Sustained treatment with a GnRH agonist (leuprorelin) affects the ultrastructural characteristics of membranous organelles in male rat pituitary gonadotropes

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Summary. Gonadotropin-releasing hormone (GnRH) agonists exert acutely stimulatory action on gonadotropes, but thereafter suppress paradoxically gonadotropin synthesis and release by receptor desensitization. To examine whether GnRH signaling affects the morphological characteristics in membranous organelles related to the synthesis of gonadotropin, we have analyzed the ultrastructural changes in the ER and Golgi apparatus of male rat pituitary gonadotropes during sustained treatment with a GnRH agonist, leuprorelin. In pituitary gonadotropes at 1 day after the onset of treatment, clusters of the tubuloreticular ER appeared, and the globular Golgi apparatus was transiently disassembled into isolated small-sized stacks. However, 1 week after the onset of the treatment, the tubuloreticular ER was seemingly converted to rough ER with regularly stacked sheets and the scattered Golgi stacks converged to form globular structures. In the following chronic phase of the treatment, the ER cisterns remained flattened and the trans-Golgi compartment appeared to be collapsed. Sustained treatment with leuprorelin could also restore the enlarged Golgi apparatus and expand the cisterns of the rough ER; a feature that was seen in hypertrophic gonadotropes of castrated rats. These findings indicated that the ultrastructure of the membranous organelles changed because of the chronic suppressive effects of leuprorelin on gonadotropes both in the physiological and stimulated states. The acute and chronic ultrastructural changes in the ER and Golgi apparatus during sustained leuprorelin treatment also suggests that GnRH signaling cross-talks with the regulation of the morphological characteristics in membranous organelles related to gonadotropin synthesis.

Introduction

The gonadotropin-releasing hormone (GnRH) is discharged from hypothalamic neurons in a pulsatile manner. GnRH binds to cognate GnRH receptors that are specifically expressed on the pituitary gonadotropes, and subsequently stimulates the cells to synthesize and secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (for reviews see, Ruf et al., 2003; Cheng and Leung, 2005; Naor 2009; Bliss et al., 2010). GnRH analogs generated by substituting residues of intrinsic GnRH bind competitively to the GnRH receptor, resulting in the augmentation (by agonists) or attenuation (by antagonists) of intracellular signaling events downstream of the receptor (Conn and Crowley, 1994; Millar et al., 2004). Sustained treatment with
powerful GnRH agonists such as buserelin (Brogden et al., 1990) and leuprorelin (Plosker and Brogden 1994), however, paradoxically suppress synthesis and secretion of gonadotropins. This effect counters their original and initial stimulatory effects on gonadotropes (McArdle et al., 2002; Millar et al., 2004). Since these paradoxical effects of GnRH agonists in the chronic phase can deplete serum androgens/estrogens levels derived from gonads, the GnRH agonists have been widely applied for the treatment of sex-steroid-dependent diseases such as endometriosis, uterine leiomyoma, prepubertal maturation and metastatic prostate cancer (Barbieri 1992, Engel et al., 2007).

Despite their wide range of applications for clinical use listed above, the intracellular mechanisms responsible for both the acute stimulatory and chronic suppressive effects of GnRH agonists remain unresolved. In particular, the ultrastructural changes in pituitary gonadotropes under the influence of GnRH agonists have been poorly investigated, although the gonadotropes are a direct target of the agonists. We previously investigated the changes in the amount of secretory granules in male rat pituitary gonadotropes during long-acting leuprorelin depot treatment, and showed that leuprorelin acts as a strong agonist on the gonadotropes in the acute phase, but then chronically suppresses both synthesis and secretion of gonadotropins (Kitahara et al., 2007). Secretory granules in endocrine cells are, however, situated downstream of the rough ER and Golgi apparatus in the secretory pathway. If there are significant changes in the secretory granules within gonadotropes, the structure and function of the ER and Golgi apparatus must be affected by the GnRH agonists.

To clarify the morphological basis for the pharmacological actions of GnRH agonists on the membranous organelles generating secretory granules, in this study we have investigated the temporal changes in the ultrastructure of the ER and Golgi apparatus in pituitary gonadotropes of male rats receiving 1-month depot formulation of leuprorelin. Based on the findings, we will discuss the putative crosstalk between GnRH signaling and the regulation of the structural properties of the ER and Golgi apparatus within gonadotropes.

**Materials and Methods**

**Antibodies**

Mouse monoclonal anti-GM130 and anti-BiP antibodies were purchased from BD Biosciences (San Jose, CA, USA; product codes: 610823 and 610978, respectively). Sheep polyclonal anti-TGN38 and rabbit polyclonal anti-calnexin antibodies were purchased from AbD Serotec (Oxford, UK; code: AHP499G) and Sigma-Aldrich Co. (St Louis, MO, USA; code: C4731), respectively.

