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REVIEW

Trafficking and signalling of gonadotrophin-releasing hormone receptors: an automated imaging approach

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Gonadotrophin-releasing hormone (GnRH) is a neuropeptide that mediates central control of reproduction by stimulating gonadotrophin secretion from the pituitary. It acts via 7 transmembrane region (7TM) receptors that lack C-terminal tails, regions that for many 7TM receptors, are necessary for agonist-induced phosphorylation and arrestin binding as well as arrestin-dependent desensitization, internalization and signalling. Recent work has revealed that human GnRH receptors (GnRHR) are poorly expressed at the cell surface. This apparently reflects inefficient exit from the endoplasmic reticulum, which is thought to be increased by pharmacological chaperones (non-peptide GnRHR antagonists that increase cell surface GnRHR expression) or reduced by point mutations that further impair GnRHR trafficking and thereby cause infertility. Here, we review recent work in this field, with emphasis on the use of semi-automated imaging to interrogate compartmentalization and trafficking of these unique 7TM receptors.
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Abbreviations: 7TM, 7 transmembrane region; GnRH, gonadotrophin-releasing hormone; GnRHR, GnRH receptor; PCSE, proportional cell surface expression; PM, plasma membrane

Introduction

Gonadotrophin-releasing hormone (GnRH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2, also known as GnRH I) stimulates secretion of the gonadotrophin hormones (luteinizing hormone and follicle-stimulating hormone) from gonadotrophs, and thereby mediates central control of reproduction. It acts primarily via Gαq-coupled 7 transmembrane region (7TM) receptors to stimulate phospholipase C, with consequent mobilization of Ca^{2+} and activation of protein kinase C isozymes, both of which are important for chronic control of gonadotropin synthesis and the acute control of exocytotic gonadotrophin secretion (Stojilkovic and Catt, 1995; Millar *et al.*, 2004; Cheng and Leung, 2005). Most vertebrates also express the highly conserved GnRH II ([His^5, Trp^7, Tyr^8]GnRH I), and ligand-selective receptors have evolved in parallel with these distinct forms of GnRH. Mammalian type I GnRH receptor (GnRHR) are selective for GnRH I and, unlike all other 7TM receptors, lack C-terminal tails (Millar *et al.*, 2004; Cheng and Leung, 2005). For many 7TM receptors, agonist-induced and G-protein receptor kinase-mediated phosphorylation within the receptor's C-terminal tail facilitates binding to β-arrestins, thereby causing arrestin-dependent receptor desensitization and internalization, as well as arrestin-mediated signalling to arrestin-scaffolded effectors (Luttrell and Lefkowitz, 2002; Pierce *et al.*, 2002). Agonist-induced phosphorylation, arrestin binding, arrestin-mediated desensitization, internalization and signalling have all been demonstrated for non-mammalian GnRHRs (i.e. for catfish or Xenopus GnRHRs, both of which have C-terminal tails with multiple potential phosphorylation sites) but not for the tailless type I GnRHRs. Where investigated, these aspects of receptor function have been conferred by addition of non-mammalian GnRHR C-terminal tails to type I GnRHRs

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supporting the notion that the molecular evolution of these receptors has led to loss of functionally relevant C-terminal tails with the advent of type I mammalian GnRHRs (Davidson *et al.*, 1994; McArdle *et al.*, 1995; 1996; Heding *et al.*, 1998; Lin *et al.*, 1998; Pawson *et al.*, 1998; Vrecl *et al.*, 1998; Blomenrohr *et al.*, 1999; Hislop *et al.*, 2000; Hislop *et al.*, 2001; Willars *et al.*, 2001; McArdle *et al.*, 2002; Millar *et al.*, 2004).

The lack of homologous desensitization of type I mammalian GnRHR is particularly intriguing in light of the fact that sustained stimulation does cause desensitization of GnRH-stimulated gonadotrophin secretion. Indeed, such desensitization underlies the therapeutic use of GnRH agonists in treatment of hormone-dependent cancers (i.e. breast and prostate cancer) and other steroid hormone-dependent conditions (i.e. endometriosis) where long-term agonist therapy ultimately reduces circulating levels of gonadotrophins and gonadal steroids (Conn *et al.*, 1987; Stojilkovic and Catt, 1995; McArdle *et al.*, 2002; Millar *et al.*, 2004; Cheng and Leung, 2005). The mechanisms underlying homologous desensitization of GnRH-stimulated gonadotrophin secretion in the absence of receptor desensitization are unknown but may well involve post-receptor adaptive processes such as IP3 receptor down-regulation (Willars *et al.*, 2001; McArdle *et al.*, 2002) and desensitization of voltage-operated Ca^{2+} channels (Stojilkovic and Catt, 1995). In the long term, depriving the cells of the pulsatile GnRH needed for efficient transcription of the genes encoding the GnRHR and gonadotropin subunits may contribute to (or explain) the chemical castration caused by GnRH agonists *in vivo* (Huhtaniemi *et al.*, 2009). In addition, early work on GnRHR trafficking suggested that agonists induce their endocytic internalization from the plasma membrane (PM), thereby reducing cell surface GnRHR number (Conn *et al.*, 1987; McArdle *et al.*, 1987; 2002; Schvartz and Hazum, 1987), so agonist-induced reduction in cell surface GnRHR number could cause desensitization of GnRH-stimulated gonadotrophin secretion even in the absence of receptor desensitization. However, this begs the question of how agonists can target GnRHR for internalization without phosphorylation, desensitization or arrestin binding, and the dogma was recently challenged by work showing that type I mammalian GnRHR undergo constitutive (but not agonist-induced) internalization when expressed in COS7 and HEK293 cells (Pawson *et al.*, 2008). In addition to work on internalization, research emphasis has recently shifted to the control of GnRHR trafficking to the PM. This was spurred by the discovery of a number of GnRHR point mutants that cause infertility (hypogonadotropic hypogonadism) in humans. Although initially thought to perturb signalling, it is now clear that most of these mutations actually impair trafficking, reducing the number of GnRHRs at the cell surface and thereby reducing responsiveness to GnRH. Here, a key observation was that a membrane permeant non-peptide GnRHR antagonist (IN3) could facilitate signalling via most of these mutant hGnRHRs (Janovick *et al.*, 2002; 2003; Ulloa-Aguirre *et al.*, 2004; Brothers *et al.*, 2006; Conn *et al.*, 2006; 2007; Conn and Janovick, 2009). By analogy with other 7TM receptor systems (Edwards *et al.*, 2000; Petaja-Repo *et al.*, 2000; 2001; Bernier *et al.*, 2004; Tan *et al.*, 2004; Dong *et al.*, 2007), this antagonist was thought to act as a pharmacological chaperone, enabling the GnRHR to fold appropriately into the conformation required for trafficking from the endoplasmic reticulum (ER) to the surface. It also caused a modest increase in signalling via the wild-type GnRHR suggesting that there is also a significant reserve of potentially functional hGnRHR within the cell. Indeed, it is now thought that hGnRHR traffic relatively inefficiently from the ER (as compared with other GnRHR) and that this makes them susceptible to effects of point mutations that further impair trafficking.

