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GnRH-I and GnRH-II-induced calcium signaling and hormone secretion in neonatal rat gonadotrophs

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Short title: Signaling by GnRH-I and GnRH-II in neonatal rat pituitary

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Abstract

Two forms of gonadotropin-releasing hormone (GnRH), GnRH-I and GnRH-II, are commonly present in mammals. The main hormone controlling reproduction is GnRH-I acting through its receptor GnRHR-I, whereas the function of GnRH-II is unknown. In primates, it has been suggested that GnRH-II is a specific agonist for the structurally distinct GnRHR-II. Here we compared effects of GnRH-I and GnRH-II, on intracellular calcium and gonadotropin hormone release in neonatal rat gonadotrophs *in vitro* and the dependence of agonist actions on cyclic nucleotide levels. Both agonists elevated intracellular calcium and stimulated gonadotropin secretion in a concentration dependent manner, with comparable peak amplitudes but GnRH-I was 3-times more potent than GnRH-II. Antide, a specific GnRHR-I antagonist, completely blocked the action of both agonists on gonadotropin release. Inhibition of adenylyl cyclase activity by melatonin and MDL significantly attenuated GnRH-I and GnRH-II-induced calcium signaling and gonadotropin release, whereas inhibition of soluble guanylyl cyclase activity was ineffective. GnRH-II also generated calcium oscillations in a fraction of gonadotrophs not expressing melatonin receptors. These results indicate the coordinate actions of GnRHR-I on gonadotropin release through intracellular calcium and cyclic nucleotide signaling, and that GnRH-II is less potent agonist for this receptor in neonatal gonadotrophs.

Key words: Gonadotropin-releasing hormone, GnRH-II, Melatonin, LH, FSH, Neonatal Rats.

Introduction

Reproductive functions in vertebrates are controlled by decapeptide GnRH-I (gonadotropin-releasing hormone-I), also known as LHRH (luteinizing hormone-releasing hormone). GnRH-I, originally isolated from ovine and porcine tissues (Matsuo et al., 1971) (Amoss et al., 1971), is synthesized by hypothalamic GnRH neurons of all mammalian species, and is secreted in a pulsatile manner into the hypophyseal portal system (Knobil, 1988). GnRH-I governs reproductive functions by regulating the synthesis, glycosylation, and release of LH (luteinizing hormone) and FSH (follicle stimulating hormone) in anterior pituitary gonadotrophs, leading to stimulation of gonadal steroidogenesis and gametogenesis (Millar et al., 2004). GnRH-I binds with high affinity to GnRHR-I, a plasma membrane receptor that belongs to the rhodopsin-like family of seven transmembrane domain receptors (Reinhart et al., 1992). The activated GnRHR-I couples to the pertussis toxin-insensitive $G_{q/11}$ proteins that stimulate phospholipase Cβ, an enzyme responsible for the generation of inositol-1,4,5-trisphosphate (IP_3) and diacyglycerol, and the IP_3-dependent oscillatory elevations in cytosolic free calcium concentrations ($[Ca^{2+}]_i$) (Hille et al., 1994; Stojilkovic et al., 1994). The GnRHR-I also couples to the cholera toxin-sensitive G_s and/or pertussis toxin-sensitive $G_{i/o}$ proteins, depending on the cell type expressing GnRHR-I and duration of agonist application (Krsmanovic et al., 2003). The functional significance of modulation of adenylyl cyclase activity by GnRHR-I in pituitary gonadotrophs has not been studied.

