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Three-dimensional properties of GnRH neuroterminals in the median eminence of young and old rats¹

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Abstract

The decapeptide GnRH that regulates reproduction in all vertebrates is stored in, and secreted from, large dense-core secretory vesicles in nerve terminals in the median eminence. GnRH is released from these terminals with biological rhythms that are critical for the maintenance of normal reproduction. During reproductive aging in female rats, there is a loss of GnRH pulses and a diminution of the GnRH surge. However, information about the specific role of GnRH nerve terminals is lacking, particularly in the context of aging. We sought to gain novel ultrastructural information about GnRH neuroterminals by performing three-dimensional (3D) reconstructions of GnRH neuroterminals and their surrounding microenvironment in the median eminence of young (4-5 month) and old (22-24 month) ovariectomized Sprague-Dawley female rats. Median eminence tissues were freeze-plunge embedded, and serial ultrathin sections were collected on slot grids for immunogold labeling of GnRH immunoreactivity. Sequential images were used to create 3D models of GnRH terminals. These reconstructions provided novel perspectives into the morphological properties of GnRH terminals, and their neural and glial environment. We also noted that the cytoarchitectural features of the median eminence became disorganized with aging. Quantitative measures showed a significant decrease in the apposition between GnRH terminal membranes and glial cells. Our data suggest reproductive aging in rats is characterized by structural organizational changes to the GnRH terminal microenvironment in the median eminence.

Keywords

three-dimensional (3D) reconstruction; gonadotropin-releasing hormone (GnRH); glia; median eminence; serial electron microscopy; reproductive aging

Introduction

Gonadotropin-releasing hormone (GnRH) is the primary control molecule of the hypothalamic-pituitary-gonadal reproductive axis of all vertebrates. The GnRH decapeptide is

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stored in large dense-core vesicles in neuroterminals and is released as pulses into the portal vasculature leading from the median eminence to the anterior pituitary. The biological pattern of GnRH release is essential for normal reproductive function (Crowley et al., 1985; Spratt et al., 1987), but the mechanisms for the coordination of GnRH pulses remain controversial. Electrophysiological recordings from GnRH somata suggest that GnRH neurons themselves have intrinsic pulse-generating activities (Kuehl-Kovarik et al., 2002; Suter et al., 2000). The coordination of GnRH pulses may also involve interactions of GnRH terminals in the median eminence (Dudas and Merchenthaler, 2006; Prevot et al., 2007; Terasawa, 1995). Indeed, explanted median eminence fragments lacking GnRH cell bodies continue to release GnRH in a pulsatile fashion (Maeda et al., 1995; Purnelle et al., 1997), showing that this is a potential site of GnRH pulse generation. In addition, inputs to GnRH cell bodies or terminals from other neurotransmitters or neuromodulators in the brain may act to coordinate GnRH release (Dudas and Merchenthaler, 2006; Kuljis and Advis, 1989; Bourguignon et al., 1989). Finally, recent evidence suggests that GnRH neurons are electrically coupled at the level of their dendrites (Roberts et al., 2008). These mechanisms are not mutually exclusive, as the coordination of reproductive function by GnRH neurons necessitates their ability to be responsive to the changing demands of their internal and external environment.

The median eminence, as the interface between the central neuroendocrine system and the peripheral endocrine system, has unique microenvironmental features such as specialized glia, large extracellular space and fenestrated portal capillaries (King and Letourneau, 1994; King and Rubin, 1994; Prevot et al., 1998; Yin et al., 2007). In this region, glial cells such as tanycytes and astrocytes have a close relationship with GnRH axons and terminals (King and Letourneau, 1994; King and Rubin, 1994; Prevot et al., 1998). This anatomical apposition changes under different hormone conditions (King and Letourneau, 1994; King and Rubin, 1994; Prevot et al., 1998) as the processes of tanycytes may retract in order to expose GnRH neuroterminals to both extracellular regulatory factors and to the portal capillaries (Prevot et al., 1999). Thus, it has been proposed that the median eminence may be a site of coordination of GnRH release (King and Rubin, 1995; Terasawa, 2001; Prevot et al., 2007).