GnRHR imaging

With the notable exception of early electron microscopy studies (Hopkins and Gregory, 1977; Duello *et al.*, 1983), biochemical approaches have been used rather than imaging for most work on GnRHR trafficking. Thus, most quantitative work on GnRHR internalization has actually monitored uptake of radiolabelled agonists in spite of the fact that it is not clear whether this reflects constitutive or agonist-induced uptake (above), and the fact that the ligand and receptor traffic differently after internalization (Cornea *et al.*, 1999). Similarly, the ability of pharmacological chaperones to increase cell surface GnRHR expression has been inferred from increased GnRH-stimulated [^{3}H]IPx accumulation (Janovick *et al.*, 2002; 2003; Ulloa-Aguirre *et al.*, 2004; Brothers *et al.*, 2006; Conn *et al.*, 2006; 2007) in spite of the fact that the precise relationship between [^{3}H]IPx accumulation and receptor number is unknown, not least because it is difficult to wash the antagonist from the cells before agonist stimulation (Janovick *et al.*, 2002; 2003). More direct measurement of GnRHR localization has been hampered by the lack of validated antibodies to normal GnRHR, and although green fluorescent protein (GFP)-tagged GnRHR have been generated these have GFP added to the C-terminus via spacers, such as the catfish GnRHR C-terminal tail (Brothers *et al.*, 2003). This raises the obvious possibility that the fluorophore and/or spacer may themselves influence receptor localization or trafficking. As an alternative strategy we have developed models based on adenovirus-mediated heterologous expression of GnRHRs with N-terminal (exofacial) epitope tags and indirect immunofluorescence staining using a semi-automated microscopy for image acquisition (IN Cell Analyzer 1000, GE Healthcare UK Ltd., Amersham, UK), and validated algorithms for image segmentation and quantification (IN Cell Analyzer version 1.0 software). Using this we simply quantified HA-GnRHR staining at the cell surface (anti-HA added to intact cells) and the whole cell (anti-HA added to permeabilized cells) for a range of GnRHR constructs and cell types incubated with or without IN3 (Finch *et al.*, 2008). We calculated whole cell and cell surface expression indices (by compounding the % of +ve cells by the mean fluorescence intensity in those cells) and used these to determine the proportional cell surface expression (PCSE) as shown in Figure 1. This revealed that a remarkably small proportion of HA-hGnRHRs are located at the cell surface (PCSE <1% in ligand naïve MCF7 cells). In contrast, the PCSE of a non-mammalian GnRHR (the XGnRHR) was much higher (40–60%), and addition of the XGnRHR C-tail to the hGnRHR (h.XGnRHR) increased PCSE approximately fivefold. We also

