Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor

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Citation

Nederpelt, I. (2017, April 6). *Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor*. Retrieved from https://hdl.handle.net/1887/47526

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Author: Nederpelt, I.
Title: Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor
Issue Date: 2017-04-06

Chapter 4

Persistent GnRH receptor activation in pituitary αT3-1 cells analyzed with a label-free technology

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Biosensors & Bioelectronics **2016** *79: 721-727*

Abstract

The gonadotropin-releasing hormone (GnRH) receptor is a drug target for certain hormone-dependent diseases such as prostate cancer. In this study, we examined the activation profiles of the endogenous ligand, GnRH and a well-known marketed analog, buserelin using a label-free assay in pituitary αT3-1 cells with endogenous GnRH receptor expression. This whole cell impedance-based technology allows for the real-time measurement of morphological cellular changes. Both agonists dose-dependently decreased the impedance as a result of GnRH receptor activation with potencies of 9.37 ± 0.1 (pEC_{50} value, buserelin) and 7.87 ± 0.06 (pEC_{50} value, GnRH). Subsequently, GnRH receptor activation was completely abolished with a selective Gαq inhibitor, thereby confirming the $G\alpha_q$-coupling of the GnRH receptor in pituitary αT3-1 cells. Additionally, we observed continued responses after agonist stimulation of αT3-1 cells indicating long-lasting cellular effects. Wash-out experiments demonstrated that the long-lasting effects induced by GnRH were most likely caused by rebinding since over 70% of the original response was abolished after wash-out. In contrast, a long receptor residence time was responsible for the prolonged effects caused by buserelin, with over 70% of the original response remaining after wash-out. In summary, we validated that impedance-based label-free technology is suited for studying receptor-mediated activation in cell lines endogenously expressing the target of interest. Moreover, this real-time monitoring allows the examination of binding kinetics and its influence on receptor activation at a cellular level.

Introduction

Label-free technologies can noninvasively monitor real-time receptor-mediated phenotypic responses in living cells encompassing all involved signaling pathways [1, 2]. Label-free whole cell assays typically use a biosensor to detect a ligand-induced cellular response by ways of acoustic, electrical or other quantifiable signals [3, 4]. The main advantage of using biosensors and cell morphology as a readout is that cells can be assessed in their native and physiologically relevant environment bypassing the potentially negative effects of engineering on cell signaling [5, 6]. Additionally, label-free assays are highly sensitive therefore making them suitable for endogenous expression systems. Label-free studies are most commonly used to examine G protein-coupled receptor (GPCR) activation and G protein-signaling profiles [7-9], but also for investigating cytotoxicity, cell adhesion, proliferation, migration and invasion [6, 10, 11].

The gonadotropin-releasing hormone receptor (GnRHR) is part of the rhodopsin family of GPCRs and is sub-classified in the β-group where all endogenous ligands are peptides [12]. Its endogenous ligand, gonadotropin-releasing hormone (GnRH) is a decapeptide synthesized in hypothalamic neurons. GnRH regulates the synthesis and secretion of luteinizing hormone (LH) and follicle stimulation hormone (FSH) by selectively stimulating pituitary gonadotropes expressing the GnRH receptor [13]. The role of GnRHR in regulation of hormone levels in both males and females makes it an important target in hormone dependent diseases such as precocious puberty, fertility disorders and cancers of the prostate, mammary, ovary and endometrium [14, 15].

In the current study we investigated GnRHR-induced signaling in a heterologous CHOhGnRH-NFAT cell line as well as the αT3-1 cell line using a label-free whole cell impedance-based assay. The gonadotrope mouse pituitary αT3-1 cell line [16] is known to have high endogenous GnRHR expression [17]. In the present study, we established that both the heterologous CHOhGnRH-FNAT cell line and the endogenous αT3-1 cell line are suitable to study GnRHR-mediated signaling using a label-free technology. In addition, we were able to, for the first time, elucidate the functional effects of GnRHR agonists with different binding kinetics. Taken together, we demonstrated the importance of monitoring integrated cellular responses to gain knowledge in receptor signaling and binding kinetics that cannot be detected with traditional endpoint assays.

