Peptides 30 (2009) 2081–2087

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Neuropeptide glutamic-isoleucine (NEI) specifically stimulates the secretory activity of gonadotrophs in primary cultures of female rat pituitary cells

Ana Lucía De Paul [a,*], Andrés Maximiliano Attademo [d], Ruben Walter Carón [e], Marta Soaje [c], Alicia Inés Torres [a], Graciela Alma Jahn [c], María Ester Celis [b]

[a] Centro de Microscopía Electrónica, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Haya de la Torre esquina Enrique Barros, Ciudad Universitaria, 5000 Córdoba, Argentina
[b] Laboratorio de Ciencias Fisiológicas, Cátedra de Bacteriología y Virología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina
[c] Laboratorio de Reproducción y Lactancia, IMBECU-CONICET, Mendoza, Argentina
[d] Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[e] Laboratorio de Hormonas y Biología del Cáncer, IMBECU-CONICET, Mendoza, Argentina

ARTICLE INFO

Article history:
Received 23 December 2008
Received in revised form 14 August 2009
Accepted 25 August 2009
Available online 1 September 2009

Keywords:
NEI
Gonadotroph
LH
Pituitary cell culture
GnRH
Electron microscopy

ABSTRACT

The neuropeptide EI (NEI) is derived from proMCH. It activates GnRH neurons, and has been shown to stimulate the LH release following intracerebroventricular administration in several experimental models. The aim of the present paper was to evaluate NEI actions on pituitary hormone secretion and cell morphology in vitro. Pituitary cells from female rats were treated with NEI for a wide range of concentrations (1–400 $\times 10^{-8}$ M) and time periods (1–5 h). The media were collected and LH, FSH, PRL, and GH measured by RIA. The interaction between NEI (1, 10 and 100 $\times 10^{-8}$ M) and GnRH (0.1 and 1 $\times 10^{-9}$ M) was also tested. Pituitary cells were harvested for electron microscopy, and the immunogold immunocytochemistry of LH was assayed after 2 and 4 h of NEI incubation. NEI (100 $\times 10^{-8}$ M) induced a significant LH secretion after 2 h of stimulus, reaching a maximum response 4 h later. A rapid and remarkable LH release was induced by NEI (400 $\times 10^{-8}$ M) 1 h after stimulus, attaining its highest level at 2 h. However, PRL, GH and FSH were not affected. NEI provoked ultrastructural changes in the gonadotrophs, which showed accumulations of LH-immunoreactive granules near the plasma membrane and exocytotic images, while the other populations exhibited no changes. Although NEI (10 $\times 10^{-8}$ M), caused no action when used alone, its co-incubation with GnRH (1 $\times 10^{-9}$ M), promoted a slight but significant increase in LH. These results demonstrate that NEI acts at the pituitary level through a direct action on gonadotrophs, as well as through interaction with GnRH.

1. Introduction

The rat neuropeptide EI (NEI) is a peptide derived from the proMCH located in neurons from the lateral hypothalamus and zona incerta, which have widespread projections throughout the central nervous system [3,10]. The structures of NEI and MCH are highly conserved among vertebrates, indicating that these peptides may have important physiological functions [13,20], among which, are the control of water and electrolyte behavior [26]. Although there is little information about the neurobiological functions of NEI, it has been demonstrated that NEI immunoreactivity (NEI-IR) can be modified by ovariectomy and treatment with 17-β estradiol benzoate [30], and it has been shown that this peptide is secreted from cultured rat hypothalamic cells [25]. Furthermore, it was demonstrated that both NEI and MCH can suppress the TRH release from hypothalamic cultures, with MCH being able to reduce the TRH-induced TSH secretion from the dispersed pituitary cell cultures [16]. In addition, we previously demonstrated that an intracerebroventricular (i.c.v.) injection of NEI, given to male and chronically ovariectomized (OVEX) female rats treated with estradiol benzoate plus progesterone, induced an increase in the serum LH levels compared to controls injected with artificial cerebrospinal fluid (ACSF) [2]. In order to identify the anatomical substrate underlying this effect, we used combined immunohistochemistry methods to analyze the forebrains of female rats. In the afternoon of the proestrus day, the NEI fibers were found to be in close apposition with the anteroventral periventricular nucleus and the GnRH neurons expressing c-Fos. In the median eminence, NEI varicosities and terminal like-structures were in near proximity to blood vessels and GnRH fibers. Related to this, it has previously been described that NEI can induce LH

* Corresponding author. Tel.: +54 351 4333021; fax: +54 351 4333021.
E-mail addresses: adepaul@cmefcm.uncor.edu (A.L. De Paul), mefisiol@mater.fcm.unc.edu.ar (M.E. Celis).

0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.08.022

secretion, by direct release into the median eminence, through the modulation of GnRH neurons located in the preoptic area, or by the modulation of GnRH terminals located in the median eminence [10].

Leading on from these results, in the present study we decided to investigate whether NEI can act directly at the pituitary level by modulating hormone secretion. To this purpose, the effect of NEI in pituitary cell cultures from female rats on the release of several pituitary hormones such as LH, FSH, GH and prolactin was studied. Furthermore, the pituitary cell morphology was evaluated by electron microscopy and immunocytochemistry, and finally, the ability of NEI to potentiate GnRH-induced LH release was tested.

2. Materials and methods

2.1. Animals

Adult female Wistar rats (200–250 g) were bred and housed at the Animal Research Facility of the National University of Córdoba, in air-conditioned quarters under a controlled photoperiod (14L:10D) with free access to commercial rodent food and tap water. Animals were kept in accordance with the National Institute Health Guide for the Care and Use of Laboratory Animals.