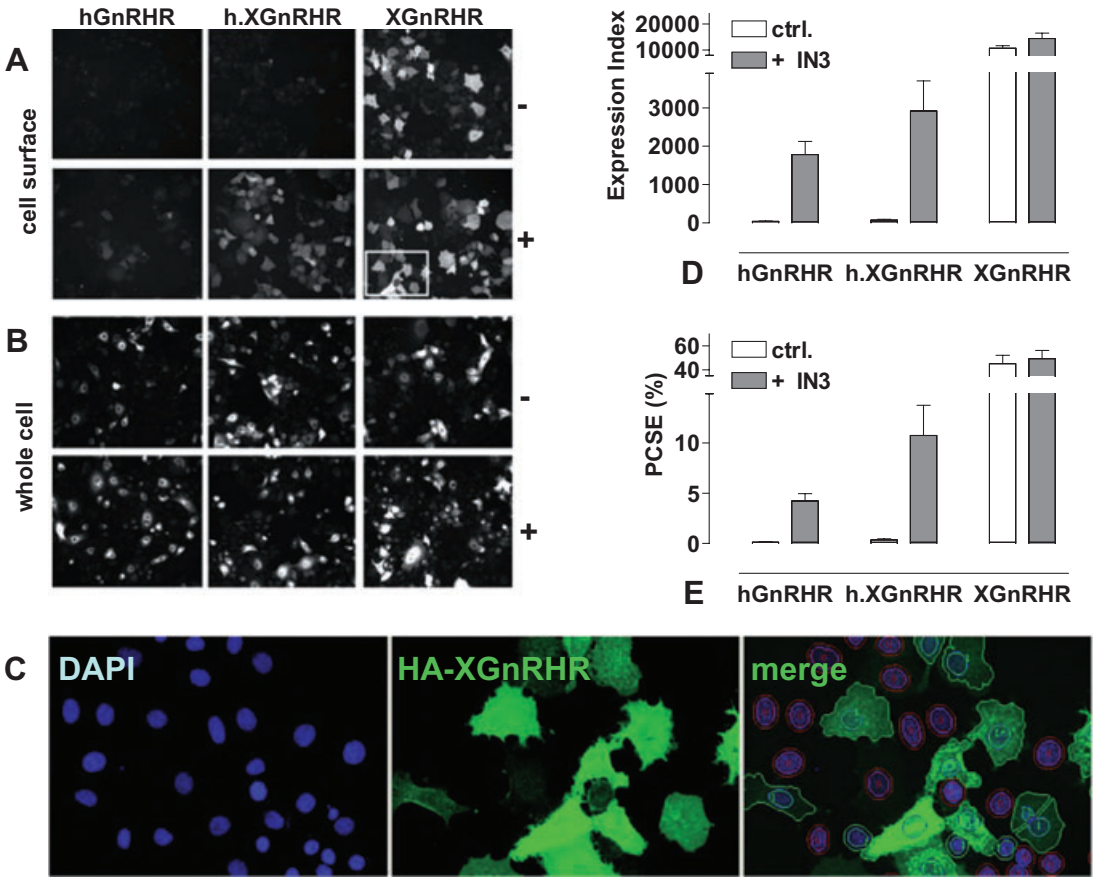

Figure 1 An automated imaging assay for gonadotrophin-releasing hormone receptors (GnRHR). MCF7 cells grown in 96 wells were transduced with Ad expressing N-terminal HA-tagged hGnRHR, XGnRHR or h.XGnRHR then incubated ~20 h with 0 or 1 μM of the non-peptide antagonist IN3 before indirect fluorescence labelling of cell surface receptors (primary antibody added to intact cells) or whole cell receptors (primary antibody added after permeabilization). Nuclei were also stained with DAPI, and digital images were captured using a 10× objective and a 0.6 mm^2 field of view. Panels A and B show representative images (each approximately 25% of the field captured) of whole cells and cell surface staining in cells transduced with the indicated receptors. Panel C shows a higher power image of nuclei, HA-XGnRHR and merged stains from the boxed region in panel A. It also illustrates the automated image segmentation used to define perimeters of nuclei (blue) and cells (green or red) and application of a filter to distinguish cells in which staining was >10% above background (green perimeters) or <10% above background (red perimeters). An expression index (EI) was calculated by multiplying the percentage of positively stained cells by the mean fluorescence intensity (arbitrary fluorescence units) in those cells, and proportional cell surface expression (PCSE) was calculated as the cell surface EI as a percentage of the whole cell EI. Panel D shows cell surface EI, and panel E shows PCSE (data from the same representative experiment as used for panels A–C).

found that the distribution of these receptors is dependent upon cellular context. hGnRHR PCSE was consistently low (<1%), and the non-peptide antagonist effect was pronounced (10–20-fold increases in PCSE) in a number of breast and prostate cancer cell lines, whereas the PCSE was higher (5–20%), and the effect of IN3 is less pronounced (maximally threefold) in gonadotroph-lineage cells (Finch *et al.*, 2008). Throughout these experiments we found that measures of cell surface GnRHR expression mirrored measures of PCSE (Figure 1). This is because whole cell expression levels were comparable for the different receptors and treatments and clearly underlines the importance of compartmentalization in determining cell surface GnRHR number.

In principle, the data outlined above could have been obtained by con-focal microscopy (compare Sedgley *et al.*, 2006 and Finch *et al.*, 2008), but the automated imaging system has the advantage of rapidly providing quantitative data from large numbers of cells without user bias on cell selection or image analysis. We routinely work with cells in 96-well plates, quantifying GnRHR in 25–100 000 cells per plate, with image acquisition and analysis each complete within 20–30 min. For most studies, interpretation is based on data pooled from multiple experiments (i.e. from thousands of cells), but imaging measures are available for each individual cell and this enables analysis on a cell by cell basis. For example, scatter plots of cell surface versus whole cell HA-GnRHR fluorescence reveal linear relationships between whole cell and cell surface expression for hGnRHR and XGnRHR and that cell surface XGnRHR expression is higher throughout the expression range (Figure 2). The availability of single cell data also facilitates analysis of sub-populations as illustrated by comparison of cell surface GnRHR expression at matched whole cell expression levels (500–1000 AFU, Figure 2 inset). Such analysis clearly argues against overexpression as a reason for the distinct localization of these receptors. Moreover, by varying adenoviral titre and comparing cell surface receptor expression, measured by radioligand binding and immunofluorescence it is possible to calibrate the latter. This

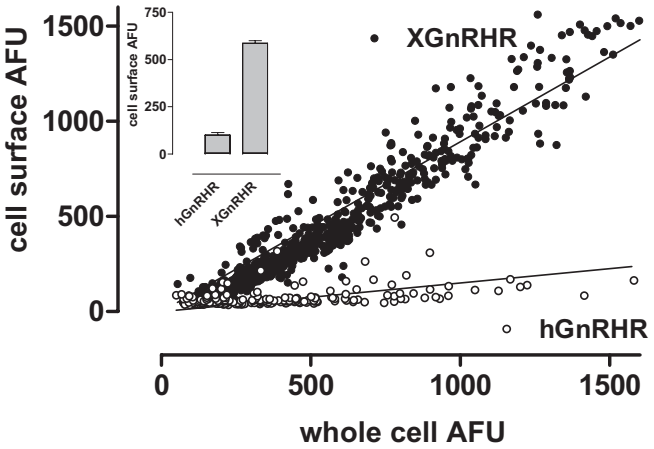

Figure 2 Single cell analysis. The automated imaging data can be analysed at the population or single cell level. To illustrate the latter, gonadotrophin-releasing hormone receptor (GnRHR)-transduced MCF7 cells were sequentially stained for cell surface receptors and whole cell receptors. The main panel shows cell surface HA-hGnRHR or HA-XGnRHR staining (Alexa fluor 564 conjugated goat anti-mouse IgG) plotted against whole cell staining (Alexa fluor 488 conjugated goat anti-mouse IgG) in arbitrary fluorescence units (AFU). The single cell data can be filtered as illustrated by the inset where the cell surface AFU is shown for cells in which whole cell expression levels were 500–1000 AFU.

