Intracellular gonadotropin-releasing hormone receptors in breast cancer and gonadotrope lineage cells

Kathleen R Sedgley*, Ann R Finch*, Christopher J Caunt and Craig A McArdle

Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, UK

(Requests for offprints should be addressed to C A McArdle; Email: craig.mcardle@bris.ac.uk)

*(K R Sedgley and A R Finch contributed equally to this work)

Abstract

Gonadotropin-releasing hormone receptors (GnRHRs) are expressed in gonadotropes and several extra-pituitary sites. They are assumed to be cell surface proteins but the human (h) GnRHR lacks features favoring plasma membrane localization and receptor location varies with cell type. When expressed in mammary (MCF7) cells, cell surface hGnRHR binding was much lower than that of mouse and sheep GnRHRs (type I GnRHRs without C-terminal tails), *Xenopus* (X) and marmoset type II GnRHRs (type II GnRHRs with C-tails) or chimeric receptors (type I GnRHRs with added XGnRHR C-tails). hGnRHR binding was higher in α T4 (gonadotrope-derived) cells and was increased less by C-tail addition. Whole cell levels of tagged human, *Xenopus* and chimeric GnRHRs were comparable (Western blotting) and confocal microscopy revealed that the hGnRHR is primarily intracellular

(distribution similar to the endoplasmic reticulum marker, calreticulin), whereas most XGnRHR is at the plasma membrane, and adding the C-tail increased cell surface hGnRHR levels. A membrane-permeant antagonist increased cell surface hGnRHR number (>4 -fold, $t_{1/2} = 4$ h) and also increased hGnRHR signaling and hGnRHR-mediated inhibition of proliferation. A more rapid increase in hGnRHR binding occurred when the temperature was raised from 4 to 37 °C (>5 -fold, $t_{1/2} = 15$ min) and this effect was prevented by mutation to prevent signaling. Thus, cell surface GnRHR expression depends on receptor and cell type and the hGnRHR is primarily an intracellular protein that traffics to the cell surface for signaling in MCF7 cells. Manipulations favoring such trafficking may facilitate selective targeting of extra-pituitary GnRHRs.

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Introduction

Gonadotropin-releasing hormone (GnRH) stimulates gonadotropin secretion from the pituitary and thereby controls gametogenesis and steroidogenesis in the gonads (Stojilkovic & Catt 1995, Millar *et al.* 2004). At the pituitary, GnRH binds 7 transmembrane region (7TM) receptors that act via $G_{q/11}$ to stimulate phospholipase C. The consequent activation of protein kinase C and increase in the cytoplasmic Ca^{2+} concentration mediate GnRH effects on gonadotropin secretion and synthesis. GnRH-stimulated gonadotropin secretion can be blocked with antagonists, or mimicked by agonists, but in the latter case, sustained stimulation causes desensitization. Thus, both treatments ultimately reduce circulating gonadotropins and gonadal steroids *in vivo*, and this 'medical castration' underlies the use of GnRH analogs to treat steroid hormone-dependent neoplasms such as those of the prostate, ovary, endometrium or mammary gland (Schally 1999). In addition to expression in the pituitary, GnRH receptors (GnRHRs) are found (often along with GnRH) in many cancers of reproductive tissues (Cheng & Leung 2005). Interest in these extra-pituitary GnRHRs stems primarily from

the fact that GnRH analogs (or their cytotoxic derivatives) can inhibit proliferation of cell lines derived from such cancers, and that direct anti-proliferative effects may therefore contribute to the therapeutic effects of GnRH analogs in cancer treatment (Eidne *et al.* 1987, Kakar *et al.* 1994, Imai *et al.* 1997, Emons *et al.* 1998, Schally & Nagy 1999, Limonta *et al.* 2003, Moretti *et al.* 2003, Cheng & Leung 2005).

The 7TM receptors are typically located in the plasma membrane, where ligand binding causes activation of cognate G-proteins. On sustained or intense stimulation this is normally followed by receptor desensitization, internalization and down-regulation, processes mediated by receptor phosphorylation and arrestin binding (Pierce & Lefkowitz 2001, Shenoy & Lefkowitz 2003). In contrast to our detailed knowledge of ligand-induced 7TM receptor trafficking, much less is known about the molecular determinants of receptor localization in the ligand naïve state. In this state, the subcellular compartmentalization of 7TM receptors is dependent upon the efficiency with which nascent receptors are delivered to and retained at the plasma membrane, parameters that are dependent not only on receptor structure, but also on receptor number and cell type (Edwards *et al.* 2000, Tan *et al.* 2004).

The cloned GnRHRs can be divided phylogenically into two distinct groups (Silver *et al.* 2005). Type I GnRHRs that mediate hypothalamic control of mammalian reproduction are preferentially activated by GnRH I. Remarkably, they lack C-terminal tails, structures that are present in all other 7TM receptors and are implicated in many aspects of receptor function (Bockaert *et al.* 2003). For many 7TM receptors, the agonist-induced phosphorylation that mediates arrestin binding (with consequent desensitization and internalization) occurs in the receptor's C-tail. However, type I GnRHRs are unique, in that where investigated, they do not bind arrestins or signal via arrestins, do not rapidly desensitize and are internalized slowly, apparently because they lack C-tails (Davidson *et al.* 1994, McArdle *et al.* 1995, 1996, 2002, Lin *et al.* 1998, Pawson *et al.* 1998, Vrecl *et al.* 1998, Blumenrohr *et al.* 1999, Willars *et al.* 1999, 2001, Heding *et al.* 2000, Hislop *et al.* 2000, 2001, Caunt *et al.* 2006). In contrast, type II GnRHRs (which include all non-mammalian GnRHRs) are preferentially activated by GnRH II and do have C-terminal tails. Where investigated, they bind arrestins, signal via arrestin (e.g. to ERK) and undergo rapid and arrestin-dependent internalization and desensitization (Davidson *et al.* 1994, McArdle *et al.* 1995, 1996, 2002, Lin *et al.* 1998, Pawson *et al.* 1998, Vrecl *et al.* 1998, Blumenrohr *et al.* 1999, Willars *et al.* 1999, 2001, Hislop *et al.* 2000, 2001, Caunt *et al.* 2006).