2.2. Pituitary cell cultures

The techniques for cell dissociation and culture of pituitary cells were previously described in detail [9]. Briefly, a pool of pituitary glands from forty female rats at random cycle stages was used for each cell culture. These rats were sacrificed by decapitation and the pituitaries rapidly excised. The posterior and intermediate lobes were discarded and the anterior pituitaries placed in Eagle's Minimal Essential Medium (SMEM). The anterior pituitaries were washed several times with SMEM and sliced into small fragments. These fragments were dispersed enzymatically by successive incubations in SMEM containing 0.4% trypsin (type I from bovine pancreas), 1 mg/ml DNase (deoxyribonuclease II, type V from porcine spleen), and 1 mg/ml trypsin inhibitor (type II-S from soybean), before being finally dispersed by extrusion through Pasteur pipettes. The cell yield was $1.5–2.0 \times 10^6$ per pituitary, and the cell viability, tested by Trypan Blue exclusion, was always better than 90%. The cells were seeded at the bottom of six-well culture plates (Corning, New York, USA) at a density of 5×10^5 cells/well, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% fetal calf serum and 8% horse serum (Invitrogen, Carlsbad, USA). These plates were placed in an oven with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3 days. Then, the media were discarded and replaced with fresh DMEM every day, while the pituitary cells derived from female rats were submitted to different experimental protocols. On the 5th day of culture, the medium was discarded, and 1 ml of fresh serum-free DMEM containing hydrocortisone (100 μg/l), 3,3′-triiodothyronine (400 ng/l), transferrin (10 mg/l) and sodium selenite (5 μg/l) was used to replaced the old one every 60 min. These cultures were allowed to stabilize for 3 h.

All culture media were filtered through 0.2 μm Nalgene membranes (Nalge Company, New York, USA). The cell culture grade reagents were obtained from Sigma (St. Louis, USA).

2.3. Cell treatments

2.3.1. Effects of NEI on the secretory activity of pituitary cells

With the aim of investigating the effects of NEI on the secretory behavior of pituitary cells in primary culture, a single stimulus of 100 or 400×10^{-8} M NEI (Bachem, Torrance, CA USA) was added to test wells for 1, 2, 3, 4, and 5 h, and this procedure was performed in triplicate. Wells incubated with medium free NEI served as controls, with the concentrations chosen based on a previous report [2,28]. Culture media samples, which represented accumulated hormone concentrations for the periods of time stated above, were collected and stored at −20 °C until LH, FSH, PRL, and GH determinations made by radioimmunoassay (RIA).

2.3.2. NEI: dose–response curve

After determining that 100 or 400×10^{-8} M NEI was able to specifically release LH from the pituitary cell cultures, with maximum effects occurring between 2 and 4 h after their addition, we performed a second set of experiments to determine a curve dose response for lower doses of NEI. To carry this out, pituitary cells were cultured for 5 days as described above, and then a single pulse with a different concentration of NEI ($1–100 \times 10^{-8}$ M) was added together with fresh serum-free DMEM for 3 h to triplicate wells. Control test wells free of NEI were also run in parallel. The culture media were then collected and stored at −20 °C for subsequent LH measurement by radioimmunoassay (RIA).

2.3.3. Treatment combining NEI with GnRH

To determine whether NEI could synergize with GnRH to stimulate LH release, we performed a GnRH dose response curve by incubating the pituitary cells on the 5th day of culture with increasing concentrations of GnRH (0.1, 1, 2, 10 and 50×10^{-9} M) for 3 h (Sigma, St. Louis, MO). Doses in this range can be considered to be physiologic, and have been previously used in other laboratories [4,14].

In the last set of experiments, the pituitary cells were co-incubated for 3 h with 0.1 or 1×10^{-9} M of GnRH, and 1, 10 or 100×10^{-8} M NEI, after which, the medium was collected and stored at −20 °C for later LH determination. The protocols also included the addition of NEI alone (10 or 100×10^{-8} M) or GnRH alone (0.1 or 1×10^{-9} M) to test wells. Controls were performed without any neuropeptides. At the end of each experimental condition, the media were collected and stored at −20 °C for LH quantification by RIA.

2.4. Hormone determinations

LH, FSH, PRL and GH were measured by double antibody radioimmunoassay, using materials generously provided by Dr. Parlow and NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radio-iodinated using the Chloramine T method, and purified by passage through Sephadex G75. The results were expressed in terms of the rat LH RP-3, FSH RP-3, PRL RP-3, TSH RP-3 or rat GH RP-2 standard preparations. Assay sensitivity was 0.5 μg/l serum, with the inter- and intra-assay coefficients of variation being less than 10% for all hormones.

2.5. Electron microscopy and immunocytochemistry

With the objective of evaluating the ultrastructural features of pituitary cells submitted to NEI treatment, the cell monolayers were scraped from the wells, washed and fixed in a mixture of 4% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h. These fixed cells were centrifuged and the pellets treated with 1% OsO_4 for 1 h, before being stained in block with 1% uranyl acetate in 0.1 M acetate buffer pH 5.2 for 20 min. After dehydration with a series of graded cold acetones, the cells were embedded in Araldite. Thin sections were cut using a JEOL ultramicrotome with a diamond knife, which were then stained with uranyl acetate/lead citrate and examined using a Zeiss Leo 906-E electron microscope (Oberkochen, Germany).