To identify gonadotropes in the pituitary tissue sections, rabbit polyclonal anti-ovine LHβ (code: HAC-OV27(β)-01RBP85; kindly provided by Dr. Matozaki, Gunma University, Japan) and commercially available goat polyclonal anti-LH (Santa Cruz Biotechnology, Santa Cruz, CA, USA; code: sc-7824) were used.

Secondary antibodies conjugated with fluorescent dyes (Alexa Fluor 488-, 594-conjugated donkey polyclonal anti-rabbit-, mouse- and sheep-IgGs) and those with colloidal gold particles (5 and 15 nm in diameter) were purchased from Invitrogen (Carlsbad, CA, USA) and British Biocell International (Cardiff, UK), respectively. Biotinylated secondary antibodies (anti-rabbit and goat IgGs; purchased from Vector Laboratories, Burlingame, CA, USA) and Alexa Fluor 405-conjugated streptavidin (Invitrogen) were also used for observation with a laser confocal microscope.

**Animals and experimental procedures**

One hundred and sixty-five adult male Wistar rats purchased at 6 weeks of age (body weight: ca. 200 g) were divided into 11 experimental groups (Groups A to K; 15 rats in each group). The rats were kept for a further 2 weeks in a well-ventilated room (temperature 23±1°C; relative humidity 55–65%; lights on, 0700–1900 h) with food and water *ad libitum*, and then used for experiments. These rats were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC, 1996) under the permission of the experimental animal welfare committee of Asahikawa Medical University (permission #09050).

At 8 weeks of age, the rats of three groups (Groups B, C, and D) were subcutaneously injected with 3 mg/kg of a GnRH agonist, leuprolide acetate, in 1-month depot formulation (leuprorelin; Takeda Pharmaceutical Co., Osaka, Japan). After the rats were maintained further for 1 day (Group B), 7 days (Group C) and 28 days (Group D), they were used for experiments as described below. Rats of the other seven groups (Groups E–K) at 8 weeks of age were bilaterally gonadectomized through a scrotal incision (castration) under ketamine/xylazine (100:10 mg/kg; IM) anesthesia. These rats were maintained for a further 2 days (Group E), 7 days (Group F), 28 days (Group G) and 56 days (Groups H–K). The rats of groups E, F, G and H were then used for experiments as described below. Three groups of rats at 56 days after castration (Groups I, J and K) received 3 mg/kg of the
leuprolerin depot suspension subcutaneously, and were further maintained for 1 day (group I), 7 days (group J) and 28 days (group K) before use in the experiments. The rats of group A were used directly for experiments as the control group at 8 weeks of age.

**Immunofluorescence microscopy**

For immunofluorescence microscopy, rats from each experimental group (n=5) were anesthetized with ketamine/xylazine (100:10 mg/kg; IM) and then perfused with 30 ml of physiological saline followed by 100 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) containing 4% sucrose. After fixation by perfusion, pituitaries were cut into small pieces, and immersed in the same fixative for 2 h at 4°C. After washing thoroughly with 0.1 M PB containing 7.5% sucrose, tissue blocks were dehydrated in graded ethanol and embedded in epoxy resin (Epon 812). Serial sections of 0.5 μm thickness were cut from the tissue blocks with an ultramicrotome and mounted on microscope glass slides.

After the removal of the resin by sodium methoxide (Grube and Kusumoto, 1986), semithin sections were treated with a 0.05% citraconic anhydride solution (Imunosaver; Nissin EM Co., Ltd., Tokyo, Japan) for 30 min at 60°C as an antigen retrieval procedure (Namimatsu et al., 2005), and then incubated with 2% normal donkey serum (30 min, 20°C) for blocking. After these pretreatments, tissue sections were incubated with a mixture of primary antibodies of different species (rabbit-, mouse-, sheep-/goat-origin) for 16 h at 20°C. The sections were subsequently incubated with a mixture of the appropriate sets of Alexa Fluor 488-, 594-labeled and biotinylated secondary antibodies for 1 h at 20°C, and then further incubated with Alexa Fluor 405-conjugated streptavidin for 1 h at 20°C. Between each step, the sections on microscopic slides were washed three times with 0.01 M PB (pH 7.4) containing 0.5 M NaCl and 0.1% Tween 20. The coverslips were then mounted on the tissue sections in 90% glycerol (vol/vol in PBS) containing 0.1% p-phenylenediamine dihydrochloride (Sigma-Aldrich). Stained sections were viewed with a laser confocal microscope (FV-1000D; Olympus, Tokyo, Japan).

For the three-dimensional reconstruction by laser confocal microscopy, cryosections of the pituitary tissues were prepared after fixation by perfusion and cryoprotection. Briefly, some pieces of fixed pituitary tissues described above were rinsed with 0.1 M PB containing 7.5% sucrose, immersed sequentially in 15% sucrose (for 6 h) and 30% sucrose (for 12 h) solutions buffered in 0.1 M PB (pH 7.4) at 4°C, and then the tissue blocks were frozen at −30°C in the Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan). Tissue sections of 15 μm thickness were cut from the frozen tissue blocks with a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and mounted on microscope glass slides. These sections were immunostained and viewed with the laser confocal microscope, as described above.