Methods

Materials and reagents

GnRH, buserelin and cetrorelix were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands), while UBO-QIC was purchased from the Institute of Pharmaceutical Biology (University of Bonn, Germany). [2-^{3}H(N)]-myoinositol (specific activity 10-25 Ci/mmol), isoplate-96™ white frame, clear well plates and YSi Poly-L-Lysine coated SPA beads were purchased from Perkin Elmer (Boston, MA). CHO cells stably expressing both the human GnRH receptor and an NFAT reporter gene (CHOhGnRH-NFAT) were obtained from Invitrogen (Carlsbad, CA). αT3-1 cells were a kind gift from Dr. Pamela L. Mellon (Salk Institute, San Diego, CA). xCELLigence E-plate 16 and 96 were obtained from Westburg (Leusden, the Netherlands). All other compounds and materials were obtained from standard commercial sources.

Cell culture

CHOhGnRH-NFAT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialyzed fetal calf serum (FCS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 µg/ml zeocin, 600 µg/ml hygromycin, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C + 5% CO_2. αT3-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4.5 g/L glucose, 548 mg/L L-glutamine, 110 mg/L pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C + 5% CO_2. Cells were cultured as a monolayer and used for whole cell experiments when a confluency of ~75% was reached.

Label-free whole-cell assays

Label-free whole-cell assays were performed using the xCELLigence RTCA system [5, 6] as described previously [18].

CHOhGnRH-NFAT cells and αT3-1 cells were cultured as a monolayer on 10-cm ø culture plates and were harvested when confluency was around 75%. The experiment was started by adding 45 µl culture medium to each well to obtain background signal. Subsequently, 50 µl of cell suspension containing $1.6*10^6$ cells/ml was added to each well to obtain approximately 40.000 cells/well. After roughly 18 hours on the recording device station in a humidified atmosphere at 37°C + 5% CO_2, cells were stimulated with increasing concentrations of GnRH, buserelin or vehicle control. For antagonistic assays, background signal was obtained with 40 µl culture medium/well and cells were incubated with an excess of the antagonist cetrorelix (160 nM) or vehicle control 30 min prior to stimulation with submaximal (EC_{80}) concentrations of GnRH (31.6 nM) or buserelin (1 nM). For inhibition of the $G\alpha_q$ signaling pathway, αT3-1 cells were pretreated with 1 µM UBO-QIC or vehicle control 30 min prior to stimulation with submaximal (EC_{80}) concentrations of GnRH (31.6 nM) or buserelin (1 nM). Submaximal (EC_{80}) concentrations of GnRH and buserelin were derived from concentration-response curves using Total Area Under the Curve (AUC) analysis (see section ‘Data analysis’).

Inositol phosphate accumulation assay

αT3-1 cells were seeded at a cell density of 100.000 cells/well with [^{3}H]-myoinositol (4 µCi/ml) overnight at 37°C and 5% CO_2. Subsequently, cells were washed twice with Buffer A containing 127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM HEPES and 0.1% BSA. Thereafter, cells were incubated for 20 minutes at 37 °C with Buffer A supplemented with 50 mM LiCl, followed by stimulation with increasing concentrations of GnRH or buserelin for 60 min at 37°C. Cells were lysed through 1 hour incubation with 10 mM formic acid at 4°C, after which 20 µL of solution was transferred to an isoplate™ 96 followed by addition of 80 µL YSi Poly-L-Lysine-coated SPA beads at 12 mg/ml. The mixture was shaken at room temperature for 60 min prior to a 5 min centrifuge step at 1500 rpm. Radioactivity of the extract/bead mixture was determined by scintillation spectrometry using the P-E 1450 Micobeta Wallac Trilux scintillation counter according to instruction manual (Perkin Elmer, Groningen, the Netherlands).

Data analysis

All experimental data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Cell index (CI) traces were normalized to time of agonist addition and then exported from RTCA Software 1.2 (Roche, Germany). Total AUC values up to 180 min after agonist addition were used for data analyses. Baseline was removed by subtracting vehicle, antagonist and inhibitor controls from corresponding normalized CI (NCI) traces.

Efficacy (E_{max}) and potency (pEC_{50}) values for GnRH and buserelin were obtained with non-linear regression of Total AUC data fitted by log(agonist) vs. response - Variable slope. Results were normalized to Total AUC induced by the maximal concentration of GnRH. Total AUC of agonist-induced cellular responses in presence of antagonist or pathway inhibitor were normalized to Total AUC obtained from αT3-1 cells responses treated with submaximal concentrations (EC_{80}) of corresponding agonist.