For immunocytochemical LH detection, pituitary cells previously stimulated with 100 or 400×10^{-8} M NEI for 2 and 4 h were scraped, washed, centrifuged and fixed in a mixture of 4% formaldehyde, 1.5% glutaraldehyde and 0.1 M cacodylate buffer pH 7.3, at room temperature for 5–6 h with osmium fixation being omitted. The pellet was dehydrated by a series of increasing concentrations of ethanol, before being embedded in a 1:1 solution of LR White (Sigma, St. Louis, USA) overnight. The following morning, samples were placed in fresh LR White, embedded in gelatin capsules, and polymerized at 50 °C for 48 h. Thin sections were then cut in a JEOL ultramicrotome with a diamond knife and incubated on a drop of β-LH rabbit polyclonal antibody overnight at 4 °C (1:1000) (NIHDDK, Bethesda, USA). These were then washed with distilled water and incubated with secondary antibody goat anti-rabbit conjugated to 15 nm colloidal gold particles (Electron Microscopy Sciences, Hatfield, USA), diluted 1:15 in blocking buffer. To validate the specificity of the immunostaining, the following controls were performed: (1) replacement of the primary antiserum with 1% BSA in 0.1 M phosphate buffer, pH 7.3, plus 0.15 M sodium chloride (PBS); (2) replacement of the primary antiserum with diluted preimmune serum followed by the secondary antibody. Sections were examined in a Zeiss LEO 906-E electron microscope and photographed with a megaview III camera.

2.6. Statistical analysis

Experimental points represented the mean ± S.E.M. of three replicates measured for each of the three independent cell cultures. The statistical analysis was carried out using ANOVA, followed by Fisher's test using the InfoStat program with the significance level chosen to be $p < 0.05$.

3. Results

3.1. Effects of NEI on pituitary hormone release

3.1.1. LH secretion

With the aim of establishing the direct actions of NEI on LH secretion from primary pituitary cell cultures, a single pulse of NEI (100 and 400×10^{-8} M) was added to test wells and the culture media were collected after 1, 2, 3, 4, or 5 h of exposition. As shown in Fig. 1, NEI induced a fast release of LH in the culture media. There were differences in the LH levels obtained for the various time periods of hormonal stimuli assayed, which were closely associated with the doses applied. The lower dose of NEI (100×10^{-8} M) induced a significant LH secretion after 2 h of stimulus, reaching a maximum response after 4 h of NEI treatment. At this time, the LH levels almost quintupled that of the control ($p < 0.01$) and maintained these values without any significant variations up to 5 h of stimulus. When the higher concentration of NEI (400×10^{-8} M) was assayed, a rapid and remarkable LH release was induced at 1 h after stimulus ($p < 0.01$), attaining the highest level after NEI incubation for 2 h (6.5-fold approximately). Subsequently, the LH secretion remained constant up to 5 h of treatment.

3.1.2. FSH, PRL and GH release

To ascertain if NEI was able to induce the secretion from the other pituitary cells, the FSH, PRL and GH levels were determined by RIA. In spite of NEI being effective in stimulating LH secretion, none of the doses assayed were capable of significantly promoting FSH secretion from gonadotroph cells. Also, no significant effects were observed on PRL or GH secretion, either with 100×10^{-8} or 400×10^{-8} M NEI in primary pituitary cell cultures, thus confirming the specificity of NEI stimuli on LH secretion (Table 1).

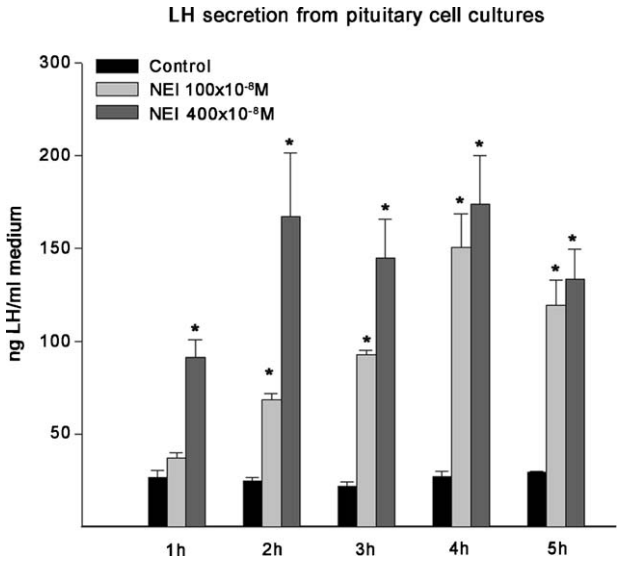

Fig. 1. Time-course study of the effects of NEI on LH secretion in the culture media. The cell cultures were treated with NEI 100 or 400×10^{-8} M for 1–5 h, in serum-free conditions. The data are represented as mean ± S.E.M. of three independent experiments. Data were evaluated by the ANOVA–Fisher test; $^{*}p < 0.01$ vs Control group.

3.2. Effects of increasing concentrations of NEI on LH secretion

In order to determine if lower doses of NEI could induce LH secretion, we assayed various concentrations of this neuropeptide (1, 7, 10, 70 and 100×10^{-8} M) on pituitary cell cultures incubated in serum-free conditions for 3 h. As shown in Fig. 2, only the two highest concentrations of NEI, 70 and 100×10^{-8} M, significantly stimulated LH release, with values rising to 36% with 70×10^{-8} M ($p < 0.01$), and this increase being even more evident after 100×10^{-8} M of NEI treatment compared to control wells. However, the other doses applied (1, 7 and 10×10^{-8} M) were unable to modify LH secretion.