Previous electron microscopic studies on GnRH terminals have revealed important information about their structural features and neural-glial relationships (Durrant and Plant, 1999; Goldsmith and Ganong, 1975; Liposits et al., 1995; Prevot et al., 1998; Silverman and Desnoyers, 1976). Nevertheless, relatively little is known about the ultrastructural properties of GnRH terminals due to limitations in immunolabeling from using pre-embedded tissues or due to two-dimensional analyses. Here, we combined serial electron microscopy with a novel cryo-embedding immunogold labeling technique and three-dimensional reconstruction computer analysis to better understand the morphological properties of GnRH neuroterminals and their surrounding neural and glial elements. For our experimental model, we compared young and old rats because decrements in pulsatile release of LH and GnRH occur in aging female rats (Scarbrough and Wise, 1990; Rubin and Bridges, 1989).

Materials and Methods

Experimental Animals

Female Sprague-Dawley rats were purchased from the Animal Resources Center at the University of Texas at Austin. Rats were housed in our laboratory colony, two per cage in a room with controlled temperature (22 °C) and light cycles (12 h/12 h light/dark cycle, lights on at 0700 h). Food and water were available *ad libitum*. All experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and followed guidelines from The Guide for the Care and Use of Experimental Animals. Rats were assigned into young (4 to 5 months, n = 5) and old-aged (23 to 24 months, n = 5) groups.

Surgical procedures and tissue preparation

Surgical procedures and tissue preparation were described previously (Yin et al., 2007). Briefly, rats were bilaterally ovariectomized (OVX) under isoflurane anesthesia and allowed to recover for 4 weeks to clear endogenous ovarian hormones from the general circulation (Gore et al., 2002). Between 0900 to 1100, rats were transcardially perfused (Gore et al., 2000; Miller and Gore, 2002) with 0.1 M phosphate buffer (PB, pH 7.4; 50 ml), followed by 4% paraformaldehyde and 0.125% glutaraldehyde in PB (500 ml) at a speed of 50 ml per minute controlled by a perfusion pump (MasterFlex, Cole-Parmer Instrument Co.). The brain was removed and postfixed in 4% paraformaldehyde overnight at 4 °C. The hypothalamus region was blocked on an adult rat coronal brain matrix (Ted Pella Inc. Redding, CA) and sectioned coronally on a vibrating blade microtome (Leica VT1000S, Bannockburn, IL). Once the median eminence was first seen in coronal sections, we collected 100 µm sections through the entire median eminence region. Sections were cryoprotected by immersion in increasing concentrations of glycerol (10%, 20%, and 30% in 0.1 M PB) and left in 30% glycerol overnight. A fragment of about 1 mm² in area was excised from the caudal median eminence under a dissecting microscope (Leica S6D stereo microscope, Switzerland). Tissue fragments were attached to a pin and rapidly plunged into liquid propane cooled to −180 °C by liquid nitrogen in a cryofixation system (Leica CPC, Vienna). Details of cryofixation and freeze-substitution have been published in detail (Yin et al., 2007). Cryofixed samples were then quickly transferred to a buffer exchange device filled with pre-cooled (−90 °C) 1.5% uranyl acetate in anhydrous methanol in an automatic freeze-substitution unit (Leica AFS, Vienna) and immersed for 24 hours. Then, the temperature in AFS was set to automatically increase in steps of 4 °C per hour from −90 °C to −45 °C. The samples were then infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA) in the ratio of resin to methanol from 1:1, 2:1 to pure Lowicryl for 2 hours each, followed by pure Lowicryl overnight at −45 °C. Finally, samples in buffer exchange capsules filled with Lowicryl resin were transferred into a sealed gel capsule. Under UV light (360 nm), Lowicryl resin was polymerized at −45 °C for 24 hours followed by 0 °C for 35 hours.

Serial sectioning of Lowicryl HM20-embedded tissue blocks

Embedded tissue blocks were first trimmed with a razor blade at a 45° angle to make a smaller block face on an ultramicrotome (Leica EM UC6, Vienna). A glass knife was used to trim the block surface and around the tissue (30° angle) to form a rectangular block face and pyramid shape base. Semithin sections (400 nm) cut with a 45° ultra diamond knife (Diatome, Hatfield, PA) were stained with 2% toluidine blue and examined under a transmitted light microscope (Leica DMLB upright microscope, Germany) for tissue orientation. Sections that contained the full surface of the lateral median eminence were collected individually on formvar-coated gold slot grids (Electron Microscopy Sciences, Fort Washington, PA) for general comparison. Due to the highly labor-intensive nature of serial sectioning and 3D reconstruction, we randomly choose two embedded tissues from each age group for serial ultrathin sectioning. The interface at the boundary between the portal capillary zone and the edge of the lateral median eminence was identified and carefully marked on the tissue block face under an ultramicrotome (Leica EM UC6, Vienna). By using a 90° trimming knife (CT388, Diatome, Hatfield, PA), we were able to trim into the block for about 50 µm in depth to form a block face of approximately 50 µm by 2 mm to cover the area of interest (Figure 1A). For serial sectioning, the long edge of the block face was carefully aligned with a diamond knife (Diatome Ultra 45). Section ribbons containing about 50 serial ultrathin sections (50 nm each) were collected on formvar-coated gold slot grids (Electron Microscopy Sciences, Fort Washington, PA) and air-dried (Figure 1B).