Efficacy (E_{max}) and potency (pEC_{50}) values for IP accumulation assay were obtained using non-linear regression of total counts upon GnRH or buserelin-induced $G\alpha_q$-activation fitted by log(agonist) vs. response - Variable slope. Results were normalized to total counts induced by the maximal concentration of GnRH.

All values obtained are means ± SEM of at least three independent experiments performed in duplicate.

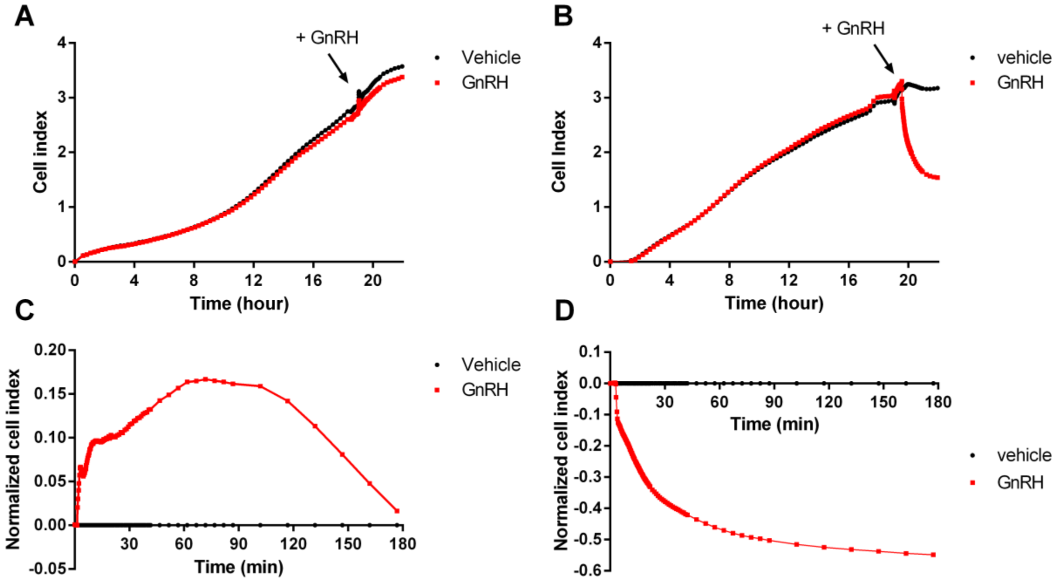

Figure 1: Real-time monitoring of the cell index as a measure of proliferation and adherence of CHOhGnRH-FNAT cells (A) and αT3-1 cells (B) before and after (18 h) addition of 1 µM GnRH. Zoom in on the effect of GnRH yielding the normalized cell index after addition of 1 µM GnRH to CHOhGnRH-NFAT cells (C) and αT3-1 cells (D).

Results

GnRHR signaling in heterologous and endogenous cells on the xCELLigence

Heterologous GnRH receptor-mediated signaling in CHOhGnRH-NFAT cells was monitored on the xCELLigence system. Overnight proliferation resulted in a cell index of approximately 4.0 (Figure 1A). Typically, the impedance increased upon agonist addition with a first peak around 10 min of approximately 0.075 NCI, followed by a second peak reaching approximately 0.15 NCI around 80 minutes. The signal decreased again back to baseline after approximately 180 min (Figure 1C).

Stimulation of CHOhGnRH-NFAT cells with increasing concentrations of GnRH and its analog buserelin resulted in a concentration-dependent increase in impedance (Figure 2A and 2C). From these impedance changes a concentration-response curve could be obtained providing pEC_{50} values of 10 ± 0.1 and 10.6 ± 0.2 for GnRH and buserelin, respectively (Figure 2E and Table 1). The efficacy of buserelin was similar to that of GnRH, namely 98 ± 4.5% (Table 1). To confirm that the observed changes in impedance are GnRHR-specific the parental CHO cell line was used as a negative control. Treatment of these cells with 1 µM of GnRH did not result in a change in impedance (Figure 3A and 3D). Additionally, the selective GnRH peptide antagonist cetrorelix was able to block receptor activation by GnRH (Figure 3B and 3D).

Table 1: potency and efficacy of GnRH and buserelin obtained with xCELLigence and IP accumulation experiments performed with CHOhGnRH-NFAT and αT3-1 cells.