In addition to their roles in signaling, the C-tails of 7TM receptors can also influence receptor stability and it has been shown that addition of the catfish GnRHR C-tail to the rat GnRHR or addition of the *Xenopus* (X) GnRHR C-tail to the human or sheep GnRHRs increases cell surface receptor number (Lin *et al.* 1998, Heding *et al.* 2000, Finch *et al.* 2004, Caunt *et al.* 2006). The oligosaccharide side-chains of glycosylated receptors can also have major effects on expression by targeting the receptors to the plasma membrane. For example, the mouse (m)GnRHR has N-linked glycosylation sites at Asn4 and Asn18 within the amino-terminal sequence and is expressed at higher levels than the human (h)GnRHR (which has only the Asn18 site). hGnRHR expression can be increased by introducing a second glycosylation site (Asn4), just as mGnRHR expression can be reduced by removal of the Asn18 site (Davidson *et al.* 1995, 1996). More recently, the primate GnRHR-specific K191 residue has been shown to reduce cell surface GnRHR expression (Arora *et al.* 1999, Janovick *et al.* 2003) and the importance of trafficking to the plasma membrane has been illustrated by the discovery of a number of GnRHR point mutants that cause hypogonadotropic hypogonadism in humans. Although these were initially thought to impair ligand binding or effector coupling, it is now recognized that loss of receptor function actually reflects misrouting of otherwise functional receptors. Here, a key observation is that exposure to a membrane-permeant GnRHR antagonist (IN3) can facilitate signaling via several of these mutant hGnRHRs (Janovick *et al.* 2002, 2003, 2006, Brothers *et al.* 2004, Ulloa-Aguirre *et al.* 2004, Knollman *et al.* 2005). This antagonist apparently acts as a pharmacological chaperone,

enabling the protein to fold appropriately into the conformation required for trafficking to the surface and thereby overcoming the inhibitory effect of the mutation. In this context, the quality control system of the endoplasmic reticulum (ER) may be particularly important, ensuring that inappropriately folded proteins do not escape the ER, or are targeted to proteasomes for degradation. It has recently been shown that over 60% of newly synthesized δ -opioid receptor fails to traffic to the cell surface but is instead degraded in the proteasome and, in this model, membrane-permeant agonists and antagonists both increased wild-type receptor trafficking to the plasma membrane (Petaja-Repo *et al.* 2002). In the case of the GnRHR, IN3 also has a modest effect on the wild-type receptor leading to the suggestion that as much as 50% of the human GnRHR does not traffic to the plasma membrane in COS7 cells (Brothers *et al.* 2004).

In recent studies, we have used recombinant adenovirus (Ad) to express GnRHRs in hormone-dependent cancer cells that lack endogenous GnRHRs (as judged by radioligand binding and functional assays). In MCF7 breast cancer cells (for example), we find that transduction with Ad sheep (s)GnRHR causes expression of high affinity G_q -coupled receptors that do not rapidly desensitize or internalize and mediate a potent antiproliferative effect of GnRH agonists that is dependent upon receptor number (Everest *et al.* 2001, Finch *et al.* 2004). In this model, antiproliferative effects mediated by the hGnRHR were consistently lower than those seen with the sGnRHR, presumably because the hGnRH was expressed at lower levels. In light of the data outlined above, we speculated that the hGnRHR may be inefficiently trafficked to and retained at the plasma membrane (as compared with other GnRHRs) and that these processes might differ for GnRHRs expressed in pituitary and extra-pituitary sites. To address these possibilities, we have generated recombinant Ad expressing epitope-tagged human, *Xenopus* and chimeric GnRHRs and used these to explore receptor compartmentalization, trafficking and function. Here we show that the vast majority of hGnRHR expressed in unstimulated MCF7 cells is actually intracellular. Consequently, signaling is dependent upon ongoing trafficking of cryptic receptors to the cell surface and it may be possible to exploit this finding in therapies targeting extra-pituitary GnRHRs.

Materials and Methods

Materials

Peptides were from Sigma except for Buserelin and [125 I]Buserelin ([T-BuSer⁶, Pro⁹ NH ethylamide]GnRH-2000 Ci/mmol) that were provided by Prof. Sandow (Aventis Pharma GmbH, Frankfurt, Germany). [125 I]GnRH II (approximately 3400 Ci/mmol) was prepared as described (Hislop *et al.* 2001). Myo-[2- 3 H]inositol (10–25 Ci/mmol) and [methyl- 3 H]-thymidine were from Perkin-Elmer, (Boston, MA,

USA). The membrane-permeant GnRHR antagonist, IN3 ((2S)-2-[5-[2-(2-axabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine) was provided by Dr Ashton Wallace (Merck and Co. Inc., Rahway, NJ, USA). Culture media and plasticware were from Gibco BRL and sera were from First Link (Brierly Hill, West Midlands, UK) and antibodies were from Invitrogen. cDNAs encoding wild-type GnRHRs were provided by Prof. R Millar (Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK).

Engineering of receptors, cell culture and transfection

Recombinant, E1-deleted Ad expressing mGnRHRs or mIIgNHRs were made as described for the Ad sGnRHR, Ad hGnRHR and Ad XGnRHR and for chimeras consisting of the sGnRHR or hGnRHR with an added XGnRHR C-terminal tail (Ad s.XGnRHR and Ad h.XGnRHR) (Hislop *et al.* 2001, Finch *et al.* 2004). The hGnRHR and the h.XGnRHR were haemagglutinin (HA)-tagged as described for the XGnRHR (Caunt *et al.* 2004) and used to generate Ad as described (Caunt *et al.* 2006). A261K mutations in HA-tagged receptor vectors were generated using the Quick Change mutagenesis kit (Stratagene) and the primers 5'-GAA CAAT AT ACC AAG AAA ACG GCT GAA GAC TCT AAA AAT GAC GG-3' and 5'-CCG TCA TTT TTA GAG TCTTCA GCC GTT TTC TTG GTA TAT TGT TC-3' according to the manufacturer's instructions prior to sequence conformation and Ad generation as above (Anderson *et al.* 2000, Caunt *et al.* 2004). MCF7 cells from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and α T4 cells from Prof. P Mellon (University of San Diego, CA, USA) were cultured as described (Hislop *et al.* 2000, Finch *et al.* 2004). For experiments, cells were transferred to culture plates and infected the following day with Ad (6 h) before medium change. They were then cultured for 4–5 days before use in [3 H]thymidine incorporation assays or for 1 day for all other assays.