3.3. Effect of co-incubation of NEI with GnRH on LH secretion

To determine whether NEI was able to synergize with GnRH in stimulating LH release, the range of GnRH concentrations that could significantly stimulate LH secretion was determined by performing a GnRH dose response curve (0.1, 1, 2, 10, 20 and 50×10^{-9} M) (data not shown). From these results, the two lowest GnRH concentrations were selected for the following protocols.

Pituitary cells were simultaneously incubated with GnRH (0.1 or 1×10^{-9} M) and different NEI concentrations (1, 10 or 100×10^{-8} M) for 3 h. Then, the media were collected and tested for LH by RIA.

No additional changes in LH secretion occurred when NEI (1, 10 or 100×10^{-8} M) was co-incubated with 0.1×10^{-9} M of GnRH compared with the effect produced by GnRH alone (0.1×10^{-9} M).

Although NEI 10×10^{-8} M had no effect on LH secretion, GnRH 1×10^{-9} M plus NEI 10×10^{-8} M induced a slight but significant increase in LH levels (16%; $p < 0.01$). A combined treatment with the highest doses of both NEI (10×10^{-8} M) and GnRH (1×10^{-9} M) significantly stimulated the secretory response, which was slightly more effective than that obtained with GnRH 1×10^{-9} M alone (30%; $p < 0.01$) (Fig. 3).

3.4. Electron microscopy

In pituitary cell cultures from female rats, all the secretory cell types were observed. These were identifiable by their ultrastruc-

Table 1
Time-course study of the effects of NEI on FSH, PRL and GH secretion accumulated in the culture media (ng/ml of culture medium). Pituitary cells were treated with NEI 100 or 400×10^{-8} M for 1–5 h. The data are shown as the mean ± S.E.M. of three independent experiments and were evaluated by the ANOVA–Fisher test.

	Time exposition	Control Mean ± S.E.M.	NEI 100×10^{-8} M Mean ± S.E.M.	NEI 400×10^{-8} M
FSH secretion	1 h	32.41 ± 4.93	38.16 ± 1.05	38.1 ± 3.30
	2 h	32.24 ± 3.22	38.46 ± 1.70	31.92 ± 5.59
	3 h	28.83 ± 3.15	36.23 ± 1.82	24.65 ± 1.35
	4 h	25.80 ± 2.22	29.53 ± 2.09	19.88 ± 1.36
	5 h	28.30 ± 3.18	28.91 ± 3.15	22.35 ± 1.30
PRL secretion	1 h	839.42 ± 47.96	998.65 ± 42.09	926.64 ± 96.40
	2 h	1079.81 ± 56.38	1204.50 ± 93.25	951.60 ± 69.38
	3 h	1182.30 ± 92.82	1338.38 ± 141.05	936.41 ± 27.40
	4 h	1567.68 ± 95.27	1703.32 ± 26.56	1373.41 ± 157.41
	5 h	1537.53 ± 66.10	1736.56 ± 129.94	130.88 ± 56.86
GH secretion	1 h	395.20 ± 56.05	309.13 ± 36.45	502.79 ± 26.09
	2 h	485.51 ± 49.54	426.75 ± 42.88	533.82 ± 36.48
	3 h	522.03 ± 70.54	585.64 ± 37.47	508.72 ± 49.67
	4 h	740.07 ± 96.60	729.82 ± 76.28	618.08 ± 71.91
	5 h	730.90 ± 38.26	897.58 ± 72.80	676.75 ± 66.53

tural characteristics, essentially by the profile of the secretory granules, which constituted a distinctive feature. The most frequent populations observed were lactotroph and somatotroph cells, which were in close contact with gonadotroph cells (Fig. 4A). In the control group, the lactotroph and somatotroph cells had numerous polymorphic and round mature secretory granules, respectively. These also had high electron densities and were stored in the cytoplasm (Fig. 4A). The gonadotrophs were characterized by a conspicuous accumulation of round secretory granules of two different sizes and by electron densities in the cytoplasm. The most abundant granules were about 150 nm in diameter and filled with homogeneous material, whereas the others were less frequent but larger in size (about 400 nm) (Fig. 4A and B).

Stimulation with NEI (100×10^{-8} and 400×10^{-8} M) for 2 and 4 h, promoted several subtle structural changes, particularly in the gonadotroph cell population. For this cell type, the most prominent changes consisted of a striking development of the rough endoplasmic reticulum (RER) and Golgi complex (Fig. 5A) and a significant reduction in secretory granules, with those remaining showing lower electron densities (Fig. 5B) when compared to the control group. Many secretory granules were located adjacent to the cell membrane and presented images of exocytosis after NEI treatment (Fig. 5C).

Other pituitary cell populations, the lactotrophs, thyrotrophs and somatotrophs, did not exhibit any features indicating a significant activation of hormone release after NEI treatment (data not shown).