Agonist	CHOhGnRH-NFAT		αT3-1			
	(xCELLigence$^{\#}$)		(xCELLigence$^{\#}$)		(IP accumulation)	
	pEC$_{50}$ (EC$_{50}$ in nM)	E_{max} (%)	pEC$_{50}$ (EC$_{50}$ in nM)	E_{max} (%)	pEC$_{50}$ (EC$_{50}$ in nM)	E_{max} (%)
GnRH	10 ± 0.1 (0.1)	100 ± 3	7.8 ± 0.06 (17)	100 ± 1	7.9 ± 0.2 (12)	100 ± 0.9
Buserelin	10.6 ± 0.2 (0.03)	98 ± 5	9.3 ± 0.1 (0.46)	90 ± 3*	10 ± 0.1 (0.09)	115 ± 1***

Values are means ± SEM of three separate experiments performed in duplicate. $^{\#}$ Values were calculated with total AUC analysis up to 180 min after agonist addition. Data were normalized to maximal response obtained for GnRH. *=p>0.05, ***=p>0.0005, compared to E_{max} GnRH determined with Student's *t* test.

To examine if the GnRHR-mediated responses in the heterologous cell line could also be observed in the endogenous pituitary αT3-1 cell line, we studied GnRH signaling with the xCELLigence on this cell line. Overnight proliferation gave a cell index of roughly 3.0 (Figure 1B). In contrast to the effect on CHOhGnRH-NFAT cells, addition of 1 µM GnRH resulted in a decrease in impedance reaching and maintaining its plateau around 0.4 NCI within 1 hour up to but not limited to 180 min after stimulation (Figure 1D).

Addition of increasing concentrations of GnRH and buserelin resulted in a concentration-dependent decrease in impedance (Figure 2B and 2D). Interestingly, low concentrations of both GnRH and buserelin resulted in a positive NCI. The concentration-response curves gave an EC$_{50}$ value of 17 nM for GnRH and a 37-fold lower EC$_{50}$ value of 0.46 nM for buserelin (Figure 2F and Table 1). Buserelin had a slightly, yet significantly, lower efficacy than GnRH of 90 ± 3.1%. The GnRHR-mediated responses were selectively blocked by pretreatment with the GnRH peptide antagonist cetrorelix (3.8 ± 0.3%), supportive of a receptor-specific effect (Figure 3C, 3D). Since the xCELLigence detects morphological changes rather than one specific intracellular signaling pathway following GPCR activation we suppressed Gα$_q$-mediated signaling with inhibitor UBO-QIC prior to GnRH treatment. This selective inhibition of the Gα$_q$ pathway completely abolished GnRHR activation (3.8± 1.6%) (Figure 3D and 3E).

