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GnRH-I and GnRH-II-Induced Calcium Signaling and Hormone Secretion in Neonatal Rat Gonadotrophs

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Summary

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 three 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 that GnRH-I and GnRH-II act on the same GnRHR to stimulate gonadotropin release through intracellular calcium and cyclic nucleotide signaling, and that GnRH-II is less potent agonist for this receptor in neonatal rat gonadotrophs.

Key words

Gonadotropin-releasing hormone • GnRH-II • Melatonin • LH • FSH • Neonatal Rats

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Introduction

Reproductive functions in vertebrates are controlled by decapeptide GnRH (gonadotropin-releasing hormone), 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 gonadotropin-releasing hormone receptor (GnRHR), 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 for 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). All decapeptides are 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 the 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, then 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 immuno-cytochemistry 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 levels of melatonin MT1 and MT2 receptors, which operate through $G_{i/o}$ signaling pathway (Vaněček 1988) (Balík *et al.* 2004). Our results are consistent with the data of Mongiat *et al.* (2004), indicating that the stimulatory action of GnRH-II on gonadotropin release is mediated by GnRHR-I and inhibited by melatonin, but that this agonist is not equipotent as GnRH-I in triggering the secretion. We also show that in neonatal gonadotrophs cAMP signaling pathway significantly contributes to 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, Belmont, CA, USA); Pluronic F-127 and Fura-2AM from Molecular Probes (Eugene, OR, USA); MDL-12,330A (hydrochloride [cis-N-(2-phenylcyclopentyl)azacyclo-tridec-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 % CO_2 at 37 °C overnight. Experiments were approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

Radioimmunoassay

LH and FSH release was determined by radioimmunoassay using antiserum and standards (RP-3) provided from the National Pituitary Agency and Dr. AF Parlow (Harbor-UCLA Medical Center, Torrance, CA).

Pituitary cells were cultured in 24-well plates. On day 2, cells were subjected in sixtuplicates to the different experimental maneuvers. After 3 h, supernatants were collected and LH or FSH concentration was measured by radioimmunoassay (Vaněček 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 ± S.E.M. 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, Eugene, OR) at 37 °C 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, 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 μm in diameter with common outlet. Solution application was controlled by microcomputer and miniature teflon solenoid valves. Complete exchange of the solution around recorded cells required less than 500 ms. Cells were examined under an x40 water immersion objective during exposure to alternating 340 nm 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 the 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