Radioligand binding, [3 H]inositol phosphate (3 H)IP_x) accumulation and [3 H]thymidine incorporation assays

Radioligand binding to GnRHRs was measured in whole cell assays, with cells grown in 24-well plates (Hislop *et al.* 2001, Finch *et al.* 2004). Briefly, 50 000 cells/well were incubated in physiological salt solution (PSS: 127 mM NaCl, 1.8 mM CaCl₂, 5 mM KCl, 2 mM MgCl₂, 0.5 mM NaH₂PO₄, 5 mM NaHCO₃, 10 mM glucose, 0.1% BSA and 10 mM HEPES, pH 7.4) containing $1\text{--}2.5 \times 10^{-10}$ M [125 I]Buserelin or [125 I]GnRH II and either 0 (total binding) or 10^{-6} M (non-specific binding) of the homologous competitor. Incubation times and temperatures are detailed in the figure legends and incubations were terminated by washing in ice-cold PSS before solubilization and quantification (Hislop *et al.* 2001, Finch *et al.* 2004). For comparison with earlier studies, the first binding experiments were performed using a 30-min incubation at 21 °C, conditions providing equilibrium binding to cell surface

GnRHRs without measurable internalization (McArdle *et al.* 1987, Hislop *et al.* 2000). Subsequent experiments were performed at 4 or 37 °C in order to prevent or permit (respectively) receptor trafficking and function. For some experiments, receptor numbers (sites/cell) were calculated from the single-point binding data using published K_d estimates (1.4 nM for [125 I]Buserelin binding to type I GnRHRs and 1.4 nM for [125 I]GnRH II binding to type II GnRHRs), the actual radioligand concentration, and assuming a Hill coefficient of 1, as described (Finch *et al.* 2004). [3 H]IP_x accumulation was used as a measure of phospholipase C activity in cells labeled with [3 H]inositol and stimulated in the presence of 10 mM LiCl (Hislop *et al.* 2001, Caunt *et al.* 2004, Finch *et al.* 2004) using the stimuli detailed in the figure legends. [3 H]Thymidine incorporation was used as an index of cell proliferation in cells grown in medium with 2% foetal calf serum (FCS), as described (Hislop *et al.* 2001, Finch *et al.* 2004) using the stimuli detailed in the figure legends.

Western blotting

Cells plated at 400 000 cells/60 mM dish were lysed 16 h after infection and extracted for Western blotting as described (Hislop *et al.* 2001). Briefly, cells were washed in ice-cold PBS before being lysed for 30 min on ice in 200 μ l extraction buffer (10 mM Tris (pH 7.6), 1 mM EDTA, 150 mM NaCl, 25 mM KCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and Protease Inhibitor Cocktail Tablets (Roche)). After separation by SDS-PAGE (10% gel), the cells were transferred to polyvinylidene difluoride membrane and blocked with 5% skimmed milk in Tris-buffered saline (10 mM Tris (pH 8.0) in 150 mM NaCl) with 0.1% Tween. The HA epitope was detected using mouse monoclonal anti-HA.11 (1:1000, clone 16B12, Cambridge Bioscience, Cambridge, UK) and visualized by enhanced chemiluminescence. Bands were scanned and quantified by densitometry (Image J, NIH, Bethesda, MA, USA) and HA-GnRHR expression was normalized to HA-hGnRHR expression in α T4 cells.

Confocal microscopy

Cells were cultured and infected with Ad GnRHRs as described (Hislop *et al.* 2005). For cell surface staining, they were incubated for 1 h at 4 °C in DMEM with 1% BSA and mouse monoclonal anti-HA (1:200), washed with ice-cold PBS, fixed (30 min in 2% paraformaldehyde/PBS) then permeabilized (10 min in PBS/0.1% Triton X-100), washed (3 $\times$), blocked (1 h in PBS/0.1% Triton X-100/1% BSA) and incubated 1 h with Alexa-Fluor 488 conjugated anti-mouse (1:500, in PBS/0.1% Triton X-100/1% BSA). They were then washed with PBS (2 $\times$), incubated with 2 μ M 4',6-diamidino-2-phenylindole (DAPI, 10 min) and washed (3 $\times$) before mounting in fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA). For whole cell staining, cells were washed with PBS, fixed, permeabilized and blocked as above before being exposed

to anti-HA (as above) with or without rabbit polyclonal anti-calreticulin (1:200, Sigma). They were then washed in PBS (3×, 5 min), incubated with Alexa Fluor 488 conjugated anti-mouse with or without Alexa Fluor 568 conjugated anti-rabbit (1:500) and DAPI, and mounted, as above. Cells were imaged using a Leica TCS-SP2 confocal laser scanning microscope attached to a Leica DM IRBE inverted epifluorescence microscope with a 63× HCX Apo BL 1:40 NA oil immersion objective and excitation using 450 and 488 nm laser lines. For internally controlled experiments, images were collected at identical photomultiplier settings and analyzed with Leica LCS Lite software. Where confocal images were quantified, ImageJ software was used to determine fluorescence intensity (e.g. in plasma membrane or cytoplasm) by drawing around areas of interest. To avoid bias, this was performed by users unaware of the experimental treatments.

Statistical analysis and data presentation

The figures show the mean $\pm$ S.E.M. of data pooled from n independent experiments (raw data or data normalized as described in the figure legends) except for the Western blot and confocal microscopy images, which are representative of those obtained in at least three experiments. Statistical analysis was by Student's t -test (accepting $P < 0.05$ as statistically significant).

Results

Expression and distribution of GnRHRs in MCF7 and α T4 cells

We have recently used recombinant Ad to express GnRHRs in MCF7 breast cancer cells and explored the relationship between Ad titer and expression of human (h), sheep (s) and *Xenopus* (X) GnRHRs as well as chimeric receptors consisting of the entire hGnRHR or sGnRHR sequence with an added XGnRHR C-tail (h.XGnRHR and s.XGnRHR) (Finch *et al.* 2004). Here, we have extended this to include the mouse (m) GnRHR and the type II marmoset (mII) GnRHR measuring cell surface receptor number by radioligand binding to intact cells for 30 min at 21 °C. In each case, maximal cell surface receptor numbers were achieved at Ad titers of 1 or 2×10^7 pfu/ml and these are illustrated in Fig. 1. As shown, binding was barely detectable for the hGnRHR (approximately 2000 sites/cell) and was much lower than that seen for the other type I GnRHRs (approximately 30 000 and 50 000 sites/cell for the mGnRHR and the sGnRHR respectively). These values are similar to those estimated for endogenous GnRHRs of rat and sheep pituitaries or murine gonadotrope-derived cell lines (approximately 20 000–80 000 sites per gonadotrope (Everest *et al.* 2001)). The XGnRHR and mII GnRHR were expressed at approximately 25 000 and 110 000 sites/cell (respectively) and addition of the XGnRHR C-tail increased binding of both type I GnRHRs (approximately 40 000 and 400 000 sites/cell for the h.XGnRHR and s.XGnRHR respectively).