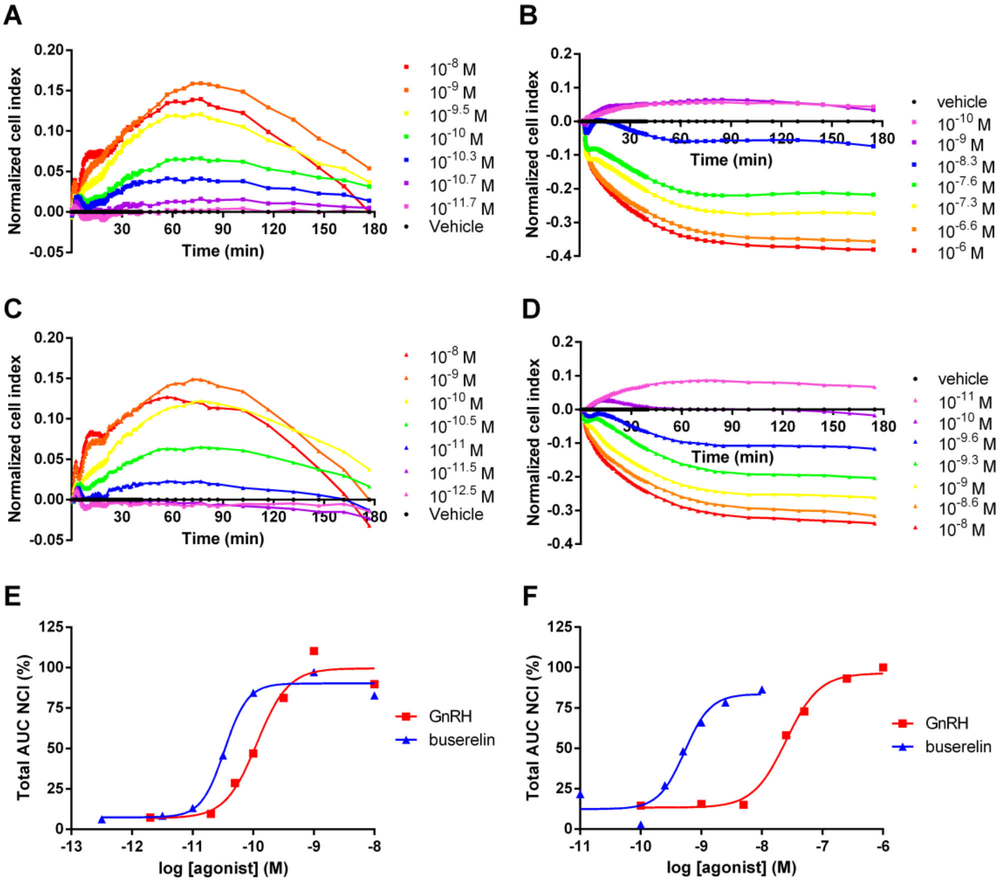

Figure 2: Concentration-dependent effects of GnRH and buserelin on CHOhGnRH-NFAT (left) and αT3-1 (right) cells. Representative graph of one experiment performed in duplicate of normalized cell index (NCI) after stimulation with increasing concentrations of GnRH (A; CHOhGnRH and B; αT3-1) or buserelin (C; CHOhGnRH and D; αT3-1). Representative concentration-effect curve of GnRH and buserelin derived from total AUC up to 180 min after agonist stimulation, data were normalized to maximal response after GnRH stimulation (E; CHOhGnRH and F; αT3-1).

GnRHR signaling in pituitary αT3-1 cells using second messenger assays

To compare our obtained potency and efficacy values for GnRH and buserelin to a more traditional second messenger assay we examined IP accumulation in αT3-1 cells upon GnRHR-activation which is a consequence of activating the $G\alpha_q$ pathway. Concentration-response curves of GnRH and buserelin yielded pEC_{50} values of 7.9 ± 0.2 and 10 ± 0.1, respectively (Supplementary Figure 1). In this assay, buserelin showed a significant increase in efficacy namely 115 ± 1% (Table 1).

Functional consequence of differential receptor binding kinetics in pituitary αT3-1 cells on the xCELLigence