4. Discussion

The study of the effects of physiological stimuli involved in the regulation of pituitary hormone secretion is facilitated by the availability of a system consisting of a suspension of single dispersed pituitary cells, in which the cell structure and function are essentially the same as in situ. The present results are the first demonstration of a specific and direct action of NEI on cultured pituitary cells, which was able to stimulate LH release, without modifying the other pituitary hormones studied (FSH, GH, and PRL). These findings indicate that the peptide acts specifically on gonadotroph cells, leading to a rapid stimulatory effect on LH release. Moreover, the analysis of the electron microscopy images taken 2 and 4 h after NEI treatment was indicative of the stimulation of LH release occurring at these times. In addition, gonadotroph cells exhibited cytological changes compatible with

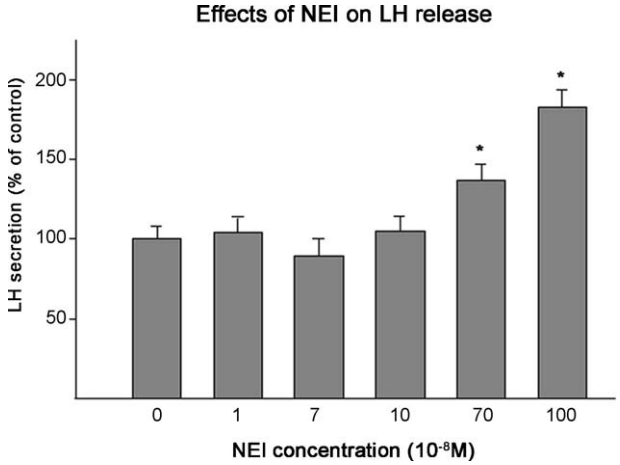

Fig. 2. Effect of different concentrations of NEI on LH secretion. Pituitary cell cultures were exposed to a dose–response curve of NEI at doses of 1, 7, 10, 70 or 100×10^{-8} M for 3 h. Data are shown as the mean ± S.E.M. of three independent experiments. ANOVA–Fisher test; $^{*}p < 0.01$ vs control group.

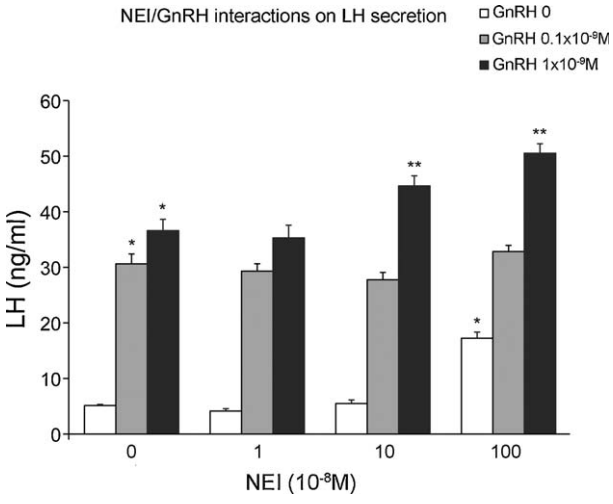

Fig. 3. NEI and GnRH combined treatments on LH secretion. The presence of 10 and 100×10^{-8} M NEI in the culture media for 3 h promoted a significant increase in LH release stimulated by GnRH 1×10^{-9} M. Data are shown as the mean ± S.E.M. of three independent experiments. ANOVA–Fisher test; $^{*}p < 0.01$ vs control group; $^{**}p < 0.01$ vs GnRH 1×10^{-9} M.

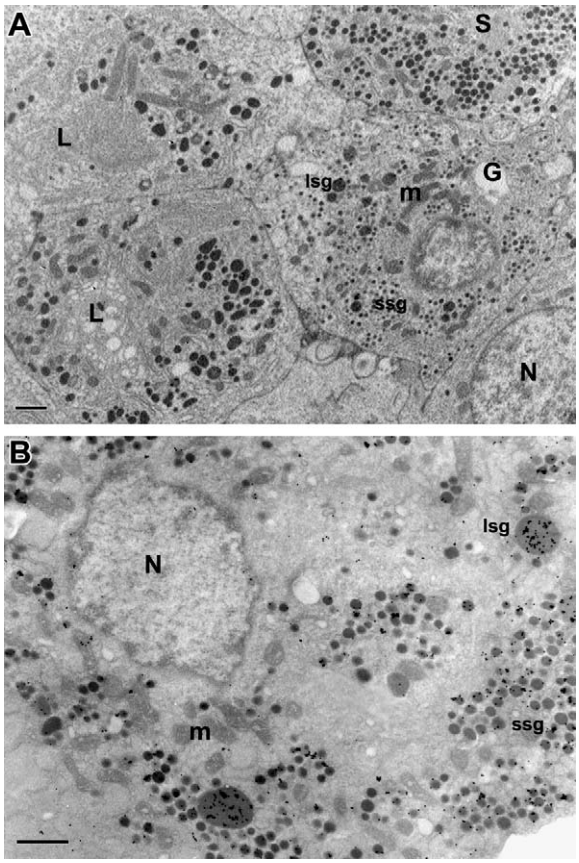

Fig. 4. (A) Electron micrograph of a pituitary cell culture from control rats illustrating different pituitary cell populations. Two lactotroph cells (L) exhibit an accumulation of large and polymorphic mature secretory granules (about 500–900 nm in diameter) in the cytoplasm. The somatotroph cell (S) can be easily recognized by the mature, round GH secretory granules ranging from 200 to 350 nm in diameter and scattered throughout the cytoplasm. In close contact with both secretory cell types, a gonadotroph cell (G) displays small (ssg) and large (lsg) round secretory granules (about 150 or 400 nm in diameter respectively, with different electron densities, homogeneously disseminated in the cytoplasm (m = mitochondria, N = nucleus). *Bar:* 1 μm. (B) Gonadotroph cell specifically immunostained for LH. The cytoplasm shows a noticeable accumulation of characteristic small (ssg) and large (lsg) round secretory granules (m = mitochondria, N = nucleus). *Bar:* 0.5 μm.

enhanced activity of LH secretion with these including a decrease in the number of LH secretory granules, their clear mobilization toward the plasma membrane and the presence of images of exocytosis of the LH containing granules. These morphological signs are clear indicators of stimulatory effects deriving from NEI, with these effects being unobserved in the rest of the pituitary cell populations. Finally, in gonadotrophs, the remarkable development of proteinopoietic organelles (RER and Golgi complex) involved in the LH synthesis suggested an enhanced metabolic activity in response to NEI stimulus.