Antibody Characterization

The antibody used in the current study was the GnRH monoclonal antibody (HU11b; Table 1), kindly provided by Dr. Henryk Urbanski (Oregon Primate Center). This antibody was extensively characterized by Dr. Urbanski using radioimmunoassay, which showed immunospecificity to the bioactive GnRH molecule, but not the GnRH free acid, the precursor molecule or to other neuropeptides (Urbanski, 1991). Further, GnRH cells are easily identified by their localization (cell bodies in preoptic area, terminals in the median eminence) and unique morphology, and the HU11b antibody only labels these few cells (Yin et al., 2007). Preabsorption control experiments have been performed with HU11b in Urbanski's laboratory and ours (Urbanski, 1991; Yin et al., 2007), and no specific labeling was found in control sections.

Immunogold labeling for serial electron microscopy

The post-embedding immunogold labeling technique was described in detail previously (Yin et al., 2007). Briefly, sections on slot grids were blocked in 2% human serum albumin (HSA in TBS) for 10 minutes and then incubated in mouse anti-GnRH monoclonal antibody (Table 1), for 4 hours. This After washing in TBS three times, sections were incubated in gold-tagged F(ab')₂ of goat anti-mouse IgG 5 nm, 1:20, Electron Microscopy Science) in TBS with 2% HSA and polyethylene glycol (PEG, MW 20,000; 5 mg/ml) for 1 hour and followed by washes in double distilled filtered water 12 times. Uranyl acetate or lead citrate counterstaining was not necessary because the ultrastructure is sufficiently defined by perfusion and freeze-plunge fixation and en bloc uranyl acetate.

Serial electron microscopy and three-dimensional (3D) reconstruction

Under the transmission electron microscope (TEM) (Philips EM208, Eindhoven, Netherlands), the neuroterminal region (10 μ m by 800 μ m) along the boundary of the portal capillary zone was identified in the lateral median eminence at 2,000x TEM magnification. Sequential images of GnRH immunopositive neuroterminals and the pericapillary region from section to section were taken with a digital camera (AMT HR 1Mb). Using the software Reconstruct (Fiala, 2005), www.synapseweb.org/tools/index.stm), specific sets of structures were identified from section to section and carefully aligned. Serial images at 11,000x TEM magnification were used to show the interaction between GnRH immunopositive neuroterminals, tanyocyte endfeet and the capillary basal lamina in three dimensions in the young and old-aged groups. Immunopositive GnRH terminal contents including large dense-core vesicle and small vesicles were identified and individually outlined in images taken at 28,000x TEM magnification to show the three dimensional distribution of vesicles in the neuroterminals and their interaction with surrounding neural and glial elements. The contrast and brightness of electron microscopy images were slightly modified using Adobe Photoshop 7.0 software, with adjustments applied uniformly to images to enhance visualization of nanogold beads and/or subcellular compartments, but not for any quantitative applications. Movies demonstrating the procedure of 3D reconstruction was captured using software Movies CamStudio version 2.00 (RenderSoft Software and Web Publishing, <http://www.rendersoftware.com/products/camstudio>).

Quantitative electron microscopy data analysis

To study the relationship of GnRH neuroterminal and glial elements, the percentage of the surface of GnRH terminals in apposition to glial membranes was quantified. Two ultrathin sections 4 to 5 μ m apart were chosen from each animal for quantification analysis to avoid repeated counting of the same terminal, which has a maximum 3 μ m diameter. The pericapillary region containing GnRH immunopositive terminals covered approximately 10 μ m by 800 μ m area along the portal capillaries. All GnRH immunopositive terminals within the 10 μ m distance from the basal lamina were individually imaged at 28,000x TEM magnification. The

surface contact of GnRH neuroterminals and glial elements was outlined and measured in each image using NIH Image J software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA. <http://rsb.info.nih.gov/ij/>). The percentage of GnRH terminal membrane in contact with glial elements was compared between young and old rats.