**Immunoelectron microscopy**

Rats from each experimental group (n=3) were anesthetized with ketamine/xylazine described above, and then perfused with physiological saline followed by 100 ml of 2% glutaraldehyde (GA)/2% PFA in 0.1 M PB (pH 7.4). After fixation by perfusion, pituitaries were cut into small pieces and immersed in the same fixative for 2 h at 4°C. After washing thoroughly with 0.1 M PB containing 7.5% sucrose, tissue blocks were further fixed with 1% OsO4 in 0.1 M PB containing 7.5% sucrose for 2 h at 4°C. The tissue blocks were then washed thoroughly with 0.1 M PB containing 7.5% sucrose, dehydrated in graded ethanol and embedded in epoxy resin (Epon 812).

After ultrathin sections from the tissue blocks embedded in Epon 812 were etched with 1% sodium methoxide for 30 sec, the sections were incubated with 5% non-immune goat serum for blocking (30 min, 20°C). After the pretreatment, the ultrathin sections were further incubated with an anti-LHβ (rabbit polyclonal, diluted 1:100) antiserum for 16 h at 20°C. They were then treated with colloidal gold-conjugated goat anti-rabbit IgG for 1 h at 20°C (size of gold particles: 15 nm in diameter). Between each step, the sections on grids were washed three times with 0.02 M Tris-HCl buffered 0.5 M saline, pH 8.2, containing 0.1% bovine serum albumin (BSA). The sections were then contrasted with saturated aqueous solutions of uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7650; Hitachi High Technologies, Tokyo, Japan).

Alternatively, similarly anesthetized rats (n=4) were perfused with physiological saline, followed by 100 ml of 0.5% GA/0.5% PFA in 0.1 M PB (pH 7.4). Immediately after fixation by perfusion, pituitaries were cut into small pieces and directly immersed in 0.5% OsO4 in 0.1 M PB for 1 h at 4°C. The tissue blocks were then washed thoroughly with 0.1 M PB containing 7.5% sucrose, dehydrated in 70% ethanol containing 1% phosphotungstic acid (Wako Pure Chemical, Osaka, Japan) three times for 20 min at 4°C, and then infiltrated into pure LR White resin monomer (London Resin Co., Hampshire, UK). The resin solution was changed three times during infiltration (20 min, each at 4°C), and finally the tissue blocks were placed at the bottom of gelatin capsules filled with fresh LR White resin and polymerized for 24 h at 60°C.
Immunogold labeling of pituitary tissues embedded in LR White resin was performed as described previously (Watanabe et al., 2012). For removal of osmium, ultrathin sections from the LR White-embedded tissues were treated in 1% sodium metaperiodate (Wako Pure Chemical) for 10 min at 20°C prior to the immunogold labeling. The sections were then incubated with 5% normal goat serum for blocking (30 min, 20°C) and further incubated with the primary antibodies for 12 h at 4°C as follows: anti-LHβ (rabbit polyclonal, diluted 1:10000); anti-BiP (mouse monoclonal, 1:100). Intracellular localization of two distinct antigens was distinguished by labeling with different sizes of colloidal gold particles (size of particles: 5 and 15 nm in diameter) conjugated to appropriate secondary antibodies. Between each step, the sections on grids were washed three times in 0.02 M Tris-HCl buffered 0.5 M saline, pH 8.2, containing 0.1% BSA. Following the immunoreactions, the sections were contrasted with saturated aqueous solutions of uranyl acetate and lead citrate, and examined with the transmission electron microscope.

**Scanning electron microscopy**

Tissue preparation for scanning electron microscopy was described previously (Tanaka and Mitsushima 1984; Koga and Ushiki 2006). Briefly, anesthetized rats (n=3 per each experimental group) were perfused with physiological saline followed by a mixture of 0.5% GA/0.5% PFA in 0.1 M PB (pH 7.4). After fixation by perfusion, pituitaries were cut into two small pieces and directly immersed in 1% OsO4 in 0.1 M PB (pH 7.4) for 2 h at 4°C. The tissue blocks were then washed thoroughly with 0.1 M PB, immersed in 25% and 50% dimethyl sulfoxide (DMSO) for 30 min each, and frozen on a metal plate that had been deeply chilled with liquid nitrogen. The frozen tissue blocks were cracked into two small pieces and directly immersed in 50% DMSO for thawing. After the freeze-cracked tissue blocks were rinsed in 0.1 M PB (pH 7.4) for 1 h at 4°C, they were placed in 0.1% OsO4 diluted with 0.1 M PB (pH 7.4) for 72 h at 20–22°C under fluorescent light illumination. During maceration of the pituitary tissues, the 0.1% OsO4 solution was renewed every 24 h. The macerated specimens were further fixed in 1% OsO4 in 0.1 M PB (pH 7.4) for 1 h, washed in 0.1 M PB for 1 h and treated with 1% tannic acid (Wako Pure Chemical) in 0.1 M PB (2 h, 20°C) and then with 1% OsO4 in 0.1 M PB (1 h, 20°C) for conductive staining. After conductive staining, the samples were dehydrated in graded ethanol, transferred into isoamyl acetate and dried in a critical point dryer (HCP-2; Hitachi Koki Co., Ltd., Tokyo, Japan) using liquid CO2. The dried samples were mounted onto a metal plate and coated with platinum-palladium in an ion-sputter coater (E1010; Hitachi Koki Co., Ltd.), and were observed in a field emission SEM (S-4100; Hitachi High Technologies).