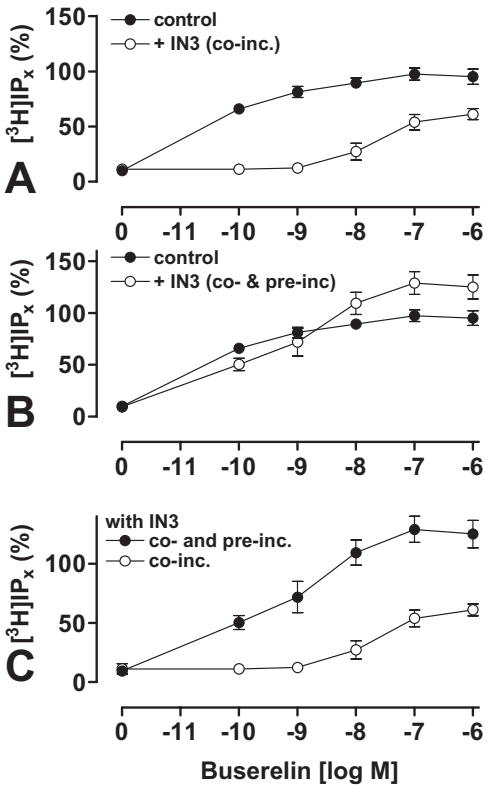

Figure 3 Effect of IN3 on gonadotrophin-releasing hormone receptor (GnRHR) signalling. [^{3}H]IPx accumulation concentration–response curves were constructed for hGnRHR-transduced MCF7 cells labelled with [^{3}H]inositol and stimulated for 45 min with agonist (buserelin) in the presence of 10 mM LiCl. When IN3 (0.1 μg·mL^{-1}) was present at the same time as the agonist (co-inc., co-incubation) it behaved as a competitive antagonist (A), but when cells were pre-incubated for approximately 20 h with the IN3 and then stimulated with Buserelin in the continued presence of IN3 (pre- and co-incubation) the inhibitory effect of IN3 was reduced or lost (B). Panel C shows concentration–response curves for cells co-incubated with IN3 or co- and pre-incubated with IN3 (data from A and B respectively). Because these curves are constructed under conditions of identical proportional receptor occupancy (i.e. both have IN3 and agonist co-incubation) the difference between the curves is due to the IN3 pretreatment. Adapted from Finch *et al.* (2008).

revealed that maximal cell surface expression of hGnRHRs in these models is <10 000 sites per cell, as compared with approximately 80 000 sites per cell for the endogenous mouse GnRHR in gonadotroph-lineage cell lines (McArdle *et al.*, 1992), so clearly the low hGnRHR PCSE is not an artefact of overexpression.

GnRHR signalling

The low proportion of HA-hGnRHR at the cell surface (<1% in MCF7 cells) is remarkable in light of the robust hGnRHR-mediated [^{3}H]IPx accumulation seen in these cells (Finch *et al.*, 2004). This led us to suspect that the HA-tag was influencing receptor function (see also Brothers *et al.*, 2003), but we have tested for this extensively and found no effect of the tag (Finch *et al.*, 2008 and Finch and McArdle, unpubl. data). In our hands it does not measurably alter binding affinity or specificity (radioligand binding assays monitoring [^{125}I]Buserelin binding to hGnRHR, hXGnRHR or mGnRHR with or without N-terminal HA-tag in HeLa or MCF7 cells), cell surface expression (measured by whole cell radioligand binding assays in cells incubated with Ad GnRHR at varied titres) or ligand potency (agonist and antagonist potency measured in various functional assays). We were also concerned that the imaging assay simply quantifies the proportion of HA-tag at the cell surface and that this might not equate to functional GnRHR expression, and therefore tested for functional correlates of IN3 effects on receptor expression in MCF7 cells (Figure 3). When IN3 (0.1 μg·mL^{-1}) was present at the same time as the agonist (co-incubation) it behaved as a competitive antagonist, but when cells were pre-incubated for approximately 20 h with the IN3 and then stimulated with Buserelin in the continued presence of IN3 (pre- and co-incubation) the inhibitory effect of IN3 was reduced or lost. The effect of the pretreatments is evident in comparison of concentration–response relationships for cells co-incubated, or co- and pre-incubated with IN3 (Figure 3C). Because these curves are constructed under conditions of identical proportional receptor occupancy (i.e. both have IN3 and agonist co-incubation) the difference between the curves is due to the IN3 pretreatment. The IN3 pretreatment clearly increased the response to Buserelin demonstrating that the pretreatment increased functional hGnRHR (as well as increasing HA-hGnRHR staining) at the cell surface. More recently we have obtained similar data using GnRHR-mediated translocation of a Ca^{2+}-sensitive reporter (NFAT1c-EFP) in HeLa cells and have found a similar IN3-induced increase in cell surface expression of functional hGnRHR irrespective of whether or not the receptors have an HA-tag (Finch and McArdle, unpublished). As an alternative approach we used the images of DAPI-stained nuclei to assess effects of Buserelin and IN3 on cell number, cell proliferation