Other forms of GnRHs are also present in individual species of most vertebrates; 16 structural variants or GnRH ligands have been isolated so far (King and Millar, 1992; Parhar, 2002; Somoza et al., 2002). They are all decapeptides sharing identical residues at 1, 4, 9, and 10 positions. Among these GnRHs, a form originally isolated from chicken brain (chicken GnRH, here termed GnRH-II) was found to be the most ubiquitous GnRH peptide with unknown function, commonly present in all vertebrates from fish to human (Somoza et al., 2002). The GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2) peptides differ in three amino acids. GnRH-II is expressed in several regions of the central and peripheral nervous systems and in non-neural tissues (White et al., 1998), indicating that it might act as a neuromodulator, in addition to possible roles in control of reproductive function (Millar, 2003). Both GnRH-I and GnRH-II peptides stimulate steroidogenesis, inhibit cell proliferation, and may play a role in ovarian tumor growth (Kang et al., 2003). In the human pituitary stalk and the mouse median eminence, GnRH-II is present together with GnRH-I, as well as in the pituitary tissues of several teleost fish (Millar, 2003). The GnRHs are not co-localized in the same cells and it has been hypothesized that these peptides

may differentially regulate LH and FSH secretion and exert their effects simultaneously (Yu et al., 1997; Bosma et al., 2000; Yu et al., 2000).

Specific receptor for GnRH-II (GnRHR-II) was first described in lower vertebrates, than it was reported in primates (Millar et al., 2001; Neill et al., 2001) (Parhar et al., 2005). Cloning of GnRHR-II from Marmoset monkey cDNA showed that this receptor has only 41% amino acid identity with the GnRHR-I and has a C-terminal tail, in contrast to GnRHR-I. Consequently, it undergoes desensitization and internalization. PCR and immunocytochemistry also showed that the GnRHR-II is expressed ubiquitously in human tissues (Grundker et al., 2002), including brain, pituitary and reproductive tissues (Millar et al., 2001; Neill et al., 2001), couples to $G_{q/11}$ proteins (White et al., 1998), and stimulates the production of IP_3 when activated by its specific agonist GnRH-II (Millar et al., 2001). So far, there is no evidence for the presence of this receptor subtype in rats and the stimulatory action of GnRH-II on IP_3 production and gonadotropin release appears to be mediated by GnRHR-I (Okada et al., 2003; Mongiat et al., 2004).

Here we compared effects of GnRH-I and GnRH-II on calcium signaling and gonadotropin release in rat neonatal pituitary gonadotrophs. Like gonadotrophs from prepubertal, peripubertal and adult animals, neonatal gonadotrophs express functional GnRHR-I. A larger fraction of neonatal gonadotrophs also expresses high level of melatonin MT1 and MT2 receptors, which operate through $G_{i/o}$ signaling pathway (Vanecek, 1988) (Balik et al., 2004). Our results are consistent with Mongiat et al. data, indicating that the stimulatory action of GnRH-II on gonadotropin release is mediated by GnRHR-I, but that this agonist is not equipotent as GnRH-I in triggering secretion. We also show that in neonatal gonadotrophs cAMP signaling pathway significantly contributes in agonist control of gonadotropin secretion, probably serving as an amplifier of calcium-mediated exocytosis.

Methods

Chemicals: GnRH-I, GnRH II and Antide were obtained from Bachem (Peninsula Laboratories, Inc., Belmont, CA, USA); Pluronic F-127 and Fura-2AM from Molecular Probes, Eugene, OR, USA; MDL-12,330A (hydrochloride [cis-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine.HCl]) from Alexis (Lausen, Switzerland); ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one) from Tocris (Bristol, UK); Melatonin and other chemicals from Sigma, St. Louis Mo., USA.