We next generated Ad expressing HA-tagged hGnRHRs, XGnRHRs and h.XGnRHRs and found the receptors to be indistinguishable from wild-type receptors in functional and competition binding assays (not shown). The epitope-tagged receptors were then expressed in MCF7 cells and in α T4 cells (gonadotrope progenitor cells that lack endogenous GnRHRs) prior to Western blotting. As shown (Fig. 2A), the HA-hGnRHR ran as two major bands of approximately 34 and 36 kDa and fainter band of 60–70 kDa. With overexposed gels, a 32 kDa band was also seen. These data are consistent with earlier studies showing that the non-glycosylated rat GnRHR shows anomalous electrophoretic mobility (running with an apparent size of 32 kDa in spite of its actual molecular weight of approximately 38 kDa) and the existence of larger forms reflecting glycosylation and/or the presence of multimers (Neill *et al.* 1997). Similar bands were seen with the HA-h.XGnRHR, although addition of the C-tail shifted the smaller bands to higher apparent molecular weights (approximately 38 and 40 kDa respectively) and these were accompanied by a more pronounced smeared band of 50–100 kDa in both cell types (Fig. 2A). The HA-XGnRHR also ran as two minor bands of approximately 40 and 42 kDa in addition to a prominent broad band of 50–100 kDa. The HA-XGnRHR separation pattern is similar to that previously reported for the catfish GnRHR (Willars *et al.* 1999) and that seen with an HA-tagged mII GnRHR (not shown). Parallel radioligand binding studies (Fig. 2C) revealed that the HA-hGnRHR was expressed at very low levels (< 0.1 fmol/well) on the surface of MCF7 cells as compared with the HA-h.XGnRHR (approximately 2.4 fmol/well) and the HA-XGnRHR (approximately 2.6 fmol/well), whereas Western blotting with whole cell proteins revealed relatively high levels of HA-hGnRHR in

Figure 1 GnRHR binding in MCF7 cells: species comparison. MCF7 cells were infected with Ad expressing the human (h), sheep (s), mouse (m), *Xenopus laevis* (X), marmoset type II (mII) receptors or chimeras with the C-tail of the XGnRHR added at the human or sheep GnRHRs (h.X and s.X respectively). They were then used in whole cell radioligand binding assays (incubation for 30 min at 21 °C) as described in Materials and Methods. The data are from a larger series in which Ad titer was varied and show maximal receptor number (e.g. that with Ad at $1\text{--}2 \times 10^7$ pfu/ml). Receptor number was calculated from specific binding, assuming equilibrium binding to a single receptor class (K_d values of 1.4 nM for each receptor-cognate radioligand pair) and a Hill coefficient of 1. The data shown are pooled from ten experiments, each with triplicate observations (mean $\pm$ S.E.M., $n=3\text{--}10$).

Figure 2 Western blotting and radioligand binding to HA-GnRHRs in MCF7 and α T4 cells. α T4 and MCF7 cells were infected with Ad HA-hGnRHR (h), Ad HA-h.XGnRHR (h.X) or Ad XGnRHR (X) at 1×10^8 pfu/ml and used in flat plate binding assays (as described under Fig. 1) and Western blotting. (A) A representative Western blot, (B) Pooled data from three such blots (mean $\pm$ S.E.M., $n=3$) quantified by densitometry and normalized to HA-hGnRHR expression in α T4 cells (internal controls) and (C) Binding data pooled from four experiments (mean $\pm$ S.E.M., $n=3-4$).

these cells (e.g. whole cell HA-hGnRHR, HA-h.XGnRHR and HA-XGnRHR levels were not significantly different, as judged by densitometry of the Western blot bands). Similar data were obtained in α T4 cells except that cell surface expression of the HA-hGnRHR was higher (approximately 1 fmol/well) so that the addition of the C-tail increased cell surface receptor number by <5 -fold, as compared with >20 -fold increase seen in MCF7 cells. Again, the higher cell surface expression of the HA-h.XGnRHR and HA-XGnRHR occurred in spite of comparable whole cell levels of all the three receptors in α T4 cells. Although cell surface receptor binding was at least tenfold higher in α T4 cells than in MCF7 cells (Fig. 2C), whole cell receptor expression was <2 -fold higher (Fig. 2A and B) suggesting that a larger proportion of hGnRHRs are membrane resident in α T4 cells. When these proteins were probed with Alexa-Fluor 488-labeled antibody, little or no HA-hGnRHR was detected on the surface of either cell type as compared with clear cell surface staining of the HA-h.XGnRHR and HA-XGnRHR (Fig. 3). In contrast, strong staining was

observed throughout the cytoplasm of permeabilized cells infected with Ad HA-hGnRHR and intracellular staining was largely restricted to a perinuclear compartment in Ad HA-XGnRHR-infected cells (Fig. 3). Staining for the tagged hGnRHRs and hXGnRHRs was similar to that of the endogenous ER marker, calreticulin, in MCF7 cells, and merging of images in dual-stained cells revealed a high degree of co-localization (Fig. 3).

