupied human GnRHR is functionally distinct from the unoccupied receptor and that cetrorelix can be a pure antagonist for GnRH I-stimulated IP accumulation and Ca^{2+} signaling, and an inverse agonist for receptor internalization. Here, the important feature is that this ligand bias is seen with a compound that is used clinically and in gonadotropes, the only proven targets for GnRHR-directed therapy.

OVERVIEW AND SUMMARY

Functional selectivity at GPCRs has a number of important implications for understanding and manipulating GnRHR signaling. It provides a likely explanation for much early work indicating reversal of efficacy or potency when comparing effects of GnRHR ligands in different cellular contexts and may prove crucial for understanding the physiological relevance of the two GnRH ligands acting via a single GnRHR in humans. It highlights the need to qualify classical pharmacological descriptors as, for example, cetrorelix has been known for decades to be a full antagonist of GnRHR-mediated gonadotropin secretion from pituitary cells but also appears to be a full agonist for GnRHR-mediated proliferation inhibition in some models and an inverse agonist for down-regulation of cell surface GnRHR in others (Fig.**1**). Similarly, non-peptide ligands that are pure antagonists for GnRHR-mediated IP accumulation and Ca^{2+} signaling may act as full agonists for GnRHR trafficking to the cell surface, providing complex pharmacological profiles dependent upon relative potency for the two effects and receptor location in ligand naïve cells (Fig. **2**). Most importantly, it raises the exciting possibility that ligands may be developed to more selectively engage therapeutically beneficial cellular responses. In the case of the GnRHR, an obvious strategy would be develop ligands that have direct G_i mediated anti-proliferative and/or pro-apoptotic effects on hormone-dependent cancers and also act as pure antagonists for G_q-mediated stimulation of gonadotropin secretion from the pituitary. The GnRH analogue 135-25 provides an example of a compound with this profile. The major challenge is now to understand the pharmacological relevance of functional GnRHR selectivity in terms of both on-target and off-target GnRHR-mediated effects *in vivo*.

DISCLOSURES

The author has nothing to disclose.

ACKNOWLEDGMENTS

Work in the McArdle lab. is supported by the Wellcome Trust (084588) and Medical Research Council (G0901763).

ABBREVIATIONS

GnRH = gonadotropin-releasing hormone also known as GnRH I

GnRHR II = gonadotropin-releasing hormone II, also known as chicken GnRH

GnRHR = GnRH receptor

GPCR = G-protein coupled receptor

LH = luteinizing hormone

FSH = follicle-stimulating hormone

GRK = G-protein coupled receptor kinase

PKC = protein kinase C

PLC = phospholipase C

ERK = extracellular signal-regulated kinase, here used to denote ERK1 and ERK2

JNK = c-Jun N-terminal kinase

MAPK = mitogen-activated protein kinase

MEK = MAPK/ERK kinase

FAK = focal adhesion kinase

NFAT = nuclear factor of activated T-cells) GFP (green fluorescent protein)

EFP = emerald fluorescent protein

cAMP = cyclic adenosine 3', 5' monophosphate

CNS = central nervous system

ER = endoplasmic reticulum

IP = inositol phosphate

PTX = pertussis toxin

EGF = epidermal growth factor

PDBu = phorbol 12, 13 dibutyrate

buserelin = [T-BuSer^6, Pro^9 NH ethylamide]GnRH

cetrorelix = Ac-D2Nal-D4Cpa-D3Pal-Ser-Tyr-DCit-Leu-Arg-Pro-DAla-NH2) and IN3 ((2S)-2-[5-[2-(2-axabicyclo[2.2.2]oct-2-yl)-1,1-dimethy-2-oxoethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine.

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Received: August 12, 2011 Revised: August 23, 2011 Accepted: September 02, 2011