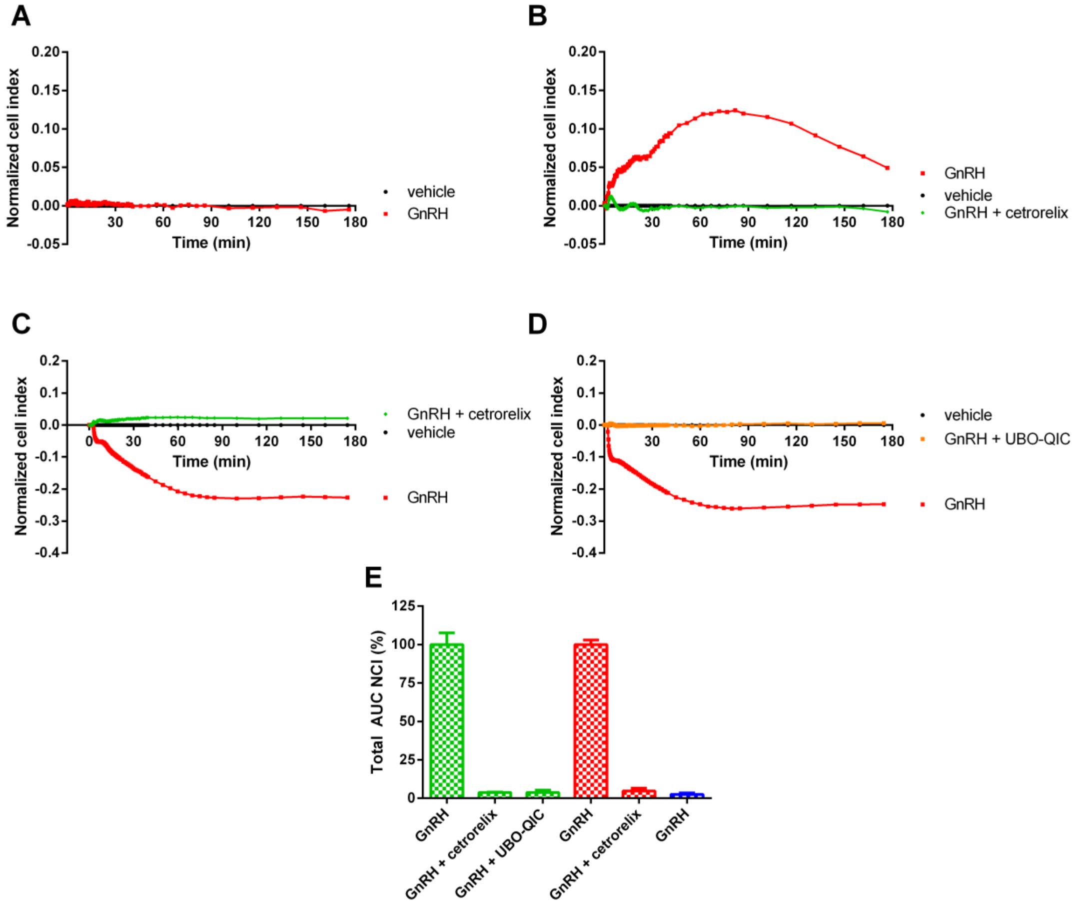

Figure 3: GnRHR mediated responses in parental CHO cells, CHOhGnRH-NFAT cells or αT3-1 cells. Representative graph of one experiment performed in duplicate of normalized cell index (NCI) after stimulation with GnRH of parental CHO cells (A), CHOhGnRH-NFAT cells (B) or αT3-1 cells (C) with or without pretreatment with cetrorelix or αT3-1 cells (D) with or without pretreatment with UBO-QIC. Bar graph of total AUC up to 180 min of cells stimulated with GnRH with or without 160 nM cetrorelix or 1 µM UBO-QIC pretreatment in αT3-1 cells (green), CHOhGnRH-NFAT cells (red) or parental CHO cells (blue), data were normalized to maximal response after GnRH stimulation without inhibitor (E).

GnRH and buserelin both showed sustained signaling on the xCELLigence, i.e. a decrease in impedance reaching and maintaining its plateau around 0.4 NCI within 1 hour up to but not limited to 180 min after stimulation, which indicates long-lasting functional effects. To examine whether these effects were due to a long drug-target residence time we performed a washout experiment. Thirty minutes after stimulating with EC_{80} concentrations of GnRH or buserelin, cells were washed followed by further label-free measurements (Figure 4A). Washout after stimulating the cells with GnRH or buserelin decreased the response relative to unwashed conditions to 43 ± 6% or 79 ± 5% after 30 min, respectively (Figure 4B and Table 2). The response continued to decline after 120 minutes for GnRH (27 ± 7%) whereas buserelin remained active at approximately 80% of the control activation (Figure 4B and Table 2).

Discussion

Label-free impedance-based xCELLigence is suitable to study GnRHR activation

Label-free whole cell assays provide new perspectives in studying GPCR signaling by continuously measuring cell morphology changes upon receptor activation [2, 6, 8, 9]. These novel phenotypic functional assays have many advantages over traditional endpoint assays, as they measure integrated cellular responses rather than signals downstream one particular G protein pathway. Label-free whole cell assays thus encompass a complete overview of cellular function and responses after receptor activation. Here, we demonstrate for the first time signaling of the GnRH receptor using the label-free whole cell impedance-based xCELLigence system in heterologous CHOhGnRH-NFAT cells as well as endogenous gonadotrope pituitary αT3-1 cells.