Cell preparation and culture: Wistar rats (4-6 days-old) were used for cell preparation. Animals were decapitated, anterior pituitaries were extracted, and cells were dissociated by papain, as described previously (Mandler et al., 1990). Purification of gonadotrophs was done using discontinuous BSA gradient, plated on glass coverslips and cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and penicillin (100 U/ml) and streptomycin (100 μg/ml) in 95% air / 5% CO2 at 37 °C overnight. Experiments were approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

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Radioimmunoassay: LH and FSH release was determined by radioimmunoassay using antiserum and standards (RP-3) provided by the National Hormone and Pituitary program, NIDDK. Pituitary cells were cultured in 24-well plates. On day 2, cells were subjected in sixtuplicates to the different experimental maneuvers. After 3 hours, supernatants were collected and LH or FSH concentration was measured by radioimmunoassay (Vanecek and Klein, 1995). The secretion was expressed as the amount of hormone released by 15 x 10^4 cells (ng/well), or as normalized mean ± SEM values. Inter- and intraassay coefficients of variation were 8.5% and 2.4%, respectively; the sensitivity of the assay was 20 pg.

Calcium measurement: Changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) were measured in single pituitary cells incubated in M199 and loaded with 2 μM Fura-2 AM (Molecular Probes, Inc., Eugene, OR) at 37 ^oC for 60 min. Coverslips with cells were then washed with Krebs-Ringer buffer and mounted on the stage of an upright microscope Olympus BX50WI-IR with fluorescence attachment (Melville, NY, USA) and METAFLUOR Imaging Software (Visitron Systems GmbH, Germany). During recording, dishes with cell cultures were continuously perfused with an extracellular solution of the following composition (in mM): 160 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH adjusted with 1 M NaOH to 7.3. Medium and drugs were applied using a fast gravity driven micro perfusion system consisting of ten glass tubes each approximately 400 m in diameter with common outlet. The solution application was controlled by microcomputer and miniature teflon solenoid valves. Complete exchange of the solution around recorded cells required <500 ms. Cells were examined under an x40 water immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light

emission at 520 nm was measured. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in Ca^{2+} concentration, was followed in several single cells at the rate of one point per second.

Results

Gonadotropin secretion in neonatal pituitary cells is more sensitive to GnRH-I than GnRH-II

LH release was measured in mixed population of pituitary cells, which have been cultured for one day. Samples were collected after 3 h incubation with different concentrations of agonists and analyzed for their LH and FSH contents. Basal LH secretion *in vitro* was low and was subtracted in measurements. Figure 1A shows concentration-response curves for GnRH-I and GnRH-II-stimulated LH release, which could be fitted by a sigmoid function. From fitted functions, done in 15 experiments, we calculated the mean EC_{50} values: GnRH-I=2.2±0.8 nM and GnRH-II=6.8±0.3 nM. Similar profiles of GnRH-I and GnRH-II-induced LH secretion was consistent with a hypothesis that GnRH-I and GnRH-II acts on the same receptor in neonatal pituitay gonadotrophs, but with different potencies. In accordance with this hypothesis, Antide in a concentration (10 nM) that only blocks GnRHR-I, inhibited LH release stimulated with both GnRHs by 82-85 %. (Fig.1A, *inset*). Furthermore, there was no significant difference between the maximum amplitude values induced by 300 nM GnRH-I, GnRH-II or mixture of both peptides, indicating that effects of both GnRHs were not additive (Fig. 1B). In five experiments, pituitary cells were stimulated with 3 nM GnRH-I or 3 nM GnRH-II and LH and FSH secretion was measured. The secretion of both hormones in response to GnRH-II application was lower by about 20% as compared with GnRH-I, but the ratio of both values was the same (Fig. 1C).

Inhibition of adenylyl cyclase attenuates GnRH-induced LH release

Because agonist stimulation of GnRHR-I involves activation of adenylyl cyclase (AC), and soluble quanylyl cyclase (sGC) (Vanecek and Vollrath, 1989), next we examined effects of MDL (5 μM), a common inhibitor of ACs, and ODQ (5 μM), a specific inhibitor of sGC (Kostic et al., 2004; Gonzalez-Iglesias et al., 2006). Cultures were incubated for 3 hours with agonists (3 nM GnRH-I and 10 nM GnRH-II) in the presence and absence of drugs (Fig. 2). We found only a slight reduction of both agonist-induced LH release by ODQ (by 5% and 7%, for GnRH-I and GnRH-II, respectively), indicating that cGMP signaling is not critical for GnRHs-induced gonadotropin release. In contrast, there was a significant inhibition of agonist-induced LH

release by MDL (by 45% and 52 %, respectively). Melatonin (1 nM) inhibited GnRH-I and GnRH-II-stimulated LH release by 49% and 48 %, respectively. These results indicate that the secretory action of both agonists depends on the status of adenylyl cyclase activity.