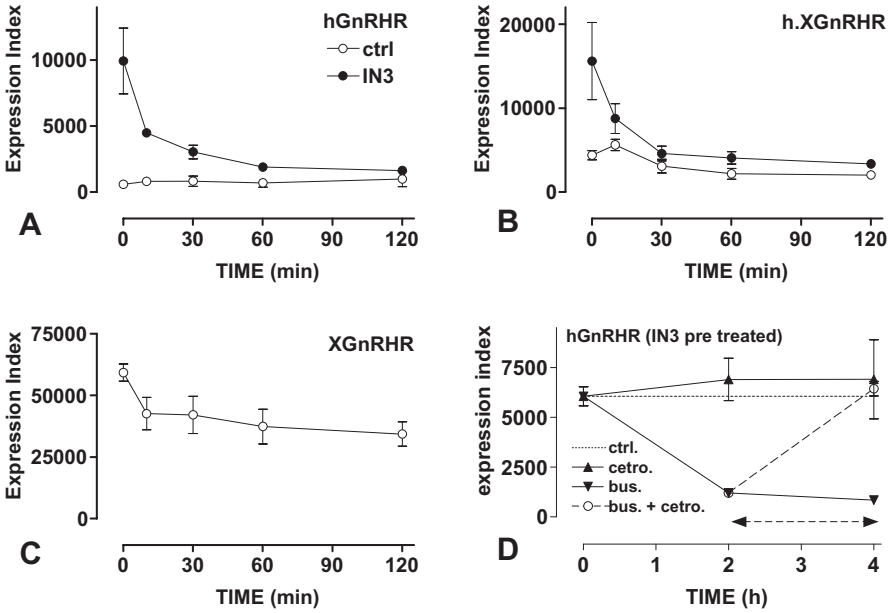

Figure 4 Agonist-induced down-regulation of cell surface gonadotrophin-releasing hormone receptor (GnRHR). (A–C) HeLa cells transduced with HA-tagged hGnRHR, h.XGnRHR or XGnRHR were incubated for ~20 h with 0 (open circles) or 1 μM (filled circles) IN3 before being washed and stimulated for the indicated period with 10^{-7} M GnRH (GnRH II for the XGnRHR). (D) Ad HA-hGnRHR transduced HeLa cells were incubated for approximately 20 h with 1 μM IN3 before being treated (for 2 or 4 h from time 0) with buserelin or cetrorelix (bus. or cetro., each at 10^{-7} M) or for 2 h with buserelin and then for 2 h with the peptide agonist and antagonist together (bus. + cetrorelix.). The expression index in control cells (maintained in medium with IN3 alone) is shown by the horizontal dotted line. All panels show data processed for automated imaging to determine cell surface GnRHR as under Figure 1 and are means (±SEM) from representative experiments.

and apoptosis in MCF7 cells (Finch *et al.*, 2008). None of these parameters were influenced by the agonist or by IN3 in control cells (lacking endogenous GnRHR), but in cells transduced with Ad hGnRHR, Buserelin reduced cell number and proliferation and increased apoptosis. These effects were not mimicked or blocked, but were actually enhanced by IN3 (Finch *et al.*, 2008) presumably because it had increased trafficking of functional GnRHR to the cell surface during the 48 h incubation period used for these experiments. Together, these functional data parallel the imaging, supporting the notion that the majority of hGnRHR are located in a potentially functional intracellular pool that can be brought to the cell surface by the non-peptide antagonist acting as a pharmacological chaperone.

Agonist-induced down-regulation and trafficking of GnRHR

As noted above, it has long been assumed that GnRH stimulates internalization and thereby reduces GnRHR at the cell surface. However, early data supporting this were largely derived from rodent models (and it is now thought that rat and mouse GnRHR are better expressed at the cell surface than hGnRHR), and recent studies reveal that type I mammalian GnRHR undergo constitutive (but not agonist-stimulated) internalization in COS7 and HEK293 cells (Pawson *et al.*, 2008). Using the automated imaging system to monitor agonist effects on cell surface HA-GnRHR levels (Figure 4) we found that GnRH II caused a rapid reduction in cell surface XGnRHR (significant reduction at 10 min, maximal effect at 120 min) and that GnRH also reduced cell surface mouse (m)GnRHR and h.XGnRHR (within 30–120 min). GnRH did not measurably alter cell surface hGnRHR, which is not surprising given the low cell surface levels for this receptor in unstimulated cells (Figure 4). As expected, pre-incubation with IN3 increased cell surface hGnRHR (>10-fold) and h.XGnRHR (>threefold) but did not alter expression of the XGnRHR (not shown). Importantly, GnRH caused a pronounced reduction in cell surface hGnRHR and h.XGnRHR in IN3-pretreated cells (Figure 4). In similar experiments, addition of a peptide antagonist (cetrorelix, added after 20 h with IN3) did not reduce hGnRHR or h.XGnRHR levels (Figure 4 and data not shown), but when added after buserelin, cetrorelix reversed the reduction in cell surface hGnRHR caused by the agonist (Figure 4). GnRH also failed to reduce cell surface receptors in cells expressing a mutant of the hGnRHR or h.XGnRHR (A261K) that does not activate its cognate G-protein (Myburgh *et al.*, 2002; Finch *et al.*, 2009).