Evidence of NEI expression and fiber projections throughout the central nervous system, has revealed the NEI physiological effects on brain circuitry [3,19,30], with NEI action on other neuropeptides also being reported. In an earlier study, Parkes and Vale [25] demonstrated an increase in MCH and NEI secretion from cultured rat hypothalamic cells after cAMP and cGMP treatments. More recently, Attademo et al. [1] showed NEI varicosities in some parts of the median eminence displaying a distribution pattern similar to that of GnRH. This morphological observation revealed a possible effect of NEI on the modulation of GnRH secretion occurring directly at the terminals, which might be one of the mechanisms by which intracerebroventricular administration of NEI causes an increase in LH secretion as was shown in a previous investigation [2]. In this context, the possibility that NEI has a pituitary gonadotrophic effect in addition to its regulatory effects on the central nervous system cannot be ruled out.

Furthermore, in view of the fact that the presence of NEI and other ppMCH-derived peptides was detected in the posterior pituitary [3], this pituitary lobe could act as a potential source of NEI stimulating gonadotrophs through the short portal vessels. Unpublished results from our laboratory show that the posterior pituitary has concentrations (of the order of 2–5 ng/mg protein) of immunoreactive NEI that are high and comparable with those found in the Arc-ME. Also, the presence of NEI terminals in the external layer of the median eminence close to the blood vessels, suggests direct secretion taking place within the portal system [1].

In the present investigation we also demonstrated that NEI can potentiate GnRH by specifically stimulating the LH secretion. This may be due to the fact that although a dose of NEI had no action by itself, when incubated with GnRH, it produced a significantly higher release of LH than the releasing hormone alone. It is possible that the interaction between NEI and GnRH occurs at the signaling level, since it has been shown that NEI can modulate the intracellular cAMP and IP_3 levels [28,29]. At the pituitary gland, GnRH drives the pulsatile release of FSH and LH mainly via the classical G (alphaq)-11-phospholipase C signal transduction pathway [4,7], but also by the activation of the MAPK cascade through PKC to stimulate the expression of gonadotrophin subunit genes [5,20,21,24]. Moreover, the existence of progesterone receptor-GnRH receptor cross-talk was demonstrated in gonadotrophs in GnRH-stimulated LH secretion [6,12]. Interaction between NEI and GnRH may also occur at the signaling level, since cAMP has been shown to be capable of inducing the expression of LH subunit genes and the release of newly synthesized LH [15]. On the other hand, it has been established that both MCH and NEI suppressed thyrotropin-releasing hormone release from hypothalamic cultures, thus demonstrating NEI effects at the hypothalamic level regulating other pituitary hormones. In the same investigation, Kennedy and co-workers also described both neuropeptides inhibiting the action of TRH on TSH release, indicating that NEI is a negative modulator of TSH release at the pituitary level [16].

Additionally, another interesting result arising from our study was that NEI exerted a differential effect on FSH and LH release in primary pituitary cell cultures. Even though both hormones are produced by the same cell type, it is known that FSH is released predominantly via a constitutive pathway, whereas the principal release of LH is through the regulated pathway, with GnRH stimulating the release of LH contained in storage granules [18]. It is important to highlight that the packaging of LH and FSH into different secretory granules is crucial for the differential secretion of these gonadotrophins. With regards to this, it was shown that the granin proteins secretogranin II and chromogranin A are commonly found co-aggregated with LH and/or FSH within specialized secretory granules in the gonadotrophs, and with this association playing an important role in the differential secretion of the gonadotrophins [8,22]. Therefore, it is not surprising that under our experimental conditions, we were able to demonstrate that gonadotroph cells responded to NEI stimuli to release LH, while FSH levels remained unchanged. However, although clear actions of NEI on LH secretion were demonstrated, none of the NEI doses applied, either alone or in co-incubation with GnRH, were able to induce significant effects on FSH secretion (data not shown). Also, the electron micrographs demonstrated that not all the secretory granules were positive for β-LH, suggesting that LH and FSH could have been localized into different secretory granules. These data provide an additional evidence of differential regulation of the secretion of both gonadotrophins, as reported by other authors [11,17,18,23,27,31].