Influence of IN3 on GnRHR localization and function

We next tested whether the membrane-permeant molecular chaperone, IN3, was able to increase cell surface receptor expression. Competition binding (not shown) revealed that IN3 has high affinity for hGnRHRs and h.XGnRHRs in MCF7 cells ($\log_{10}$ molar IC_{50} values for competition with [125 I]Buserelin were -9.1 ± 0.2 and -9.4 ± 0.2 respectively) and confocal microscopy revealed that IN3 increases cell surface HA-hGnRHR expression (Fig. 4). Indeed, 24 h with 1 μ g/ml IN3 increased plasma membrane HA-hGnRHR >4 -fold and this effect was both dose- (50% effective concentration, $EC_{50}=0.003$ μ g/ml) and time-dependent ($t_{1/2}=4$ h) (Fig. 4). We also attempted to demonstrate the ability of IN3 to increase cell surface GnRHR number by radioligand binding but were unable to reliably wash IN3 pre-treatments from the cells (not shown). We therefore developed an indirect method based upon quantification of Buserelin-stimulated [3 H]IP $_x$ accumulation in cells either co-incubated with IN3 or pre-incubated and co-incubated with IN3. As shown, IN3 inhibited Buserelin-stimulated [3 H]IP $_x$ accumulation, shifting the Buserelin dose-response curve to the right as expected for a competitive antagonist (Fig. 5A). In contrast, when cells were pre-incubated for 24 h with the same concentrations of IN3 prior to stimulation with Buserelin (again in the presence of IN3), the inhibitory effect of the 1 μ g/ml IN3 was reduced and the lower concentrations of IN3 actually increased maximal Buserelin-stimulated [3 H]IP accumulation (Fig. 5B). The IN3 pre-treatment effect is most evident from comparison of Buserelin-stimulated [3 H]IP $_x$ accumulation curves constructed with cells either co-incubated with a given concentration of IN3 or co- and pre-incubated with the same IN3 concentration (e.g. compare open and filled symbols in Fig. 5C). Since these curves are constructed under conditions of identical proportional receptor occupancy, the difference between them reflects the increment in response due to increasing receptor number during the pre-incubation. This increase in [3 H]IP $_x$ response caused by pre-treatment with IN3 was paralleled by an increase in hGnRHR-mediated Ca^{2+} mobilization (not shown).

hGnRHR-mediated inhibition of proliferation

Since the magnitude of GnRHR-mediated antiproliferative effects are dependent on receptor number (Finch *et al.* 2004) and maximal Buserelin-stimulated [3 H]IP $_x$ accumulation was increased by IN3 pre-treatment (Fig. 5B), we suspected that this antagonist might actually increase the antiproliferative

effect of hGnRHRs. To test this, control and Ad hGnRHR-infected MCF7 cells were cultured for 16–24 h with 0, 0.01 or 0.1 $\mu\text{g/ml}$ IN3 and then for 4–6 days with or without 10^{-7} M Buserelin (and the same concentration of IN3) prior

to assessment of [^3H]thymidine incorporation. As expected, Buserelin had no measurable effect on [^3H]thymidine incorporation in control cells but it had a clear inhibitory effect in cells infected with Ad hGnRHR (Fig. 6). IN3 had no measurable effect on [^3H]thymidine incorporation in control cells without Ad hGnRHR or in Ad hGnRHR-infected cells in the absence of Buserelin (not shown). However, pre-treatment with IN3 significantly increased the antiproliferative effect of Buserelin (e.g. in cells infected with Ad hGnRHR at 20 pfu/nl, Buserelin reduced [^3H]thymidine incorporation to approximately 40% of control without IN3 and to 20% of control with 0.01 or 0.1 $\mu\text{g/ml}$ IN3).

Temperature-dependent trafficking of GnRHRs to the cell surface

The observation that the hGnRHR mediates robust [^3H]IP_x accumulation in spite of the very low cell surface receptor number implies that hGnRHR signaling might be dependent upon ongoing trafficking of the receptor to the plasma membrane. To test this, we first monitored the receptor-mediated uptake of Alexa-Fluor 488-labeled anti-HA by confocal microscopy. In Ad hGnRHR-infected cells incubated for 60 min at 37 °C, the antibody accumulated in vesicles (presumably endosomes) whereas much less uptake was seen in control cells that had not been infected with Ad HA-hGnRHR (not shown). Thus, although very little of the HA-hGnRHR is resident at the plasma membrane (Figs 2 and 3), it clearly does pass through this compartment and should therefore have the potential to bind radioligand at a temperature permissive for trafficking. To test this, we examined the temperature dependence of binding and observed very little binding of [^{125}I]Buserelin to Ad hGnRHR-infected MCF7 cells at 4 °C, a temperature used to prevent trafficking of GnRHRs to or from the plasma membrane (Fig. 7). However, raising the temperature to 37 °C (after 120 min at 4 °C) caused a rapid increase in binding ($t_{1/2} < 15$ min) to a maximum that was five- to tenfold greater than that in control cells remaining at 4 °C. In contrast, in Ad h.XGnRHR-infected cells, considerable [^{125}I]Buserelin binding was observed at 4 °C and increasing the temperature to 37 °C caused only a modest (50%) increase in binding (Fig. 7). The increase in binding seen on transferring Ad hGnRHR-infected cells from 4 to 37 °C was retained when the cells were then returned to 4 °C (not shown). These data are consistent with the existence of

Figure 3 Confocal imaging of HA-tagged GnRHRs in MCF7 and αT4 cells. αT4 and MCF7 cells on glass cover slips were infected with Ad HA-hGnRHR, Ad HA-h.XGnRHR or Ad XGnRHR (10^8 pfu/ml) and then used for confocal imaging of cell surface receptors (A) or whole cell receptor expression (B) as described in Materials and Methods. Alternatively, MCF7 cells were infected with Ad HA-hGnRHR and permeabilized before staining for HA-hGnRHR (C, left image) and for endogenous calreticulin (C, right image). Co-localization of the two antigens is evident in the merged view (C, lower image). A bar = 10 μm , C bar = 20 μm .

Figure 4 Effect of IN3 on cell surface expression of HA-hGnRHRs. MCF7 cells were infected with Ad HA-hGnRHR (10^8 pfu/ml) and cultured for 24 h in medium with 0 or 0.001–1 µg/ml IN3 (upper panels) or for 0–24 h with 1 µg/ml IN3 (lower panels) as indicated. They were then used for confocal imaging of cell surface receptors as above (Fig. 3). IN3 effects on plasma membrane HA-hGnRHR expression were quantified using ImageJ software to define fluorescence intensity at the plasma membrane for each cell. These data were not corrected for background fluorescence and therefore provide accurate measures of potency (EC_{50} 0.003 µg/ml at 24 h) and kinetics (half-time approximately 4 h with 1 µg/ml IN3) but underestimate the fold increase caused by IN3 (>4-fold increase with 1 µg/ml IN3 at 24 h).

intracellular GnRHR pools that can traffic rapidly to the cell surface to bind ligand at 37 °C.