The data above reveal that agonist-induced down-regulation of hGnRHR occurs in HeLa cells, and that such down-regulation is dependent upon activation (rather than just occupancy) of cell surface GnRHR and is reversed when receptor activation is stopped. Suspecting that this reflects agonist-induced internalization and recycling of internalized receptors to the cell surface (when activation is terminated) we developed additional automated imaging assays to test for this. In the first experiments we simply transduced cells with Ad GnRHR (with or without exofacial HA-tag), then incubated them with a primary anti-HA for various periods at 37°C (with or without agonist) before fixation, permeabilization and staining. Non-specific staining (HA detection in cells

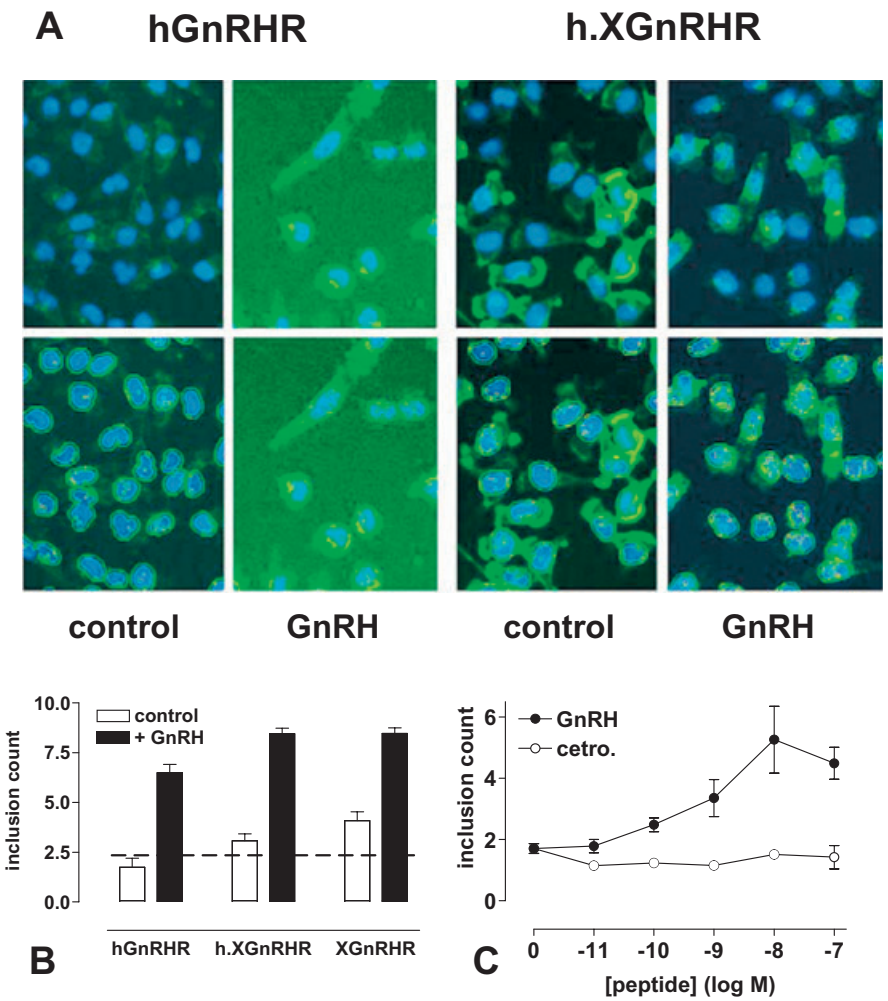

Figure 5 Anti-HA loading assays. Panel A shows data from a receptor trafficking assay in which HeLa cells transduced with HA-hGnRHR or HA-h.XGnRHR were incubated with mouse anti-HA for 60 min at 37°C with 0 (control) or 10^{-7} M GnRH as indicated. They were then fixed, permeabilized and stained with Alexa fluor 488 conjugated goat anti-mouse IgG and DAPI before image acquisition (10× objective). Merged images are shown for small regions (approximately 10% of a single field of view). The lower images illustrate the automated segmentation procedure (Multi-target Analysis algorithm with Analyzer software) used to define the nuclear perimeter and then add a 2 μm collar (green lines) and quantify the bright inclusions (yellow lines) in the cross-sectional area defined by the expanded nucleus. Panel B shows data from a representative experiment in which inclusion counts were increased by agonist stimulation of HA-tagged hGnRHR, h.XGnRHR and XGnRHR. Also shown (horizontal dotted bar) is the inclusion count seen in cells expressing receptors without HA-tags (i.e. background staining and/or receptor-independent uptake). Panel C shows data from an internalization assay in which HeLa cells transduced with HA-hGnRHR or HA-h.XGnRHR then loaded with mouse anti-HA for 60 min at room temperature, then washed and stimulated for 60 min at 37°C in medium with the indicated concentration of GnRH before being fixed, permeabilized and stained with Alexa fluor 488 conjugated goat anti-mouse IgG and DAPI as above. Note that GnRH stimulated HA-hGnRHR trafficking (B) but that inclusion counts were too low for assessment of GnRH effects on HA-hGnRHR internalization (C). cetro., cetrorelix; GnRH, gonadotrophin-releasing hormone; GnRHR, GnRH receptor.

expressing non-tagged h.XGnRHR) was negligible but clear HA-h.XGnRHR staining was observed after 60 min incubation with anti-HA (Figure 5 images and Finch *et al*., 2009). In unstimulated cells HA-h.XGnRHR was relatively evenly distributed over the cells with more intense staining evident at the perimeter of many cells. Addition of GnRH caused a pronounced redistribution of HA-h.XGnRHR or HA-hGnRHR to intense punctate regions that were often prevalent around the nucleus. Similar staining was seen in cells expressing HA-XGnRHR (Figure 5 and Finch *et al*., 2009). These staining patterns are suggestive of agonist-induced internalization, and this was confirmed by monitoring uptake of fluorophore-labelled transferrin (which undergoes receptor-mediated uptake into endosomes and is then trafficked to post-endosomal sorting compartments) under similar conditions. In control cells there was little co-localization of HA-GnRHR and transferrin but in GnRH stimulated cells, the fluorophores were often co-localized in punctate regions (Finch *et al*., 2009). This agonist-induced redistribution and co-localization with transferrin receptors is similar to that previously seen by confocal microscopy (i.e. for HA-tagged rat GnRHR in HEK293 cells, Vrecl *et al*., 1998) supporting the notion that GnRH also stimulates the redistribution of HA-GnRHR from the cell surface to endosomes in the HeLa cell model . This redistribution was quantified in a 'granularity assay' in which an image segmentation algorithm was used to determine the number of small intensely stained 'inclusions' in the area defined by adding a 2 μm collar to the