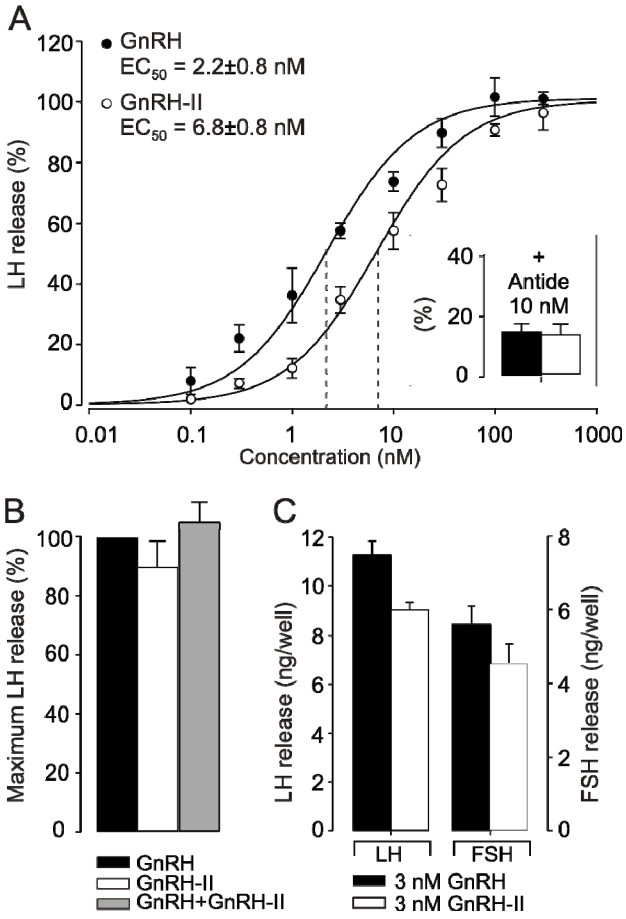

Fig. 1. LH and FSH release stimulated by GnRH-I and GnRH-II in primary culture of neonatal pituitary cells. **A.** The release of LH was stimulated by both GnRH-I (here and in all figures marked as GnRH) and GnRH-II in a dose-dependent manner. The GnRH-II dose-response curve (open circles) was shifted 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. **C.** The ratio of secretion of both hormones in response to GnRH-II and GnRH-I application was comparable.

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 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 act on the same receptor in neonatal pituitary gonadotrophs, but with different potencies.

In accordance with this hypothesis, Antide in a concentration (10 nM) that only blocks GnRHR-I,

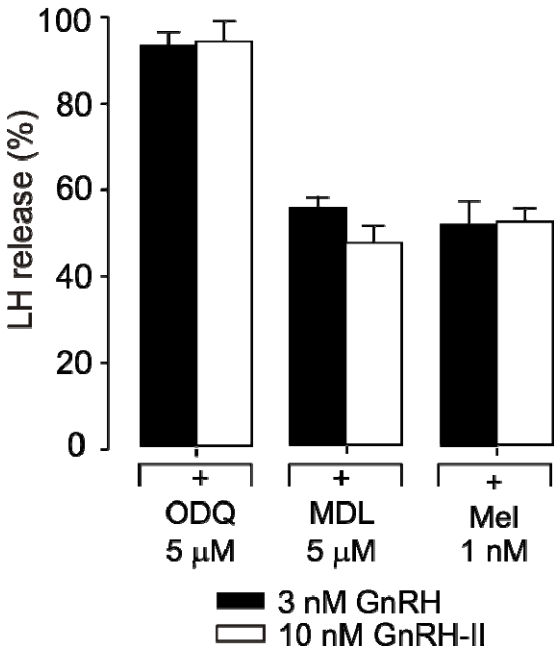

Fig. 2. The effect of adenylyl cyclase inhibitors on LH secretion stimulated by GnRH-I and GnRH-II. The GnRH-I- and GnRH-II-induced LH release in primary culture of neonatal pituitary cells was inhibited by common adenylyl cyclase inhibitor MDL (5 μM) and pineal hormone melatonin (Mel; 1 nM). The effect of inhibition of soluble guanylyl cyclase by ODQ (5 μM) was insignificant in both cases.

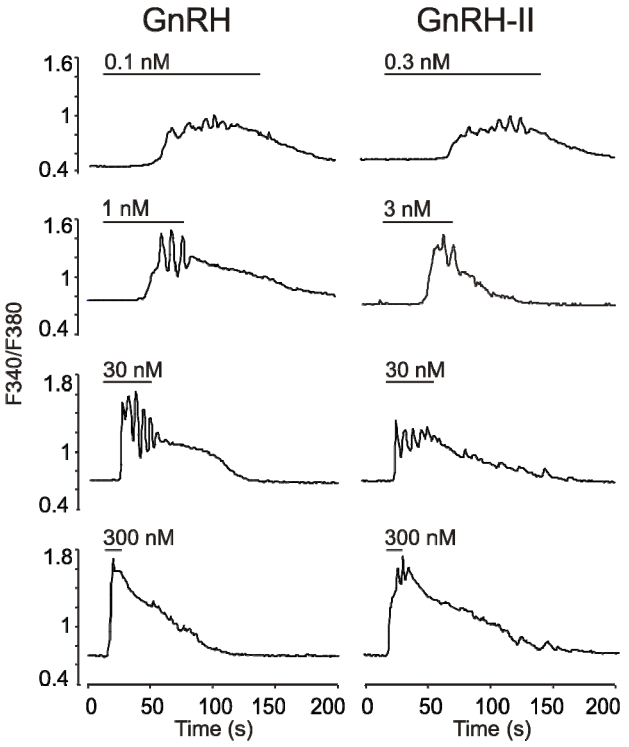

Fig. 3. The effect of GnRH-II on calcium oscillations in neonatal gonadotrophs. GnRH-II stimulated oscillatory increase in $[Ca^{2+}]_i$ in the same subpopulation of neonatal gonadotrophs, which also responded to GnRH-I, but the threshold concentration was different GnRH-I-induced calcium responses could be evoked at 0.1 nM (*left)*, whereas GnRH-II-induced oscillations started after stimulation with 0.3 nM (*right*). Horizontal bars above traces indicate the time of agonist application.