Single cell calcium response by GnRH-I and GnRH-II.

It is well established that GnRH-I acts on its Ca^{2+}-mobilizing receptors in gonadotrophs, leading to oscillatory release of calcium from intracellular stores. In neonatal gonadotrophs, GnRH-I induced various types of Ca^{2+}-oscillations and the oscillatory pattern of these responses differed considerably from cell to cell but is very similar when the same cell is stimulated repeatedly with the same concentration of GnRH-I (Iida et al., 1991; Tomic et al., 1994; Zemkova and Vanecek, 2000). It is also well established that calcium is the major intracellular signaling molecule mediating the action of GnRH-I on gonadotropin release (Stojilkovic and Catt, 1992; Tse and Hille, 1992). Single-cell calcium measurements revealed that GnRH-II, like GnRH-I, increased $[Ca^{2+}]_i$ when applied for 5-120 sec. An increase in GnRH-I concentration from 0.1 to 30 nM also increased the frequency of superimposed oscillations and shortened the latency preceding the GnRH-I-induced response, as indicated in Fig. 3, *left*, by the time of beginning of agonist application and onset of response, but had little effect on the maximum response. An increase in GnRH-I concentration above 100 nM usually produces a transition from oscillatory to non-oscillatory spike response. The same pattern of calcium responses was observed when the cells were stimulated with increasing concentrations of GnRH-II (Fig. 3, *right*). However, the threshold concentration was higher for GnRH-II (~ 0.3 nM) as compared with that for GnRH-I (~ 0.1 nM).

Melatonin (1 nM), applied together with GnRH-I or GnTh-II, prolonged latency (not shown) or completely inhibited agonist-induced calcium responses when cells were stimulated with lower GnRH concentrations (Fig. 4A). However, both effects were observed only in 52% of neonatal gonadotrophs, which is in accordance with literature showing the expression of functional receptors in 40-70% of neonatal gonadotrophs (Vanecek and Klein, 1992; Zemkova and Vanecek, 1997). Remaining cells were melatonin-insensitive, and these cells also responded to GnRH-II (Fig. 4B). At higher agonist concentrations, melatonin was ineffective (data not shown). These results indicate that melatonin inhibits GnRH-I and GnRH-II-induced gonadotropin release by inhibiting initiation of calcium signaling and in a calcium-dependent (Zemkova and Vanecek, 2000) and probably cAMP/protein kinase C-dependent manner.

Discussion

Here we show that GnRH-II has comparable effects as GnRH-I in pituitary cells from 4 to 6-day-old rats. Both agonists evoked comparable $[Ca^{2+}]_i$ signals and secretory outputs in a dose-dependent manner, a finding consistent with a major role of calcium signals in controlling exocytosis of dense core vesicles in these cells (Hille et al., 1994). Three lines of evidence argue against the functional expression of GnRHR-II in neonatal gonadotrophs: 1. There was no difference in the maximum LH and FSH response when cells were stimulated with the supramaximal agonist concentrations (300 nM); 2. The action of two agonists was not additive; 3. Antide inhibits GnRHR-I and GnRHR-II signaling with the EC_{50} concentrations of 1 and 10000 nM, respectively (Neill, 2002). In our hands, the action of both agonists was abolished by 10 nM of this GnRHR-I-specific blocker.