nuclear perimeter. Using this 'inclusion count' as a measure of GnRHR trafficking from the PM to endosomes, we found that this was relatively low for each of the receptors used but was increased by stimulation with GnRH (HA-hGnRHR and HA-h.XGnRHR) or GnRH II (HA-XGnRHR) with maximal inclusion counts after 30–60 min of activation in each case (Finch *et al.*, 2009). Very few inclusions were seen in cells expressing non-tagged GnRHRs, and the agonist had no measurable effect on distribution of the G-protein signalling deficient HA-A261K-h.XGnRHR (Finch *et al.*, 2009).

The protocol used above to demonstrate agonist-induced trafficking of HA-tagged hGnRHR, h.XGnRHR and XGnRHR is dependent upon antibody (Ab) binding to cell surface receptors in the presence of agonist, so the stimulus could conceivably be increasing trafficking to and/or from the PM. To monitor internalization more directly, cell surface HA-GnRHR were Ab loaded by incubation for 60 min at 21°C, before washing and incubation for varied times at 37°C in the presence or absence of agonist. HA-GnRHR stained inclusions were then quantified by imaging as above. This revealed a clear time- and dose-dependent agonist-stimulated internalization of HA-tagged h.XGnRHR (effect maximal at 30–60 min, pEC50 9.5), but inclusion counts were too low for measurement of hGnRHR internalization with this assay (Figure 5 Finch *et al.*, 2009). Taken together, these data reveal that agonists can stimulate the trafficking of hGnRHR and h.XGnRHR, the internalization of h.XGnRHR and the down-regulation of cell surface of h.XGnRHR, mGnRHR and XGnRHR, but that internalization and/or down-regulation of hGnRHR are only evident when steps are first taken to increase cell surface expression (IN3 pretreatment or C-tail addition).

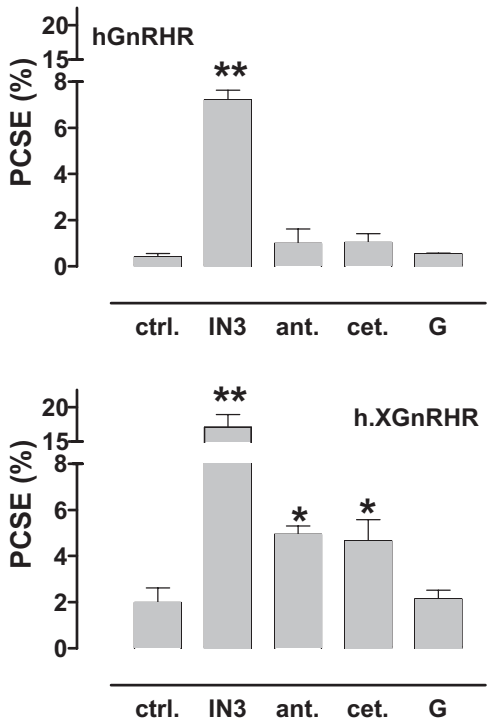

Figure 6 Peptide and non-peptide antagonist effects on gonadotrophin-releasing hormone receptor (GnRHR) expression. Ad HA-hGnRHR or HA-h.XGnRHR-transduced MCF7 cells were incubated for approximately 20 h in control medium (ctrl.) or in medium with IN3, cetrorelix (cet.), antide (ant.) or GnRH (G), each at 10^{-7} M, before determining the proportional cell surface expression (PCSE) as in Figure 1. Note that IN3 increased hGnRHR and hXGnRHR PCSE and this effect was not mimicked by cetrorelix or antide in hGnRHR expressing cells. However, these peptide antagonists did increase PCSE and expression index (not shown) in h.XGnRHR expressing cells. *$P < 0.05$, **$P < 0.01$. Adapted from Finch *et al.* (2008).

Peptide and non-peptide antagonist effects

Pharmacological chaperones are thought to bind receptors within the ER, holding them in a conformation suitable for ER exit, thereby facilitating their delivery to the PM. Key evidence for this includes ER localization of the receptor, dependence of the chaperone effect on ER to golgi transit and the inability of membrane-impermeant peptide ligands to mimic effects of the membrane permeant pharmacological chaperone (Kopito and Ron, 2000; Bernier *et al.*, 2004). In accord with this we have shown that HA-hGnRHR is largely co-localized with calreticulin (an ER marker) in MCF7 cells (Sedgley *et al.*, 2006). Moreover, knock-down of calnexin (a molecular chaperone mediating ER exit of many proteins) can reduce hGnRHR-mediated [^{3}H]IPx accumulation and radioligand binding to cell surface hGnRHRs in COS7 cells (Brothers *et al.*, 2006). We also compared the effects of IN3 with two peptide antagonists and as expected, IN3 increased the HA-hGnRHR PCSE whereas the peptides had no such effect (Figure 6) (Finch *et al.*, 2008). The clear implication is that IN3 acts as a pharmacological chaperone, increasing HA-hGnRHR PCSE by binding nascent receptors within the cell and that the peptides fail to do so because they do not access these intracellular receptors. We also found that these peptide antagonists failed to block the effect of IN3 on HA-hGnRHR PCSE, again arguing that they do not have access to the intracellular site at which IN3 acts (not shown). However, we were surprised to find that the peptide antagonists did increase PCSE of HA-h.XGnRHRs. Although their effects were much less than that of IN3 (Figure 6), they were statistically significant. This difference in efficacy was not due to difference in time courses because effects of IN3 and the peptide antagonists were both maximal at 24–48 h of incubation, and differences in potency can also be excluded because maximally effective concentrations of these peptides (10^{-7} and 10^{-8} M) were less effective than IN3 (data not shown). Moreover, the effects were specific to the peptide antagonists because the agonists GnRH and buserelin failed to increase the PCSE of HA-h.XGnRHRs (Figure 6). Theoretically, the proportion of GnRHRs PCSE could be elevated by increasing ER to PM trafficking, by reducing internalization from the PM or by accelerating recycling of internalized receptors back to the PM. The IN3 effect is largely (or entirely) attributable to the first of these mechanisms but, as GnRH analogues are taken up by receptor-mediated endocytosis and do not pass freely across the PM (Schvartz and Hazum, 1987), and both peptides failed to inhibit IN3 effects on HA-hGnRHR PCSE, they are unlikely to have access to GnRHRs within the ER. We therefore suggest that the peptide antagonists could act at the cell surface to slow internalization in the face of ongoing