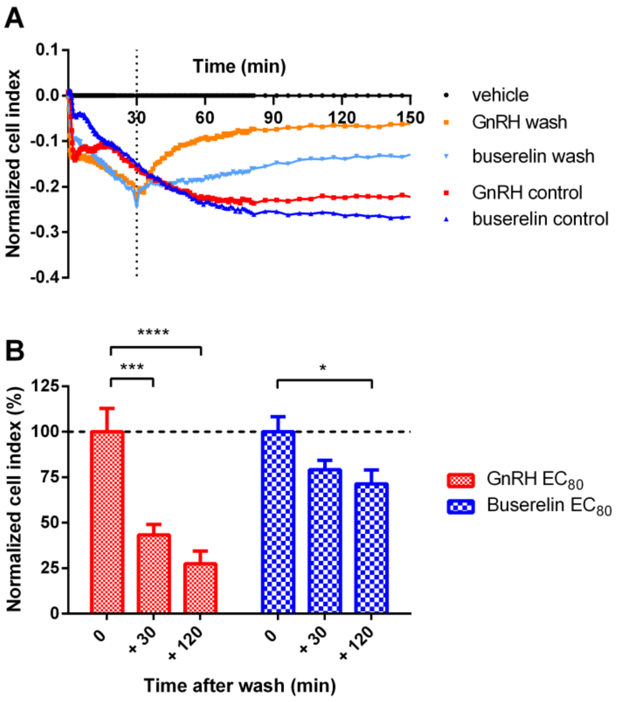

Figure 4: Wash out of αT3-1 cells stimulated with GnRH or buserelin. (A) Representative graphs of one experiment performed in duplicate after stimulation with either GnRH or buserelin (control) or followed by wash out at 30 min indicated by vertical dashed line (wash). (B) Bar graph of normalized cell index agonist induced effects 0 min, 30 min and 120 min after wash out (B). Data are mean ± SEM from three separate experiments performed in duplicate. Data were normalized to maximal response obtained before wash out, indicated as ‘’0’’, *= p<0.05, ***= p<0.001, ****= p<0.0001.

Initial experiments with CHOhGnRH-NFAT cells demonstrated that the xCELLigence system is suitable to study GnRHR-mediated responses in a heterologous expression system. Concentration-response curves provided a pEC50 value of 10 ± 0.1 for GnRH, which is comparable to previously reported data [19]. Control experiments with the parental CHO cell line and the selective GnRHR peptide antagonist cetrorelix showed absence of GnRHR responses, further verifying that the observed responses were GnRHR-mediated.

Subsequent experiments established that the endogenous pituitary αT3-1 cell line is also well suited for studying GnRHR signaling on the xCELLigence. GnRH-mediated signals were selectively blocked by the peptide antagonist cetrorelix, confirming the agonist responses were GnRHR specific. Additionally, we observed that silencing of the $G\alpha_q$ pathway with the selective inhibitor UBO-QIC completely abrogated agonist-mediated αT3-1 cell responses. This illustrates that GnRH and buserelin signal through the $G\alpha_q$ pathway, which is in agreement with previously reported data [14, 20-22].

Considering that the endogenous mouse pituitary αT3-1 cell line is derived from immortalized anterior pituitary gonadotrope cells [16] and the mouse and human GnRHR are 99 % homologous, all further experiments were continued with the endogenous pituitary αT3-1 cell line.

Table 2: Receptor activation after wash out of EC_{80} concentrations of GnRH or buserelin

	EC_{80} response (%)	
Time after wash out	GnRH	Buserelin
0 min	100 ± 13	100 ± 8
+ 30 min	43 ± 6***	79 ± 5
+ 120 min	27 ± 7****	71 ± 8*

Values are means ± SEM of three separate experiments performed in duplicate. Cells were washed after 30 min agonist stimulation and values were calculated using normalized cell index at 0 min, 30 min and 120 min after wash out. Data were normalized to maximal response obtained before wash out, indicated as ''0 min''. *= p<0.05, ***= p<0.001, ****= p<0.0001, compared to 0 min determined using one-way ANOVA with Dunnett's post-test.

Two well-known GnRH receptor agonists were studied in this research; the endogenous ligand GnRH and buserelin a GnRHR agonist that slowly dissociates from the receptor [23]. In the label-free whole cell assays both agonists were able to activate the GnRH receptor in a concentration-dependent manner, albeit that buserelin had a 37-fold higher potency than GnRH (0.46 nM and 17 nM, respectively). Similar findings were observed in more traditional IP accumulation assays; here buserelin was 133-fold more potent than GnRH (0.09 nM and 12 nM, respectively). A much smaller (7-fold) potency shift between GnRH and buserelin has been reported before (3.4 nM and 0.47 nM) with total inositol phosphate production measurements using αT3-1 cells [24]. A possible explanation could be that the incubation time in their assay was only 30 min, which could result in an underestimated potency since buserelin might not yet have reached equilibrium. In our hands, the potency of GnRH was in the same range between the impedance-based and IP accumulation assays while buserelin was 5-fold more potent in the latter. Differences in potency between label-free whole cell assays and traditional second messenger functional assays have been observed before [25-28].