Signal-dependent trafficking of GnRHRs to the cell surface

To test the possible relevance of receptor signaling for this effect, we also determined the temperature dependence of binding to intact cells infected with Ad expressing the hGnRHR, h.XGnRHR and corresponding receptors with a mutation (A261K) in the hGnRHR that prevents signaling to phospholipase C in COS-1 cells (Myburgh *et al.* 1998). In preliminary experiments, we found that the A261K mutation did not measurably alter cell surface receptor number, receptor affinity or ligand specificity for either the hGnRHR or the h.XGnRHR, and that these point-mutated receptors did not signal to phospholipase C (as judged by Ca^{2+} mobilization and [3H]IP_x accumulation assays) in MCF7 cells (not shown). As expected, maximal binding to intact Ad hGnRHR- or Ad h.XGnRHR-infected cells was achieved after 1–3 h at 4 °C or 15–30 min at 37 °C and the increment between maximal 37 °C binding and maximal 4 °C binding was greater for the hGnRHR than for the h.XGnRHR (Fig. 8 upper panels). However, maximal 37 °C binding did not exceed maximal 4 °C binding for either of the point-mutated receptors (Fig. 8 lower panel), implying that the temperature-dependent trafficking occurring during the incubation is dependent upon receptor signaling.

Discussion

Here we have explored the cellular compartmentalization of a number of wild-type and chimeric GnRHRs, and have observed remarkable differences in cell surface receptor

numbers when recombinant Ad is used to express these receptors in MCF7 breast cancer and $\alpha T4$ gonadotrope lineage cells (Fig. 1). Notably, radioligand binding at 21 °C in MCF7 cells revealed maximal expression levels of hGnRHRs to be approximately 2000 sites/cell, as compared with 5– to 20-fold higher levels of other type I and type II GnRHRs and 20- to 100-fold higher levels of chimeric receptors (human or sheep GnRHRs with added C-tails from XGnRHRs). These infections were performed with identically prepared E1-deleted Ad in which GnRHR expression is driven by the cytomegalovirus promoter, and at Ad GnRHR titers of $1\text{--}2 \times 10^7$ pfu/ml (equivalent to a multiplicity of infection of 30–60) which results in transfection of over 90% of these cells (Everest *et al.* 2001). Accordingly, the differences in cell surface receptor number likely reflect differences in receptor structure, rather than differences in viral tropism, transfection efficiency or transcription rate. Similar differences were seen with HA-tagged hGnRHRs, HA-h.XGnRHRs and HA-XGnRHRs (Fig. 2) but they do not correlate with independent measures of whole cell receptor expression (Figs 2 and 3). Indeed, densitometric analysis of Western blots revealed indistinguishable levels of all the three HA-tagged GnRHRs and confocal microscopy revealed abundant intracellular staining for HA-hGnRHR (whereas HA-XGnRHR staining was predominantly in the plasma membrane). Thus, our data suggest that the hGnRHR is primarily an intracellular protein in MCF7 (breast cancer) and $\alpha T4$ (gonadotrope lineage) cells.

Although little is known about the determinants of 7TM receptor compartmentalization in the ligand naïve state, a number of linear motifs (e.g. tyrosine-based, dileucine, polyproline and FxxxFxxx motifs) have been implicated in trafficking of 7TM receptors to the plasma membrane (Tan

Figure 5 Effect of IN3 co- and pre-incubation on busserelin-stimulated $[^3\text{H}]\text{JIP}_x$ accumulation. MCF7 cells were infected with Ad hGnRH-R (2×10^7 pfu/ml) then transferred to media with either 0 or 0.01–1 µg/ml IN3 and $[^3\text{H}]\text{inositol}$ (for 16 h), after which they were washed and stimulated for 1 h with 0 or 10^{-11} – 10^{-6} M Buserelin with 10 mM LiCl and either 0 (control) or 0.01–1 µg/ml IN3. (A) shows data for cells stimulated with busserelin and IN3 (e.g. co-incubated) and (B) shows data from cells pre-treated with the indicated concentration of IN3 prior to stimulation with busserelin and the same IN3 concentration (pre- and co-incubated). The lower panel shows data extracted from the upper panels to simplify comparison of responses in cells either co-incubated or pre- and co-incubated with IN3 at 0.1 µg/ml. The data shown are mean $\pm$ S.E.M. ($n=3$) from three experiments, each with triplicate observations and each normalized as a percentage of the maximal response in control cells (e.g. that seen with 10^{-7} or 10^{-6} M Buserelin in control cells having no IN3).

et al. 2004) and many 7TM receptor binding proteins (e.g. chaperones, scaffolds and other 7TM receptors) can influence targeting to, retention at, or compartmentalization within, the plasma membrane (Edwards *et al.* 2000, Tan *et al.* 2004). To our knowledge, these motifs have not been implicated in GnRHR trafficking and accessory proteins or chaperones influencing compartmentalization of type I GnRHR have not been described. However, a primate-specific GnRHR residue (K191) has been identified that reduces plasma membrane expression of GnRHRs (Arora *et al.* 1999, Janovick *et al.* 2003) and cell surface GnRHR number can

Figure 6 Effect of IN3 on hGnRHR-mediated inhibition of $[^3\text{H}]\text{thymidine}$ incorporation in MCF7 cells. Cells plated at a low density in 96-well plates were infected with Ad hGnRHR at 0, 10 or 20 pfu/ml then pre-incubated for 16–24 h with 0 or 0.01–0.1 µg/ml IN3 before stimulation for 4–6 days with 0 or 10^{-7} M Buserelin and assessment of $[^3\text{H}]\text{thymidine}$ incorporation on the final day of culture. The figure shows data pooled from four separate experiments (mean $\pm$ S.E.M., $n=3-4$), each of which had four to eight replicate observations. Pooling was achieved by normalizing the data as a percentage of $[^3\text{H}]\text{thymidine}$ incorporation seen in Ad titer matched control cells without peptide. IN3 had no measurable effect on $[^3\text{H}]\text{thymidine}$ incorporation in the absence of buserelin (not shown) but enhanced the antiproliferative effect of buserelin in Ad hGnRHR-infected cells. * $P < 0.05$, † $P < 0.001$ compared with buserelin alone (control).