**Results**

**Acute effects of the depot formulation of leuprolelin on the morphology of the ER in gonadotropes**

As we previously reported, the levels of immunoreactive LH in the blood plasma were markedly increased after 1 day of the leuprolelin depot treatment, and then promptly decreased by 7 days of the treatment and remained below the basal level for 28 days from the onset of the treatment (Kitahara et al., 2007). In parallel to the acute elevation of the plasma LH mentioned above, patch-like accumulation of two representative ER chaperones, calnexin and BiP, was discerned in gonadotropes at day 1 of the leuprolelin depot treatment (Group B; Fig. 1B), whereas the chaperones were not localized as patch-like structures in gonadotropes of the control rats (Group A; Fig. 1A). The membrane-bound chaperone calnexin had more prominently accumulated within gonadotropes than the soluble chaperone BiP in the ER cisterns. Similar but lighter accumulation of the ER chaperones was also observed in the stimulated gonadotropes 2 days after castration (Group E; Fig. 1C), suggesting that the patch-like accumulation of the ER chaperones commonly occurs in the gonadotropes immediately in response to the specific stimuli. Observation by electron microscopy revealed that the atypical accumulation of the ER chaperones in the stimulated gonadotropes resulted from anomalous clusters of tubuloreticular membranes (Group B; Fig. 2C–E), which was not observed in gonadotropes of the control rats (Group A; Fig. 2A, B). The tubular networks of membranes, on which ribosomes were sparsely distributed, were occasionally connected with the nuclear envelope (Fig. 2D). The immunocytochemical localization of BiP (Fig. 2E) and calnexin (data not shown) on the structures indicates that the tubuloreticular membrane is part of the ER transiently increased in the stimulated gonadotropes.

**Chronic effects of the depot formulation of leuprolelin on the morphology of the ER in gonadotropes**

After the acute stimulatory phase, leuprolelin exerts, in turn, strong inhibitory effects on gonadotropes by desensitizing surface GnRH receptors (Conn and Crowley 1994; Millar...
Fig. 1. Changes in the intracellular distribution of BiP and calnexin (CNX) in the pituitary gonadotrope of rats immediately following leuprorelin depot treatment (B) or castration (C). Semithin sections (thickness: 0.5 μm) of the anterior pituitary glands, prepared from the control rats (A), rats 1 day after receiving 1 month of the depot formulation of leuprorelin (B; Leu_1d) and rats 2 days after castration (C; Cast_2d), were immunostained simultaneously with mouse monoclonal anti-BiP (visualized with Alexa Fluor 488; green pseudocolor in A1–C1), rabbit polyclonal anti-CNX (Alexa Fluor 594; red) and sheep polyclonal anti-LH (Alexa Fluor 350; blue) antibodies. The immunocytochemical localizations of BiP and calnexin are separately demonstrated in A2–C2 and A3–C3, respectively. Arrows indicate patch-like accumulations of BiP and CNX in gonadotropes. Bar: 10 μm.
Fig. 2. Ultrastructure of the ER in the pituitary gonadotropes of rats treated with leuprolrelin for 1 day. Gonadotropes of the non-treated control rats (A, B) and rats 1 day after receiving the depot formulation of leuprolrelin (C–E; Leu_1d) were identified in the ultrathin sections of the Epon 812-embedded pituitary tissue by immunolabeling with a rabbit polyclonal anti-LH antiserum (visualized with 15 nm immunogold particles). Partial areas indicated in A and C were further photographed at a higher magnification (B and D, respectively). Anomalous clusters of tubuloreticular membranes (asterisks in C and D) were occasionally continuous with the outer membrane of the nuclear envelope (indicated with an arrow in D), and are immunolabeled with an anti-BiP antibody (E: an LR White-embedded ultrathin section). Bars: 500 nm
et al., 2004). At 7 days of treatment, the gonadotropes of leuprorelin-administrated rats (Group C; Fig. 3A) became smaller in size than that of the control rats, whereas those of castrated rats (Group F; Fig. 3B) were slightly enlarged. The clusters of anomalous tubuloreticular structures accumulating ER chaperones disappeared from the cytoplasm and the typical rough ER with regularly stacked sheet arrangements was predominately observed in the gonadotropes of both groups C (Fig. 3C) and F (Fig. 3D).