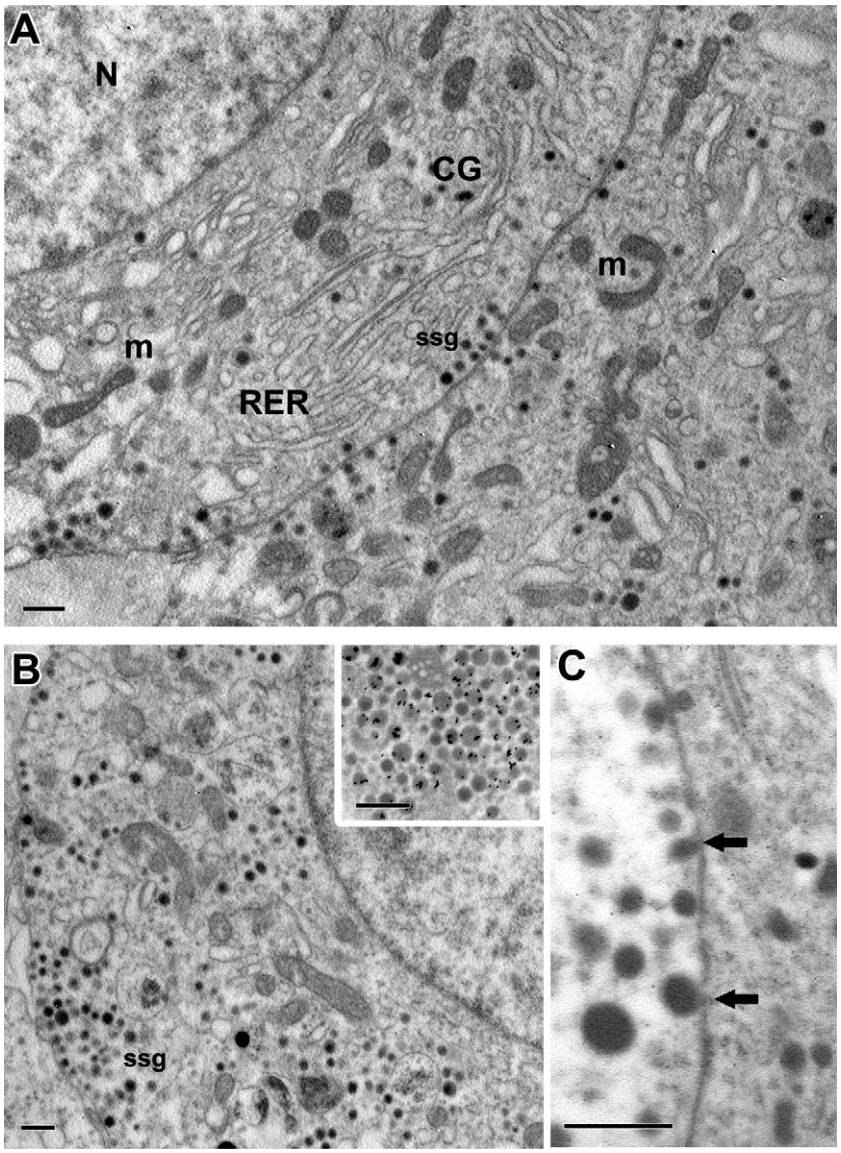

Fig. 5. (A) Electron microscopy of two cultured gonadotroph cells treated with 400×10^{-8} M NEI for 4 h. The cytoplasm contains a remarkably well developed rough endoplasmic reticulum (RER) and Golgi complex (GC) and also scarce small secretory granules (ssg) that are mostly in contact with the plasma membrane. *Bar:* 0.5 μm. (B) Electron micrograph of a cultured gonadotroph cell (G) after exposition to 100×10^{-8} M NEI for 2 h which is shown exhibiting small secretory granules (ssg) mobilized toward the plasmalemma, where they will then be subsequently discharged by exocytosis. *Bar:* 0.5 μm. Inset: small round secretory granules from a gonadotroph cell specifically identified by immunocytochemistry for LH. *Bar:* 0.5 μm. (C) Detail of two adjacent gonadotroph cells after NEI treatment displaying evidence of secretory activity. The secretory granules are aligned alongside the cell membrane and are in the process of exocytosis (arrows). *Bar:* 0.5 μm.

Regarding the potential physiological role of NEI at the pituitary level, Viale and co-worker studied the interrelationship between NEI and the preovulatory LH surge [30]. They described the presence of NEI immunoreactive fibers and terminals at hypothalamic locations related to the control of the LH surge, such as the medial preoptic area, where GnRH neurons were also found. Furthermore, the levels of both MCH-IR and NEI-IR increased 72 h post-estradiol administration, at the same time as LH release, suggesting the possible involvement of these peptides in the regulation of preovulatory midcycle LH release [30].

Although many investigations support the physiological role of NEI acting mainly at the central nervous system, in addition we cannot disregard its direct action at the pituitary level, as demonstrated in the present study. This paper gives the first evidence that NEI may act directly on the anterior pituitary, thus stimulating the LH secretion not only through a direct action on the gonadotroph cells, but also by interaction with GnRH. In this way, the study provides new insight into a potential interaction between regulating hypothalamic peptides and pituitary hormones, and emphasizes the increasing complexity of the neuroendocrine system involved in regulating hormone secretion.

Acknowledgments

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fondo para la Investigación Científica y Tecnológica (FONCyT-ANPCyT) and the Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT-UNC). ADP, RC, MS, GAJ, AIT and MEC are career scientists from CONICET and AMA is a postgraduate student from the National University of Córdoba. The authors wish to thank Prof. Norma Carreño, Mrs Elena Pereyra and Mrs. Mercedes Guevara for her excellent technical assistance and native English speaker Prof. Paul Hobson for revising the English of the manuscript.

References

[1] Attademo AM, Rondini TA, Rodrigues BC, Bittencourt JC, Celis ME, Elias CF. Neuropeptide glutamic acid-isoleucine may induce luteinizing hormone secretion via multiple pathways. Neuroendocrinology 2006;83:313–24.

[2] Attademo AM, Sanchez-Borzone M, Lasaga M, Celis ME. Intracerebroventricular injection of neuropeptide EI increases serum LH in male and female rats. Peptides 2004;25:1995–9.

[3] Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, et al. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 1992;319:218–45.

[4] Burger L, Dalkin A, Aylor K, Haisenleder D, Marshall J. GnRH pulse frequency modulation of gonadotropin subunit gene transcription in normal gonadotropes—assessment by primary transcript assay provides evidence for roles of GnRH and follistatin. Endocrinology 2002;143:3243–9.