be influenced by glycosylation within the amino-terminal sequence (apparently because a second glycosylation site increases trafficking to the plasma membrane, above), and by the presence or absence of a C-terminal tail. Indeed, it is

Figure 7 Temperature dependence of radioligand binding in MCF7 cells. MCF7 cells were infected with Ad hGnRHR and Ad h.XGnRHR ($1-2 \times 10^7$ pfu/ml) and used in flat plate binding assays as described in Materials and Methods using an initial incubation at 4 °C for 120 min. They were then either incubated for a further 30 or 60 min at 4 °C (open symbols), or were transferred to 37 °C for a further 5–60 min (filled symbols), prior to termination by washing on ice. The data are specific binding pooled from three experiments each with duplicate or triplicate observations (mean $\pm$ S.E.M., $n=3$). In order to pool data from multiple experiments, they were first normalized to the maximal binding (e.g. that observed after 30 or 60 min at 37 °C). These control values were 0.53 ± 0.19 and 3.14 ± 0.43 fmol/well for the hGnRHR and h.XGnRHR respectively and the actual increments seen on raising the temperature from 4 to 37 °C were approximately 0.5 and 1.5 fmol/well for the hGnRHR and h.XGnRHR respectively.

Figure 8 Temperature dependence of radioligand binding to wild-type, chimeric and point-mutated receptors in MCF7 cells. The upper panels show data from MCF7 cells infected with Ad hGnRHR or Ad h.XGnRHR ($1-2 \times 10^7$ pfu/ml) and used in flat plate binding assays as described in Materials and Methods using incubations that were entirely at 4 (open symbols) or 37 °C (filled symbols). The data are specific binding pooled from four experiments each with duplicate or triplicate observations (mean $\pm$ S.E.M., $n = 3-4$) normalized to the maximal binding observed at 4 °C for each receptor. The lower panel shows data from similar experiments with cells infected with Ad hGnRHR or Ad h.XGnRHR as above (–) or with the A261K mutation (+). For each receptor, specific binding after 30 min at 37 °C is normalized to maximal specific binding at 4 °C (indicated by the dashed line at 100%). The data are pooled from four experiments each with duplicate or triplicate observations (mean $\pm$ S.E.M., $n = 3-4$). The 37 °C binding was significantly greater than maximal 4 °C binding for the hGnRHR (* $P < 0.01$) and the h.XGnRHR ($^{\dagger}P < 0.05$) but not for either of the point-mutated receptors.

thought that two cysteins within the C-tail of the catfish GnRHR provide a palmitoylation site used to generate a fourth intracellular loop that stabilizes the receptor at the cell surface (Blonrohr *et al.* 1999). Although the XGnRHR C-tail is not palmitoylated, it does have the potential for stabilization at the cell surface by an amphiphilic α -helical section within the C-tail, as described for rhodopsin (Ballesteros *et al.* 2001, Caunt *et al.* 2004). Thus, it appears that the hGnRHR may be primarily intracellular in these cells because it lacks these structural features favoring trafficking to, and retention at, the cell surface. Interestingly, the Western blotting revealed distinct banding patterns for the receptors used herein. The HA-hGnRHR ran as two major bands of approximately 34 and 36 kDa and similar bands were seen with the HA-h.XGnRHR, although these were shifted to higher apparent molecular weight by addition of the C-tail and an

additional broad band of 50–100 kDa was also observed in both cell types (Fig. 2). The HA-XGnRHR ran predominantly as a broad band of 50–100 kDa. Since the predicted MWs of these receptors are all below 50 kDa, the high MW bands could reflect C-tail-dependent formation of SDS-resistant multimers (including receptor oligomers) as reported for other G protein coupled receptors (GPCRs) (Carrillo *et al.* 2004).

Although we have not explored the intracellular localization in detail, the staining for tagged hGnRHRs and hXGnRHRs was similar to that of the ER marker, calreticulin. This is consistent with the possibility that a large proportion of the nascent hGnRHR fails to pass ER exit quality control checks and is therefore retained in the ER. We also found that the molecular chaperone, IN3, increased hGnRHR expression > 4 -fold (Fig. 4) and caused a parallel increase in signaling (Fig. 5) in MCF7 cells, confirming that the intracellular epitope is functional GnRHR and providing further evidence that inefficient ER exit contributes to the poor cell surface expression of hGnRHRs in MCF7 and α T4 cells, as it does in HEK and COS cells (Janovick *et al.* 2003, Brothers *et al.* 2004, Ulluo-Aguirre *et al.* 2004, Janovic *et al.* 2006). The finding that hGnRHRs are largely intracellular in gonadotrope lineage α T4 cells is in apparent contradiction of early fractionation studies showing that pituitary GnRHRs are primarily located in the plasma membrane (Hopkins & Gregory 1977, Marian & Conn 1983, Zolman & Theodoropoulos 1984, Badr & Pelletier 1988). However, most of the earlier work was with rat and mouse GnRHRs, which lack the primate-specific K191 residue and have a second amino-terminal glycosylation site, features absent from the hGnRHR that favor plasma membrane expression. To our knowledge, the compartmentalization of human pituitary GnRHRs has not been defined in this way but subpopulations of intracellular GnRHRs have been reported in some models (Halmos & Schally 2002) and immunohistochemical approaches have revealed cytoplasmic hGnRHRs in tissues including breast carcinomas (Moriya *et al.* 2001, Reich *et al.* 2005). Such observations have remained controversial, largely because of the lack of reliable and well-characterized GnRHR antibodies, but are entirely compatible with our data that hGnRHRs are predominantly intracellular in MCF7 breast cancer cells.