The differences in ultrastructure between castrated and leuprorelin-treated rats became pronounced at 28 days after the onset of the treatment; gonadotropes treated with the leuprorelin depot predominantly shrank with an irregular contour (Group D; Fig. 4A), whereas most of gonadotropes of castrated rats were markedly expanded and immunolabeled intensely with BiP (Group G; Fig. 4B). In gonadotropes of leuprorelin-treated rats, the expression of BiP had apparently decreased (Fig. 4A2), whereas that of calnexin had not drastically changed (Fig. 4A3). At the electron microscope level, the cisterns of the rough ER were flattened and regularly stacked in the gonadotropes of leuprorelin-treated rats (Group D; Fig. 4C), whereas the gonadotropes of castrated rats were filled with the prominently dilated rough ER (Group G; Fig. 4D). These clear differences in ultrastructure of the rough ER confirmed that the treatment with the depot formulation of leuprorelin chronically exerts quite opposite effects on the secretory protein biosynthesis processes in pituitary gonadotropes to that seen following castration.

Changes in the Golgi apparatus within gonadotropes after administration of the depot formulation of leuprorelin

In addition to the changes in the ER, the configuration and ultrastructure of the Golgi apparatus were largely affected by the treatment with the depot formulation of leuprorelin. The Golgi apparatus, which was globular in shape within gonadotropes of the control rats (Group A; Fig. 5A), was transiently dispersed in the cytoplasm at day 1 after receiving the depot formulation of leuprorelin (Group B; Fig. 5B) and 2 days after castration (Group E; data not shown). Although the Golgi apparatus then converged again at the perinuclear region by day 7 of the sustained leuprorelin treatment (Group C; Fig. 5C), the size of the Golgi apparatus had clearly decreased and was not restored to that of the control rats by 28 days of the leuprorelin treatment (Group D; Fig. 5D). In contrast, the size of the Golgi apparatus continuously increased in size following castration (Fig. 5E and 5F; 7 days (Group F) and 28 days (Group G) after castration, respectively).

By scanning electron microscopy, the dispersed Golgi apparatus in a mini-stack form (Fig. 6B; arrowheads) and the tubuloreticular ER clusters (Fig. 6B; asterisks) were observed in gonadotropes 1 day after the leuprorelin depot administration (Group B). Such structures were not seen in the control rats (Group A; Fig. 6A). Observation of ultrathin sections immunolabeled with an LH antiserum confirmed the dispersion of the Golgi apparatus (Fig. 6C; arrowheads) in gonadotropes 1 day after the onset of the leuprorelin treatment (Group B). The dispersed Golgi stacks were laterally merged once more after 7 days of the leuprorelin depot administration (data not shown); however, the trans-Golgi compartment inside the merged Golgi apparatus (Fig. 6D; arrowheads) remained collapsed 28 days after sustained leuprorelin treatment (Group D), and this observation contrasted with that of the gonadotropes of non-treated control rats (Group A; Figs. 2A and 6A).

The dilated rough ER in gonadotropes after castration could be restored by a successive treatment with leuprorelin

Since the castration and leuprorelin depot treatment chronically exerted opposite effects on the ultrastructure of the rough ER in gonadotropes, we next examined whether the expanded rough ER cisterns of hypertrophic gonadotropes after castration could be restored to the flattened ones in regularly stacked sheets by sustained leuprorelin treatment.