We also found that the EC_{50} value for GnRH-II was about 3 times rightward shifted compared to GnRH-I, which is consistent with heterologous activation of the GnRHR-I in neonatal rat pituitary. Theoretically, an activation of receptor with ligand of lower affinity could induce differences in receptor conformation changes and lower effect. In the case G protein-coupled receptors, domain that determine agonist specificity does not affect interaction with G protein on the intracellular side of the receptor (Colquhoun, 1998) which could explain why maximum effect stimulated by both GnRHs was identical. These results are in agreement with previous finding obtained on pituitary cells from adult monkey or rats (Okada et al., 2003) and from 15-day-old rats (Mongiat et al., 2004) showing that GnRH-II increases production of intracellular IP_3, and gonadotropin secretion by activating GnRH-I receptors.

In vertebrates, including rats, GnRH-II is more widely distributed than GnRH-I, indicating that it might have several roles (Millar et al., 2001). However, gene for GnRHR-II could not be detected in peripubertal rats (Mongiat et al., 2004) and we also have failed to produce direct evidence of mRNA transcript for GnRHR-II in total RNA from neonatal pituitary (Balik, unpublished). The finding that GnRH-II can activate GnRH-I receptor in neonatal rat pituitary, and was a less effective stimulator oft both LH and FSH secretion than was GnRH-I, support the idea that it may have main biological effects in nonreproductive functions in the rat .

Earlier studies have revealed that activation of melatonin receptors in pituitary cells leads to inhibition of cAMP production (Vanecek and Vollrath, 1989). MDL, a broad AC inhibitor, also down regulates cAMP production in pituitary cells (Gonzalez-Iglesias et al., 2006). However, the role of cAMP in GnRH-induced gonadotropin secretion has not been clarified. Here we show that in neonatal gonadotrophs, the GnRH-I and GnRH-II-stimulated secretion of hormones is sensitive to both receptor- and non-receptor-mediated inhibition of AC. In part, the

inhibitory action of melatonin and MDL could be related to the control of voltage-dependent Ca channels and calcium entry, which is important for agonist-induced calcium signaling (Zemkova and Vanecek, 2000). This in turn affects gonadotropin secretion, as demonstrated earlier (Stojilkovic and Catt, 1995). However, melatonin was ineffective in blocking high agonist-induced calcium signals, indicating that it also inhibits secretion independently of the status of calcium signaling. This is consistent with findings in other secretory anterior pituitary cells showing effects of cAMP, presumably through protein kinase A, on exocytosis of dense core vesicles (Zorec et al., 1991).

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Text to Figures

Fig.1

LH and FSH release stimulated by GnRH-I and GnRH-II in primary culture of neonatal pituitary cells.

(A) The release of LH was stimulated by both GnRH-I and GnRH-II in a dose-dependent manner. The GnRH-II dose-response curve (open circles) was shifted to the right when compared to GnRH-I dose-response curve (closed circles). Concentrations inducing half maximal effects (EC_{50} values) are shown above traces. Both GnRH-I- and GnRH-II- induced LH-release, stimulated by EC_{50} concentrations, was inhibited by specific inhibitor of GnRHR-I Antide (inset). (B) Maximum LH release stimulated by GnRH-I (300 nM), GnRH-II (300 nM) and both GnRHs was comparable.

Fig.2

The effect of adenylyl cyclase inhibitors on LH secretion stimulated by GnRH-I and GnRH-II.

The GnRH-I- and GnRH-II- induced LH-release in primary culture of neonatal pituitary cells was inhibited by common AC inhibitor MDL (5 μM) and pineal hormone melatonin (1 nM). The effect of inhibition of soluble guanylyl cyclase by ODQ (5 μM) was insignificant in both cases.

Fig.3

The effect of GnRH-II on calcium oscillations in neonatal gonadotrophs.

GnRH-II stimulated oscillatory increase in $[Ca^{2+}]_i$ in the same subpopulation of neonatal gonadotrophs, which also responded to GnRH-I, but the threshold concentration was different GnRH-I-induced calcium responses could be evoked at 0.1 nM (*left),* wheras GnRH-II-induced oscillations started after stimulation with 0.3 nM (*right*). Horizontal bars above traces indicate the time of agonist application.