[5] Burger L, Haisenleder D, Aylor K, Marshall J. Regulation of intracellular signaling cascades by GNRH pulse frequency in the rat pituitary: roles for CaMK II, ERK, and JNK activation. Biol Reprod 2008;79:947–53.

[6] Cheung LW, Wong AS. Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues. FEBS J 2008;275:5479–95.

[7] Counis R, Laverrière JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, et al. Gonadotropin-releasing hormone and the control of gonadotrope function. Reprod Nutr Dev 2005;45:243–54.

[8] Crawford J, McNeilly A. Co-localisation of gonadotrophins and granins in gonadotrophs at different stages of the oestrous cycle in sheep. J Endocrinol 2002;174:179–94.

[9] De Paul AL, Pons P, Aoki A, Torres A. Different behavior of lactotroph cell in response to angiotensin II and thyrotropin-releasing hormone. Cell Mol Neurobiol 1997;17:245–58.

[10] Elias CF, Bittencourt JC. Study of the origins of melanin-concentrating hormone and neuropeptide EI immunoreactive projections to the periaqueductal gray matter. Brain Res 1998;755:255–71.

[11] Fujii Y, Okada Y, Moore Jr JP, Dalkin AC, Winters SJ. Evidence that PACAP and GnRH down-regulate follicle-stimulating hormone-beta mRNA levels by stimulating follistatin gene expression: effects on folliculostellate cells, gonadotrophs and LbetaT2 gonadotroph cells. Mol Cell Endocrinol 2002;192:55–64.

[12] Garrido-Gracia J, Bellido C, Aguilar R, Sánchez-Criado J. Protein kinase C crosstalk with gonadotrope progesterone receptor is involved in GnRH-induced LH secretion. J Physiol Biochem 2006;62:35–42.

[13] Griffond B, Baker BI. Cell and molecular cell biology of melanin-concentrating hormone. Int Rev Cytol 2002;213:233–77.

[14] Haisenleder D, Burger L, Walsh H, Stevens J, Aylor K, Shupnik M, et al. Pulsatile gonadotropin-releasing hormone stimulation of gonadotropin subunit transcription in rat pituitaries: evidence for the involvement of Jun N-terminal kinase but not p38. Endocrinology 2008;149:139–45.

[15] Horton CD, Halvorson LM. The cAMP signaling system regulates LHβ gene expression: roles of early growth response protein-1, SP1 and steroidogenic factor-1. J Mol Endocrinol 2004;32:291–306.

[16] Kennedy AR, Todd JF, Stanley SA, Abbott CR, Small CJ, Ghatei MA, et al. Melanin-concentrating hormone (MCH) suppresses thyroid stimulating hormone (TSH) release, in vivo and in vitro, via the hypothalamus and the pituitary. Endocrinology 2001;142:3265–8.

[17] Kumru S, Simsek M, Yilmaz B, Sapmaz E, Kutlu S, Sandal S, et al. Differential regulation of preovulatory luteinizing hormone and follicle-stimulating hormone release by opioids in the proestrous rat. Physiol Res 2001;50:397–403.

[18] McNeilly AS, Crawford JL, Taragnat C, Nicol L, McNeilly JR. The differential secretion of FSH and LH: regulation through genes, feedback and packaging. Reprod Suppl 2003;61:463–76.

[19] Nahon J, Presse F, Bittencourt J, Sawchenko P, Vale W. The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. Endocrinology 1989;125:2056–65.

[20] Nahon JL. The melanin-concentrating hormone: from the peptide to the gene. Crit Rev Neurobiol 1994;8:221–62.

[21] Naor Z, Benard O, Seger R. Activation of MAPK cascades by G protein-coupled receptors: the case of gonadotropin-releasing hormone. Trends Endocrinol Metab 2000;11:91–9.

[22] Nicol L, McNeilly J, Stridsberg M, Crawford J, McNeilly A. Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in LbetaT2 gonadotroph cells. J Endocrinol 2002;174:473–83.

[23] Nicol L, McNeilly JR, Stridsberg M, McNeilly AS. Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in LbetaT2 cells. J Mol Endocrinol 2004;32:467–80.

[24] Olcese J, Sikes HE, Resuehr D. Induction of PER1 mRNA expression in immortalized gonadotropes by gonadotropin-releasing hormone (GnRH): involvement of protein kinase C and MAP kinase signaling. Chronobiol Int 2006;23:143–50.

[25] Parkes D, Vale W. Secretion of melanin-concentrating hormone and neuropeptide-EI from cultured rat hypothalamic cells. Endocrinology 1992;131:1826–31.

[26] Parkes DG. Diuretic and natriuretic actions of melanin concentrating hormone in conscious sheep. J Neuroendocrinol 1996;8:57–63.

[27] Pawson AJ, McNeilly AS. The pituitary effects of GnRH. Anim Reprod Sci 2005;88:75–94.

[28] Sanchez MS, Berberian V, Celis ME. Effects of MCH/NEI in the striatum and interactions between them and with alpha-MSH on IP3 levels. Peptides 2002;23:877–80.

[29] Sanchez MS, Cremer MC, Celis ME. Effects and interactions between alpha-MSH and MCH/NEI upon striatal cAMP levels. Peptides 1999;20:611–4.

[30] Viale A, Kerdelhue B, Nahon JL. 17beta-estradiol regulation of melanin-concentrating hormone and neuropeptide-E-I contents in cynomolgus monkeys: a preliminary study. Peptides 1999;20:553–9.

[31] Walczewska A, Yu WH, Karanth S, McCann SM. Estrogen and leptin have differential effects on FSH and LH release in female rats. Proc Soc Exp Biol Med 1999;222:170–7.