Eight weeks after castration, the hypertrophic gonadotropes exhibited the so-called “signet-ring appearance” containing an extraordinarily expanded cavity of the ER, of which contour and lumen were immunolabeled with anti-calnexin and anti-BiP antibodies, respectively (Group H; Fig. 7A). After 1 day of additional treatment with the depot formulation of leuprorelin, the expanded ER lumen of the hypertrophic gonadotropes of the castrated rats was highly lobulated (Group I; Fig. 7B; see also Fig. 8A). At the same time, patch-like accumulations of the calnexin signal were occasionally observed in gonadotropes (Fig. 7B, insets), just like those in the solely leuprorelin-treated rats (Group B; Fig. 1B). Following 7 days of the sustained leuprorelin treatment on the castrated rats, anomalous accumulations of the calnexin signal disappeared and the dilated cisterns of the ER were reduced in size in the gonadotropes (Group J; Fig. 7C). The immunocytochemical signals indicative of BiP in the ER cisterns were gradually diminished and further dispersed throughout the cytoplasm of irregularly shrunk gonadotropes following 28 days of the sustained leuprorelin-treatment (Group K; Fig. 7D).
Fig. 3. Pituitary gonadotrope of rats treated with leuprorelin (A, C; Leu_1w) or castrated (B, D; Cast_1w) for 1 week. (A and B) Intracellular distribution of BiP and CNX. Semithin sections (thickness: 0.5 μm) of the anterior pituitary glands, prepared from rats 7 days after receiving the depot formulation of leuprorelin (A) or castration (B), were immunostained simultaneously with mouse monoclonal anti-BiP (visualized with Alexa Fluor 488; green pseudocolor in A1–B1), rabbit polyclonal anti-CNX (Alexa Fluor 594; red) and sheep polyclonal anti-LH (Alexa Fluor 350; blue) antibodies. The immunocytochemical localizations of BiP and CNX are separately demonstrated in A2–B2 and A3–B3, respectively. Gonadotropes are indicated with arrows. Bar: 10 μm. (C and D) The ultrastructure of the rough ER in the gonadotropes of rats 7 days after receiving the depot formulation of leuprorelin (C), or castration (D). Gonadotropes were identified by immunolabeling as described in the legend of Figure 2. Bars: 500 nm.
Fig. 4. Pituitary gonadotropes of rats treated with leuprorelin (A, C; Leu_4w) or castrated (B, D; Cast_4w) for 4 weeks. (A and B) Intracellular distribution of BiP and CNX. Semithin sections (thickness: 0.5 μm) of the anterior pituitary glands, prepared from rats 28 days after receiving the depot formulation of leuprorelin (A) or castration (B), were immunostained simultaneously with mouse monoclonal anti-BiP (visualized with Alexa Fluor 488; green pseudocolor in A1–B1), rabbit polyclonal anti-CNX (Alexa Fluor 594; red) and sheep polyclonal anti-LH (Alexa Fluor 350; blue) antibodies. The immunocytochemical localizations of BiP and CNX are separately demonstrated in A2–B2 and A3–B3, respectively. Gonadotropes are indicated with arrows. Bar: 10 μm. (C and D) The ultrastructure of the rough ER in the gonadotropes of rats 28 days after receiving the depot formulation of leuprorelin (C), or castration (D). Gonadotropes were identified by immunolabeling as described in the legend of Figure 2. Bars: 500 nm.
Fig. 5. Intracellular distribution of Golgi-associated proteins in gonadotropes of leuprolelin-treated (B–D) and castrated (E, F) rats. Semithin sections (thickness: 0.5 μm) of the anterior pituitary glands, prepared from control rats (A), rats 1 day (B; Leu_1d), 7 days (C; Leu_1w) and 28 days (D; Leu_4w) after receiving the depot formulation of leuprolelin, or rats 7 days (E; Cast_1w) and 28 days (F; Cast_4w) after castration, were immunostained simultaneously with mouse monoclonal anti-GM130 (visualized with Alexa Fluor 488; green pseudocolor), sheep polyclonal anti-TGN38 (Alexa Fluor 594; red) and rabbit polyclonal anti-LH (Alexa Fluor 350; blue) antibodies. Gonadotropes are indicated with arrows. Bar: 10 μm.
Fig. 6. Changes in the ultrastructure of the Golgi apparatus in gonadotropes of leuprorelin-treated rats. (A and B) Osmium-macerated pituitary tissues of control rats (A) and rats 1 day after receiving the depot formulation of leuprorelin (B; Leu_1d) were viewed with a scanning electron microscope. Typical gonadotropes of non-treated control rats contain a single ball-like Golgi apparatus (A; colored green), whereas gonadotropes of leuprorelin-treated rats, in which anomalous clusters of the tubuloreticular membrane (B; asterisks) are seen, contain numerous isolated Golgi mini-stacks scattered in the cytoplasm (B; arrowheads). Bars: 1 μm. (C and D) The Golgi apparatus in the gonadotropes of rats 1 day (C; Leu_1d) and 28 days (D; Leu_4w) after receiving the depot formulation of leuprorelin. Gonadotropes were identified by immunolabeling as described in the legend of Figure 2. Note that an anomalous cluster of the tubuloreticular membrane (C; asterisk) and scattered Golgi mini-stacks (C; arrowheads) were seen in the gonadotrope 1 day after receiving the depot formulation of leuprorelin, but the Golgi stacks converged again and were arranged in a collapsed ellipsoid at 28 days after the onset of the leuprorelin treatment (D; arrowheads). Bars: 500 nm
Fig. 7. Changes in the intracellular distribution of BiP and CNX in gonadotrope of castrated rats concomitantly receiving the depot formulation of leuprorelin. Semithin sections (thickness: 0.5 μm) of the anterior pituitary glands, prepared from rats 8 weeks after castration and with no additional treatment (A; Cast_8w), or similarly castrated rats but 1 day (B; C+Leu_1d), 7 days (C; C+Leu_1w) and 28 days (D; C+Leu_4w) after receiving an additional 1-month depot formulation of leuprorelin, were immunostained simultaneously with mouse monoclonal anti-BiP (visualized with Alexa Fluor 488; green pseudocolor in A1-D1), rabbit polyclonal anti-CNX (Alexa Fluor 594; red) and sheep polyclonal anti-LH (Alexa Fluor 350; blue) antibodies. The immunocytochemical localizations of BiP and calnexin are separately demonstrated in A2–D2 and A3–D3, respectively. Gonadotropes are indicated with arrows. Note that the patch-like accumulation of CNX was occasionally discerned in the gonadotrope at 1 day after the onset of the additional leuprorelin treatment (insets in B). Bar: 10 μm.
The fate of the expanded cavity of the ER seen in the hypertrophic gonadotropes of the castrated rats was further examined in detail by confocal laser scanning microscopy and electron microscopy. The administration of the depot formulation of leuprorelin caused rapid exocytosis of secretory granules located in the cell periphery of the hypertrophic gonadotropes of castrated rats, and the remaining gonadotropins only accumulated in the lumen of the ER (Fig. 8A). Although the large cavity of the ER in hypertrophic gonadotropes became highly lobulated after 1 day of additional treatment with the leuprorelin depot formulation, the continuity of the ER lumen likely remained, judging from the three-dimensional reconstructed images of the gonadotropes with a confocal laser scanning microscope (Fig. 8A). The outer surface of the extremely expanded cistern of the rough ER seen in typical hypertrophic gonadotropes 8 weeks after castration (Fig. 8B) was highly invaginated immediately after the onset of an additional treatment with the leuprorelin depot (Fig. 8D). Scanning electron microscopy showed that a smooth inner surface of the rough ER observed in the gonadotropes of castrated rats (Fig. 8C) became irregularly convoluted with ridges and grooves 1 day after receiving the depot formulation of leuprorelin (Fig. 8E).