Fig.4

Melatonin sensitivity of GnRH-I and GnRH-II-induced calcium responses.

(A), Melatonin inhibited Ca^{2+} oscillations evoked by both GnRH-I and GnRH-II-induced in the same cell. Melatonin-sensitive cells represent a subpopulation of neonatal goadotrophs. (B) GnRH-II-induced oscillations also in melatonin-insensitive gonadotrophs.

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Fig. 1

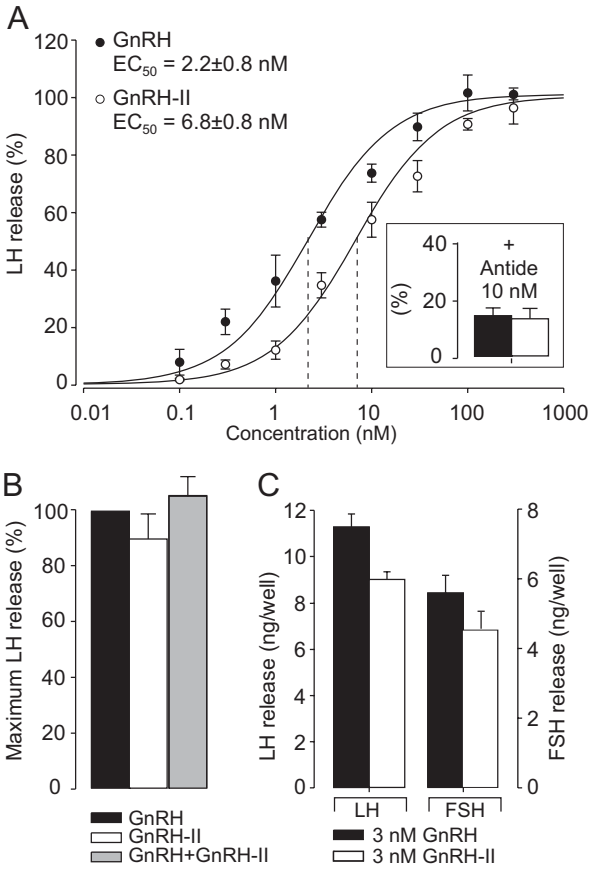

A
GnRH
EC50 = 2.2±0.8 nM
GnRH-II
EC50 = 6.8±0.8 nM
LH release (%)
Concentration (nM)
+
Antide
10 nM
(%)
B
Maximum LH release (%)
GnRH
GnRH-II
GnRH+GnRH-II
C
LH release (ng/well)
FSH release (ng/well)
LH
FSH
3 nM GnRH
3 nM GnRH-II

Fig. 2

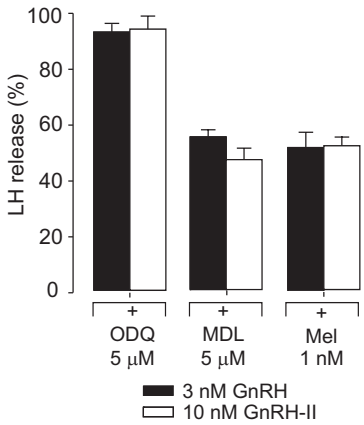

100
80
60
40
20
0
LH release (%)
+
+
+
ODQ
5 μM
MDL
5 μM
Mel
1 nM
3 nM GnRH
10 nM GnRH-II