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) (Vaněček 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 h 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 was very similar when the same cell was stimulated repeatedly with the same concentration of GnRH-I (Iida *et al.* 1991, Tomic *et al.* 1994, Zemková and Vaněček 2000). It is well established that calcium is the major intracellular signaling molecule mediating the action of

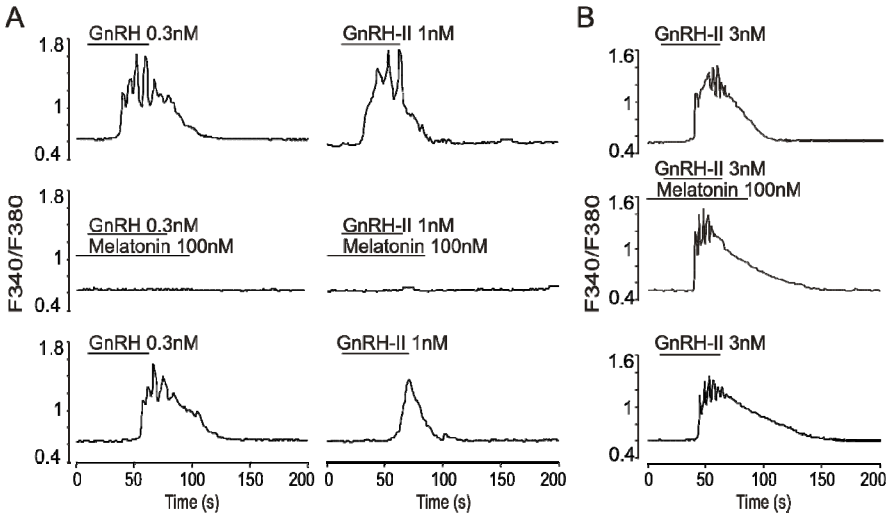

Fig. 4. Melatonin sensitivity of GnRH-I and GnRH-II-induced calcium responses. **A.** Melatonin inhibited Ca^{2+} oscillations evoked by both GnRH-I and GnRH-II-induced in the same cell. Melatonin-sensitive cells represent a subpopulation of neonatal gonadotrophs. **B.** GnRH-II also induced oscillations in melatonin-insensitive gonadotrophs.

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 s. 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 (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 (Vaněček and Klein 1992, Zemková and Vaněček 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 (Zemková and Vaněček 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, and 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 shifted about three times rightward 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 of G protein-coupled receptors, the domain that determines agonist specificity does not affect interaction with G protein on the intracellular side of the receptor (Colquhoun 1998). This could explain why maximum effect of stimulation with both GnRHs was identical. These results are in agreement with previous findings obtained in 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 have also failed to obtain direct evidence of mRNA transcript for GnRHR-II in total RNA from neonatal pituitary (Balík, unpublished). The findings that GnRH-II can activate GnRH-I receptor in neonatal rat pituitary, and that GnRH-II was a less effective stimulator of both LH and FSH secretion than was GnRH-I, support the idea that it may have main biological effects in non-reproductive 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 adenylyl cyclase 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 adenylyl cyclase. The inhibitory action of melatonin and MDL could be partially related to the control of voltage-dependent Ca^{2+} channels and calcium entry, which is important for agonist-induced calcium signaling (Zemková and Vaněček 2000). This in turn affects gonadotropin secretion as demonstrated earlier (Stojilkovic and Catt 1995). However, melatonin was ineffective in blocking calcium signals stimulated with high doses of GnRH-I (Zemkova and Vanecek 2001) or GnRH-II (here, data not shown), indicating that it also inhibits secretion by calcium–independent mechanism. 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).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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