trafficking of the HA-h.XGnRHR to and from the PM. This observation has important implications for GnRHR function. Conventional receptor theory assumes that there are single inactive and active receptor conformations that are induced or stabilized by antagonists and agonists respectively. It is now clear, however, that there are multiple active conformations for many 7TM receptors, including GnRHRs (Caunt *et al.*, 2004; Maudsley *et al.*, 2004). Existence of such conformations underlies the ligand bias seen with many 7TM receptors (Galandrin and Bouvier, 2006; T Kenakin, 2007). The pharmacological chaperone effect of IN3 implies that there are also distinct unoccupied and antagonist-occupied conformations (the unoccupied hGnRHR conformation that fails to exit the ER, and the IN3-occupied conformation that is trafficked to the cell surface). However, conformations within the ER could presumably relate to protein maturation and would not necessarily equate to multiple inactive conformations of the mature receptor at the cell surface. Most importantly, the effect of the peptide antagonists reveals the existence of an antagonist-occupied GnRHR conformation at the cell surface that differs from that of the unoccupied receptor (and causes the cetrorelix-induced increase in PCSE). Accordingly, it appears that multiple functionally distinct type I mammalian GnRHR conformations can exist at the cell surface that are inactive in terms of phospholipase C activation. Such conformations may prove relevant to GnRHR signalling in non-pituitary sites (i.e. in hormone-dependent cancers) where coupling to effectors other than Gq/11 may occur, and effects of agonists have been found to be mimicked rather than blocked by peptide antagonists (Eidne *et al.*, 1987; Imai *et al.*, 1997; Emons *et al.*, 1998; Limonta *et al.*, 2003; Moretti *et al.*, 2003; Maudsley *et al.*, 2004).

Conclusions and future directions

Many studies over the last 15 years have revealed GnRHRs as structurally and functionally unique. Notably, they have undergone a relatively recent period of accelerated molecular evolution in which the advent of mammals has coincided with the loss of C-terminal tails and associated functions including rapid homologous desensitization, agonist-induced phosphorylation and arrestin-mediated signalling (McArdle *et al.*, 2002; Millar *et al.*, 2004; Cheng and Leung, 2005; Caunt *et al.*, 2006). The discovery that type I mammalian GnRHRs do not desensitize underlines the importance of cell surface GnRHR number in determining responsiveness to GnRH, just as the more recent discovery that most hGnRHRs are intracellular underlines the importance of trafficking to (as well as from) the PM in determining cell surface GnRHR number. We have begun to explore the regulation of these issues using automated cell imaging methods that offer a number of advantages including: (i) the removal of user bias on selection of cells or regions of interest; (ii) the high throughput achievable in assays of whole cell expression, cell surface expression and granularity (i.e. endocytic trafficking); (iii) the availability of single cell data for frequency distribution study or for sub-population analysis; and (iv) the potential to combine receptor imaging with imaging of signalling reporters at the single cell and cell population level. Using these assays we have found the proportion of hGnRHRs at the cell surface is remarkably low (<1% in MCF7 cells) and that GnRHR compartmentalization is dependent upon receptor structure (as evidenced by the greater PCSE for XGnRHRs) and conformation (as evidenced by the >10-fold increase in hGnRHR PCSE caused by a pharmacological chaperone). We also found that the proportion of h.XGnRHRs at the cell surface can be increased by two (membrane impermeant) peptide antagonists in MCF7 cells. This is compatible with the different classes of antagonist having distinct sites and mechanisms of action, the non-peptide antagonist IN3 acting intracellularly to increase receptor trafficking to the PM and the peptide antagonist cetrorelix acting at the cell surface to slow receptor internalization. If so, this would mean that a peptide antagonist that is used to treat various hormone-dependent conditions and is well established as a pure GnRHR antagonist in many assays (Schally, 1999) is actually an inverse agonist in terms of internalization. In spite of such developments, it is remarkable how little is actually known about the control of GnRHR trafficking. We know that agonists stimulate type I mammalian GnRHR internalization and down-regulation but do not know how the agonist targets them for internalization without receptor phosphorylation or arrestin binding. Similarly, we know that the vast majority of hGnRHR are intracellular in heterologous expression systems but (in the absence of well validated antibodies) the proportion of endogenous GnRHR at the cell surface and the possibility that this is regulated physiologically remain unexplored. Finally, we know that the proportion of GnRHR at the cell surface is dependent upon receptor structure and conformation and that this most likely reflects differences in efficiency of ER exit, but we know very little about which components of the molecular machinery for ER exit quality control machinery restrict or permit GnRHR exit, and nothing about how they sense GnRHR structure and conformation. We suggest that the high throughput imaging techniques described here should facilitate RNA inhibition screens for exploration of the molecular mechanisms controlling GnRHR trafficking to and from the cell surface.

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Statement of conflicts of interest

None.

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