Fig.3

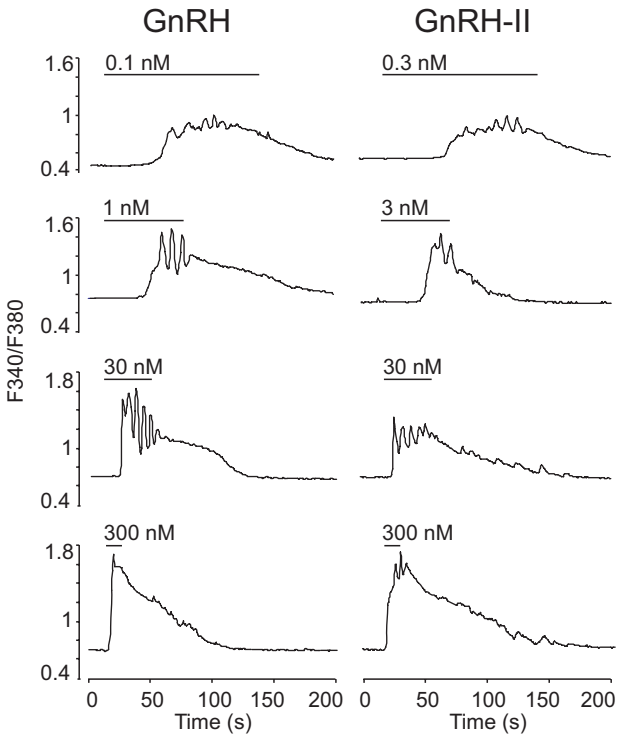
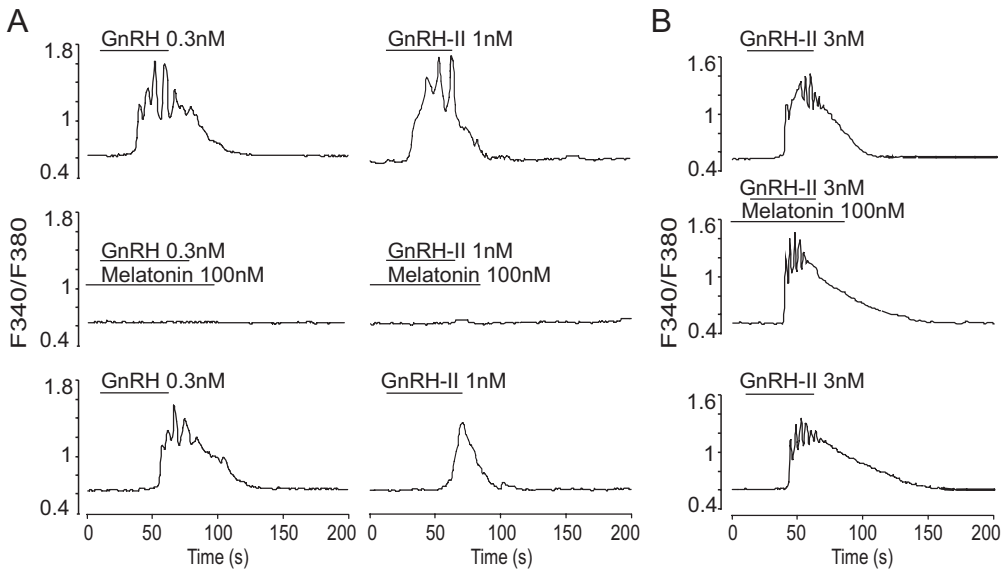

GnRH
GnRH-II
0.1 nM
0.3 nM
1 nM
3 nM
30 nM
30 nM
300 nM
300 nM
F340/F380
1.6
1
0.4
1.8
0
50
100
150
200
Time (s)

Fig. 4


A
GnRH 0.3nM
GnRH-II 1nM
GnRH 0.3nM
Melatonin 100nM
GnRH-II 1nM
Melatonin 100nM
GnRH 0.3nM
GnRH-II 1nM
F340/F380
1.8
1
0.4
0
50
100
150
200
Time (s)
B
GnRH-II 3nM
GnRH-II 3nM
Melatonin 100nM
GnRH-II 3nM
1.6
1
0.4
F340/F380
0
50
100
150
200
Time (s)