Serum luteinizing hormone (LH) concentrations

At the time of perfusion, a blood sample was collected from each rat, centrifuged, and the serum stored at -80°C for subsequent radioimmunoassay. Serum LH concentrations were measured in duplicate samples (50 μl) in the laboratory of Dr. Michael J. Woller (University of Wisconsin, Whitewater, WI). The rat LH RP-3 standard was used, kindly provided by Dr. A.F. Parlow through the National Hormone and Pituitary Program of the NIDDK. The primary antibody was complexed to goat anti-rabbit gamma globulin for precipitation. Assay sensitivity was 0.03 ng/tube at 85% binding. Intra-assay variability was 2.36%.

Statistics

Statistical analyses of serum LH levels and numbers of GnRH terminals in the 10 μm by 800 μm area were performed by t-test using SPSS 13.0 software. For the GnRH terminal analysis, a biostatistician, Dr. W. Y. Wendy Lou (University of Toronto, Toronto, ON, Canada), compared differences between young and old rats using a mixed model approach and the generalized estimating equation approach (Laird & Ware, 1982). This statistic takes into consideration that there are multiple measurements within an animal and calculates a composite value for each animal. Therefore, the unit of analysis is the animal, and the final sample size is the number of animals per group ($n = 5$ each for young and old groups). In all cases, differences were considered significant at $P < 0.05$.

Results

The organization of the median eminence

Using the immunogold labeling technique, we were able to locate immunopositive GnRH terminals in the pericapillary area of the lateral median eminence under the electron microscope as reported previously (Yin et al., 2007). Under low magnification, the pericapillary region in the lateral median eminence could easily be discerned (Figure 1A), and we were able to collect serial sections through this region (Figure 1B). The organization of the lateral median eminence is shown in Figure 1C and 1D for a representative young and old rat, respectively. Glial elements could be distinguished by their elongated shape and, at higher magnification, a high density of electron-dense glycogen granules in the cytosol (Gruetter, 2003). Glia were seen in close relationship to both GnRH immunopositive and immunonegative terminals. When the general glial organization in the pericapillary zone was assessed in the young rats, we consistently observed radial glial processes from the third ventricle to the base of the median eminence that tended to be linearly oriented toward the basal lamina of the portal capillary system (Figure 1C). By contrast, this linear glial pattern of the median eminence was lost in old rats. In these latter animals, the glial cells appeared thicker and were more irregularly positioned in the median eminence (Figure 1D). In addition, the basal lamina, which was relatively smooth in young rats, became less delineated and was extremely convoluted in the old group (Figure 1 D).

Ultrastructural properties of GnRH terminals in three dimensions

In order to gain further insights into the relationship among the neural, glial and portal capillary components of the median eminence, serial sectioning and immunogold labeling were used to reconstruct several representative GnRH neuroterminals in two- and three-dimensional detail. In young rats, these techniques confirmed the two dimensional results, and also revealed novel

organizational features. Figure 2 shows a GnRH-immunoreactive neuroterminal of a young OVX rat that is in close proximity to several other immunonegative nerve terminals, tanycytic endfeet and the basal lamina of the portal capillaries. Although the immunogold labeling cannot be seen at the magnification shown in panels 2A, panels 2E and F are serial sections of this GnRH terminal higher magnification to enable visualization of immunogold labeling in the large-dense core vesicles. At lower magnification (Figure 2A) the relatively linear organization of the median eminence glia, and the evenness of the basal lamina, can be seen two dimensions (Figure 2A) and reconstructed in three dimensions (Figure 2B-D and Supplemental Animation 1). GnRH-immunoreactive terminals as identified by immunogold labeled large dense-core vesicles were frequently seen within a few microns of the basal lamina. Some GnRH-immunonegative terminals were seen in direct contact with the basal lamina but due to a lack of large dense-core vesicles, presumably because the vesicles were already released, we were unable to distinguish whether they formerly contained GnRH or not (Figure 2 A).