Although type I GnRHR transcripts detected in breast and ovarian cancers are identical to those of the pituitary, the receptors may differ functionally (Eidne *et al.* 1987, Kakar *et al.* 1994, Imai *et al.* 1997, Emons *et al.* 1998, Schally & Nagy 1999, Limonta *et al.* 2003, Moretti *et al.* 2003, Cheng & Leung 2005). Pituitary GnRHRs have high affinity for agonists such as Buserelin, whereas the vast majority of GnRHRs in extra-pituitary sites are low affinity and there are also apparent differences in signaling. Thus, it appears that the functional characteristics of the type I hGnRHR can vary according to the cell type in which it is expressed. Although the molecular determinants of such context-dependence are essentially unknown, one intriguing possibility is that this is

related to the need for trafficking of intracellular receptors to the cell surface for signaling. In accord with this, the maximal cell surface hGnRHR number was tenfold lower in MCF7 cells than in α T4 cells (Fig. 2) and addition of the XGnRHR C-tail increased receptor binding >20-fold in MCF7 cells as compared with <5-fold in α T4 cells (Fig. 2). In addition to its major effect on expression of 'trafficking-deficient' point mutants (Janovick *et al.* 2002, 2003, 2006, Brothers *et al.* 2004, Ulloa-Aguirre *et al.* 2004, Knollman *et al.* 2005), IN3 caused a modest increase in cell surface expression of a hGnRHR-GFP fusion protein in COS7 cells, leading to the suggestion that as much as 50% of wild-type hGnRHRs are not transported to the cell surface in this model (Brothers *et al.* 2004). Our data suggest that the proportion of plasma membrane-resident hGnRHRs is much lower than this in MCF7 cells. We have also found that hGnRHR expression is extremely low (<2000 sites per cell) in Ad hGnRHR-infected Ishikawa endometrial cells as well as PC3 and LNCaP prostate cells (not shown), implying that cell surface hGnRHR expression may be particularly inefficient in hormone-dependent cancer cells. If so, this could underlie functional differences between GnRHRs in pituitary and extra-pituitary sites. Although the molecular determinants of cell-specific plasma membrane expression of hGnRHRs are unknown, we speculate that proteins facilitating such expression (e.g. molecular chaperones) could be relatively poorly expressed in cells other than gonadotropes. These data also raise the question of whether wild-type hGnRHR trafficking can be manipulated for therapeutic benefit. We have found that the >2-fold increase in cell surface hGnRHRs caused by 0.1 μ g/ml IN3 in MCF7 cells (Fig. 4), is associated with a two- to threefold increase in maximal hGnRHR-mediated [3 H]IP_x accumulation at matched proportional receptor occupancy (Fig. 5C), and an increase in hGnRHR-mediated inhibition of [3 H]thymidine incorporation (Fig. 6). This enhancement of Buserelin-mediated inhibition of proliferation by the membrane-permeant antagonist is in contrast to the prevention of proliferation inhibition caused by antide and Cetrorelix (conventional membrane-impermeant antagonists) in this model (Everest *et al.* 2001). Thus, this membrane-permeant antagonist can, indeed, be used to enhance the antiproliferative effect of an agonist in this model.

The data outlined above are in accord with earlier work showing that cell surface expression of hGnRHRs is low compared with that of other GnRHRs because of relatively inefficient exit from the ER (as evidenced by the stimulatory effect of IN3) as well as inefficient retention at the cell surface (because it lacks the C-tail thought to increase cell surface receptor expression of type II GnRHRs by insertion into or association with the plasma membrane). However, we were puzzled by the observation that the hGnRHR-mediated robust stimulation of [3 H]IP_x accumulation in spite of the expression of <2000 sites per cell, and suspected that a proportion of the intracellular receptors might traffic to the cell surface for signaling during stimulation. In accord with

this, we found that when Ad HA-hGnRHR-infected cells were incubated for 37 °C with anti-HA, the antibody accumulated in punctate regions in the cytoplasm (not shown). This trafficking was also seen in experiments in which raising the temperature from 4 to 37 °C increased hGnRHR binding >5-fold but increased h.XGnRHR binding by only 50%. Apparently, the intracellular hGnRHR must traffic to the surface for binding (at the permissive temperature), whereas the higher cell surface level of h.XGnRHR negates this requirement (Fig. 7). Moreover, the temperature-dependent trafficking of these receptors to the cell surface was prevented by introduction of a point mutation that prevents signaling to phospholipase C, implying that a small pool of activated cell surface receptors may actually recruit receptors from the intracellular pool (Fig. 8). This temperature-dependent increase in hGnRHR binding does not simply reflect a temperature-dependent change in association or dissociation rate constants because the ligand binding pockets of the hGnRHR and h.XGnRHR are identical in spite of the fact that the pronounced (>5-fold) increase in binding is only seen with the hGnRHR. Similarly, these differences in temperature dependence of binding do not reflect differences in receptor internalization because when assessed using a standard acid wash protocol (Hislop *et al.* 2001) internalization rates of the hGnRHR and h.XGnRHR were indistinguishable (data not shown, see also Finch *et al.* (2004) for similar experiments with the sGnRHR and s.XGnRHR). Importantly, the effect of IN3 on cell surface receptor number (half-time approximately 4 h) was much slower than the increase in binding (seen within 5 min of raising the temperature of MCF7 cells from 4 to 37 °C). This is presumably too fast to reflect accelerated exit from the ER and implies that an undefined proportion of the intracellular hGnRHRs is actually not ER-resident but instead, forms a receptor pool that can be rapidly trafficked to the plasma membrane to facilitate signaling. The mechanism by which these receptors are brought to the cell surface is unknown but no temperature-dependent increase was seen with the A261K mutants of the hGnRHR or the h.XGnRHR, implying that signaling via the small number of cell surface hGnRHRs may recruit cryptic intracellular hGnRHRs to the cell surface.

In summary, we have found that the hGnRHR is largely intracellular when expressed in MCF7 breast cancer and α T4 gonadotrope lineage cells. Our data suggest that a large proportion of these receptors are retained in the ER so that cell surface expression can be increased by sustained treatment with IN3, a molecular chaperone that increases exit from the ER with a half-time of approximately 4 h. In addition, we show that cell surface hGnRHR binding can be rapidly increased (half-time approximately 15 min) by increasing temperature from 4 to 37 °C in order to facilitate receptor trafficking. This raises the possibility that signaling is dependent upon rapid recruitment of hGnRHRs from a cryptic intracellular pool (which may well be distinct from the IN3-sensitive ER pool). We also suggest that the proportion

of membrane-resident hGnRHRs may be particularly low in hormone-dependent cancers, in which case manipulations that increase receptor trafficking to the surface could be exploited to selectively enhance the effectiveness of therapies targeting extra-pituitary GnRHRs. Since membrane-permeant agonists can act as chaperones for other 7TM receptors, membrane-permeant GnRH agonists could be particularly effective stimuli for extra-pituitary GnRHRs.

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