The lobulated cavity of the expanded rough ER then gradually decreased as the treatment with leuprorelin continued (Group J; Fig. 9A, B), and finally the cisterns of the rough ER of castrated rats were flattened in regularly stacked sheets following 28 days of treatment with the depot formulation of leuprorelin (Group K; Figs. 9C, D). These findings suggest that sustained treatment with leuprorelin may suppress biosynthetic activity of gonadotropes in both the physiological and hypertrophic states.

Discussion

In addition to our previous study demonstrating the changes in secretory granules (Kitahara et al., 2007), we have further demonstrated in this study the temporal changes in the ultrastructures of the ER and Golgi apparatus in the pituitary gonadotropes of leuprorelin-treated rats, and compared these observations with rats that have been castrated.

One day after the start of the leuprorelin treatment anomalous tubuloreticular clusters of the ER were frequently observed in the pituitary gonadotropes. This ultrastructural change was also observed in gonadotropes 2 days following castration (Koga et al., unpublished data; see also Fig. 1C). Besides the observation of the anomalous tubuloreticular clusters in the ER, the globular Golgi apparatus seen within gonadotropes of the control rats (Koga and Ushiki, 2006; Watanabe et al. 2012) was found to have transiently disassembled into numerous small-sized Golgi stacks scattered throughout the cell both at 1 day after leuprorelin depot administration and 2 days following castration. These findings indicated that the increased stimuli on the GnRH receptor commonly led to the appearance of the tubuloreticular ER clusters and disorganization of the Golgi apparatus. The anomalous tubuloreticular clusters of the ER were observed more frequently in the leuprorelin-treated rats, and the size of the structure appeared to be larger than that seen in the pituitary gonadotropes of the castrated rats. Since the stimulation by leuprorelin is more reproducible and intense on the GnRH receptor when compared with that caused by castration, the continuous administration of leuprorelin possibly provides an experimental animal model suitable for further analyzing the relationship between GnRH signaling and the ultrastructural changes in pituitary gonadotropes.

Although the ER and Golgi apparatus within the pituitary gonadotropes in the acute phase by 1 week after both treatments exhibited similar structural changes, the morphological effects of leuprorelin on the membranous organelle then diverged from those following castration. Following the disappearance of the anomalous clusters of the tubuloreticular ER after 1 week of either the drug treatment or castration, the cisterns of the rough ER in the gonadotropes of leuprorelin-treated rats were arranged in stacked sheets and remained flattened after 4 weeks of treatment. In contrast, those of the castrated rats became gradually dilated over the 4 weeks. The disassembled Golgi stacks converged again after 1 week of leuprorelin treatment or castration. Subsequently, the trans-Golgi compartment surrounded by the Golgi stacks was observed to gradually collapse and appear distorted in the gonadotropes of leuprorelin-treated rats, whereas that of the castrated rats became spherically expanded. These differences in the changes of the ultrastructures between the two experimental conditions indicated that the continuous administration of leuprorelin does not simply exert agonistic effects on the GnRH receptor.