Figure 3 shows serial sections and reconstructions from two additional young OVX rats. The same GnRH terminal and its environment is shown at low and higher magnification (Figure 3A and 3B) in two dimensions, and reconstructed in three dimensions from two perspectives (Figure 3C and 3D; Supplemental Animation 2). Another set of serial sections from a different young OVX rat is shown (Figure 3E and 3F). Several observations were revealed by these series. First, they demonstrate that GnRH immunogold labels are present in all of the large dense-core vesicles in GnRH terminals. Although in one section of this pair (Figure 3E, 3F, each approximately 50 nm in thickness) some large dense-core vesicles (approximately 100 - 120 nm in diameter) may appear unlabeled, the next sequential section confirms immunogold labeling of the vesicles. Occasionally, gold beads were seen in cytosol or in association with mitochondria, but these were relatively infrequent and not seen in serial sections. Therefore, the vast majority of GnRH immunolabeling was in association with large dense-core vesicles. Glial processes were also noted to be in frequent contact with GnRH immunopositive terminals. Finally, small vesicles were abundant within GnRH-immunoreactive terminals (Figure 3, Supplemental Animation 2; also see Figure 2E).

Figure 4 shows GnRH terminals and their microenvironment from two representative old OVX rats (Figure 4A-C correspond to Supplemental Animation 3, and Figure 4D-F correspond to Supplemental Animation 4). The basal lamina from these old rats was highly convoluted (Figure 4A and 4D), therefore we were unable to trace the basal lamina for making 3D models in low magnification as we did in young rats (e.g., Figure 2). In old OVX rats, GnRH immunopositive terminals were in close proximity to the basal lamina and some were in direct contact (Figure 4B and 4E). The terminal shown in Figure 4E and 4F had the highest density of GnRH vesicles observed in our study. However, we did not quantify vesicle density in this study, as such measures cannot easily be made in the 3D reconstructions. In Figure 4G-I, a portion of the same terminal branch is shown at higher magnification, ensheathed by glial elements, and demonstrating that all large dense-core vesicles are immunolabeled for GnRH through the series.

We also noted the presence of extruded membrane structures with low electron density in terminals in the median eminence (Figure 5). These structures had been noted in numerous two-dimensional TEM images (Figure 5A-C), but only three-dimensional reconstructions (Figure 5D) reveal the possibility that these “empty” regions may represent those where terminal contents such as secretory vesicles have been released. Although the region shown in Figure 5A-D was not immunolabeled, and therefore we do not know if it is a GnRH terminal, the extruded membrane structures were seen in GnRH immunopositive terminals in both young and old OVX rats (Figure 5 E and F).

Quantitative TEM data on GnRH terminal numbers

The numbers of GnRH immunopositive terminals within the 10 μm by 800 μm area along the portal capillaries were counted in two ultrathin sections per rat. In young OVX rats, an average of 21 ± 2 GnRH immunoreactive (ir) terminals were found. In old OVX rats, 32 ± 4 GnRH-ir terminals were found in this same sized region. A trend for higher numbers of GnRH-ir terminals in old compared to the young group was found, but it did not attain significance ($t = 2.217$, $p = 0.057$).

The close relationship between GnRH immunopositive terminal and glial elements was quantified by measuring the percentage of the GnRH terminal membrane that was in contact with the glial membrane as depicted in Figure 3F. Comparisons were made between young and old female OVX rats using high magnification electron microscopy images. A total of 105 and 158 GnRH immunopositive terminals were analyzed in young and old female rats, respectively ($N = 5$ rats per group). An average of $81.30 \pm 3.38\%$ and $68.60 \pm 4.35\%$ of membrane apposition was measured in young and old rats, respectively. Using a mixed model approach and the generalized estimating equation statistic, a small (13%) but significant decrease in membrane apposition with age was detected ($p < 0.005$).

Serum LH concentrations

Serum LH concentrations were assayed by radioimmunoassay. In the young OVX rats, concentrations were 22.28 ± 3.60 ng/ml. In old OVX rats, serum LH levels were 6.60 ± 2.58 ng/ml. The levels in the young rats were significantly higher than those in the old animals ($t = 3.542$; $p < 0.01$).

Discussion

Hypophysiotropic neurons project terminals to the median eminence, where neurohormones are released into the portal capillary vasculature leading to the anterior pituitary gland. Previous studies have focused on this complex neuroterminal region, but as yet, the spatial and functional relationships among neuroterminals, glia and capillaries has not been fully visualized or understood due to limitations in working in two dimensions, and other technical obstacles. Our current study used serial electron microscopy in concert with computer graphics to enable us to perform 3D reconstruction. This approach has previously proven to be a powerful tool to collect detailed ultrastructural anatomical information on synaptogenesis in hippocampus (Carlsson et al., 1994; Sorra et al., 2006; Ventura and Harris, 1999), but to our knowledge it has never been applied to the hypothalamus. We extended these methods by applying the technique to cryo-substituted tissues prepared at low temperature to preserve antigenicity, enabling post-embedding immunogold labeling. In this way, we were able to study the hypophysiotropic GnRH neural system and demonstrate detailed ultracellular information.