Long-lasting residence time can be translated to a persistent activation profile in vitro

In contrast to previously published xCELLigence data on GPCR activation [25, 29], we observed long-lasting signaling events of GnRH and buserelin on αT3-1 cells implying persisted GnRHR activation. This finding might be explained by either rebinding of the agonist to the receptor or long-lasting target binding [30]. Long-lasting target residence time has already been reported for buserelin, however GnRH was found to have a shorter residence time [23]. It has been postulated that the *in vivo* high potency and long duration of action of buserelin was caused by long-lasting GnRH receptor binding [31-33]. Therefore, we hypothesized that the long-lasting effects caused by GnRH are due to rebinding of this molecule to the receptor while the long-lasting effects caused by buserelin are a result of this compound's prolonged target binding. To test this hypothesis we designed a washout experiment to minimize rebinding where we examined the remaining cellular response by GnRH and buserelin after washing. Wash out of GnRH or buserelin at EC_{80} concentrations showed a decreased signaling response for both agonists. Conversely, this decreased response in signaling was much more outspoken for GnRH than for buserelin. These results confirm our hypothesis, being that the observed persisted signaling profile of GnRH was due to rebinding while the persisted signaling profile of buserelin is a combination of both rebinding and prolonged drug-target occupancy caused by long drug-receptor residence time. Casarosa et al. [34], reported on a washout second messenger assay, examining cAMP production. In this assay the long residence time β_2 adrenoceptor (β_2-AR) agonist olodaterol remained associated with the receptor, while the short residence time β_2-AR agonist salbutamol was readily washed out. Lindstrom and coworkers performed washout experiments with U373MG cells endogenously expressing the Tachykinin 1 receptor (NK1R) measuring intracellular Ca^{2+} levels. They demonstrated that the response to the endogenous agonist Substance P was not restored after a 60 min wash out of the slowly dissociating NK1R antagonist aprepitant [35].

Conclusions

We have validated the label-free whole cell xCELLigence system as a valuable biosensor to investigate GnRHR-mediated signaling in endogenous pituitary αT3-1 cells. For the first time in a label-free assay environment we showed prolonged receptor signaling due to drug-target binding kinetics by wash out experiments. Our results illustrate the importance of monitoring phenotypic and integrative responses using label-free whole cell assays, since traditional end-point assays are less suited to acquire information on drug-target binding kinetics in real-time. Incorporating whole cell label-free technologies in drug development will provide a more complete overview of the functional properties of a ligand and hopefully improve future drug discovery.

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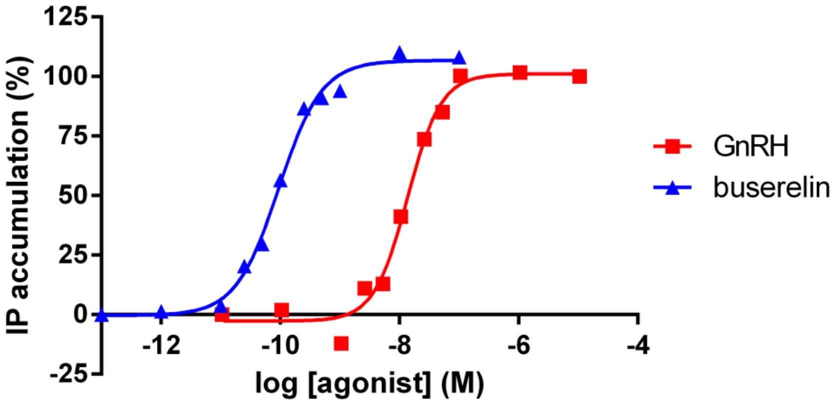
Supplemental Data



Supplementary Figure 1: Concentration-dependent effects of GnRHR agonists in αT3-1 cells. IP accumulation upon stimulation with increasing concentrations of GnRH (red) or buserelin (blue). A representative graph is shown of one experiment performed in duplicate, data were normalized to maximal response after GnRH stimulation.