Removal of gonadal steroids by surgical castration results in loss of negative feedback control at the hypothalamic level, increasing the pulsatile discharge of intrinsic GnRH from the neuron (Pielecka and Moenter 2006). The pulsatile release of GnRH maintains appropriately stimulating pituitary gonadotropes to synthesize and secrete gonadotropins. In contrast, continuous exposure of powerful GnRH agonists, including leuprorelin, to the pituitary gonadotropes deeply suppresses the signaling
Fig. 8. Three-dimensional structure of the lobulated rough ER in gonadotropes of castrated rats at 1 day after receiving the depot formulation of leuprolrelin. (A) A tissue section (thickness: 15 μm) of the anterior pituitary gland of the castrated rats at 1 day after receiving the depot formulation of leuprolrelin (C+Leu_1d) was immunostained simultaneously with a goat polyclonal anti-LH (labeled with Alexa Fluor 488; green pseudocolor) and rabbit polyclonal anti-CNX (Alexa Fluor 594; red) antibodies. The cell nucleus was also stained with DAPI (Sigma-Aldrich) and viewed with a 405 nm laser source (blue). A three-dimensional reconstructed image is demonstrated in an orthogonal representation; in addition to the x-y view of the representative datum plane obtained originally with the confocal laser scanning microscope (main panel), two reconstructed views (y-z and x-z views) are demonstrated in the left and lower smaller panels, respectively. White lines indicate the position of each slice. At the right of the orthogonal representation, the series of the original data indicative of the immunolocalization of LH for three-dimensional reconstruction are additionally shown. The thickness of each optical section is 1 μm, and the z-axial distance of each optically sliced section from the datum plane is indicated in the left-lower corner of the data. Bar: 10 μm. (B–E) The profiles (B, D) and inner surfaces (C, E) of the rough ER membrane in gonadotropes of rats 8 weeks after castration with no additional treatment (B, C; Cast_8w), or similarly castrated rats with the additional leuprolrelin treatment for 1 day (D, E; C+Leu_1d) were viewed with transmission and scanning electron microscopes, respectively. Gonadotropes in the ultrathin sections (B, D) were identified by immunolabeling as described in the legend of Figure 2. The tissue specimens viewed with the scanning electron microscope (C, E) have been macerated with diluted OsO₄ as described in the Materials and Methods. Arrows in D and E indicated invagination of the cytoplasm toward the expanded lumen of the rough ER. Bars: 500 nm
Fig. 9. Ultrastructure of the rough ER and the Golgi apparatus in gonadotropes of castrated rats concomitantly receiving the depot formulation of leuprorelin for 1 week (A, B; C+Leu 1w) and 4 weeks (C, D; C+Leu 4w). Gonadotropes were identified by immunolabeling, as described in the legend of Figure 2. Partial areas indicated in A and C were further photographed at a higher magnification (B and D, respectively). Note that the spherical Golgi apparatus seen in gonadotropes of castrated rats at 7 days after the onset of the additional treatment with leuprorelin (A; arrowheads) collapsed into a flattened ellipsoidal one at 28 days after the onset (C; arrowheads). In parallel, the dilated cisterns of the rough ER at 7 days after (B) are shrunk and flattened by 28 days after the onset of the additional treatment with leuprorelin (D). Bars: 500 nm
events downstream of the GnRH receptor, by mechanisms referred to as receptor/post-receptor desensitization (McArdle et al., 2002; Millar et al., 2004; Naor 2009). The present study further confirmed long-lasting suppressive effects of the depot formulation of leuprorelin on biosynthesis and release of gonadotropins (Okada et al., 1991; Murase et al., 2005), by demonstrating the chronic changes in the ultrastructure of the Golgi apparatus and ER within pituitary gonadotropes.

By using the chronic effects of the leuprorelin depot formulation, we further examined whether the GnRH agonist could also suppress the hypertrophic state of gonadotropes long after castration. Treatment with the depot formulation of leuprorelin could convert extremely dilated cisterns of the rough ER in the pituitary gonadotropes of castrated rats into first lobulated and finally flattened ones. These ultrastructure findings likely reflect the suppressive effects of leuprorelin on the synthesis of gonadotropins in the markedly stimulated gonadotropes 8 weeks after castration. Although the sustained treatment with GnRH agonists has been conventionally used to prevent the synthesis of gonadal sex steroids which potentially aggravate androgen- or estrogen-dependent diseases such as prostate cancer, endometriosis and uterine myomas (Barbieri 1992, Engel et al., 2007), the findings described above clearly proved the putative efficacy of GnRH agonists on the clinical application for suppressing the excessive secretion state of pituitary gonadotropins in diseases such as central precocious puberty in juveniles (Antoniazzi and Zamboni, 2004; Mul and Hughes, 2008).

In summary, our present study demonstrated in detail the ultrastructural changes in the ER and Golgi apparatus of pituitary gonadotropes under the influence of a long-acting depot formulation of the GnRH agonist, leuprorelin. Apart from the clinical significance discussed above, perturbation of the rat gonadotropes by leuprorelin depot treatment and castration possibly provide intriguing animal models for analyzing the morpho-functional relationship in the ER and Golgi apparatus. The changes in expression and intracellular localization of the molecules related to the Golgi apparatus and the ER should be further analyzed under the experimental conditions established in the present study.

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