The results of 3D reconstructions provide a much more meaningful depiction of the shape and structural relationships between glia and neuroterminals, which are otherwise difficult to infer from single or multiple isolated 2D sections. Using serial electron microscopy we can positively identify previously ambiguous structures by investigating adjacent sections. For example, we identified extruded membrane structures in hypophysiotropic nerve terminals, including GnRH-immunoreactive terminals (Figure 5). To our knowledge, these structures have not previously been described. Considering that these are neurosecretory cells, we speculate that the regions of extrusion represent areas where large dense-core vesicles may have been released. Furthermore, by combining serial electron microscopy with immunogold labeling, the labeling specificity can be confirmed by virtue of corresponding labels on the same structures on adjacent sections (Figure 2E and F, Figure 3E and F, Figure 4 G-I). These results showed that GnRH immunogold labels were present in all large dense-core vesicles in GnRH

terminals in both young (Figure 2E and F) and old (Figure 4 G-I) female rats, again a finding that to our knowledge has never been shown by previous electron microscopy studies. Along with the large dense-core vesicles, our 3D images revealed that GnRH neuroterminals are rich in small vesicles (Figure 3C and D, Supplemental Animation 2). However, the nature of the contents of small vesicles, presumably neurotransmitters, is still unknown. We are beginning to perform immunohistochemical studies to ascertain the contents of these small vesicles, which are consistent in size with those containing a monoaminergic neurotransmitter such as GABA, glutamate, a catecholamine, or indoleamine (Nirenberg et al., 1995) although limitations with antibodies to these molecules have thus far prevented us from positively identifying the contents. Recently, Hrabovszky and Liposits (Hrabovszky and Liposits, 2008) showed that vesicular glutamate transporter 2 immunoreactivity is associated with small vesicles in unidentified terminals in the external median eminence, suggesting that they may be glutamatergic. It would be interesting to determine whether this is the case specifically in GnRH terminals.

The median eminence is a circumventricular organ with a relatively porous blood-brain barrier, thereby enabling the neuroendocrine systems to communicate with the peripheral circulation (Okon and Koch, 1977). This unique feature is maintained by specialized tanycyte and astrocyte cells (Baroncini et al., 2007; Durrant and Plant, 1999; Kozłowski and Coates, 1985; Nozaki et al., 1979; Ojeda et al., 2008; Prevot et al., 1998; Theodosis and MacVicar, 1996). In the capillary zone of the median eminence, tanycyte processes covers the basal lamina of the portal capillary and were frequently seen in contact with neural axons and terminals. In our study, a high density of glycogen granules was seen in the cytosol of tanycyte processes. Because glucose is the major energy source in the brain, the glycogen granules in glial processes probably subserve the high-energy demand of neural-glial activity (Gruetter, 2003).

Previous morphological studies demonstrated the close interaction between glial cells and GnRH neurons in bird (Yamamura et al., 2004), rodent (King and Letourneau, 1994; Kozłowski and Coates, 1985; Romero et al., 1994; Prevot et al., 2007), nonhuman primate (Perera and Plant, 1997; Witkin et al., 1991) and human hypothalamus (Baroncini et al., 2007) and these relationships are thought to relate to the regulation of GnRH secretion (Dhandapani et al., 2003; Garcia-Segura et al., 2008; Ojeda et al., 2003; Prevot et al., 1998). Our study on the organization of the capillary zone in the median eminence showed clear evidence for overall changes in its cytoarchitecture, and morphological changes in glial cells, during aging. Most interestingly, the general organization of the median eminence became less structured in aged rats. In young rats, the tanycytes were long and narrow and oriented perpendicularly from the third ventricle to the portal capillary plexus (Figure 1 C and Figure 2 A). By contrast, this linearity was largely lost in aging females, and the tanycytes appeared wider in shape (Figure 1 D, Figure 4 A and D). In addition, the boundary between the neuroterminals of the pericapillary zone with the portal capillaries became more convoluted in aged female rats (Figure 1 D, Figure 4 A and D). We interpret these findings to mean that the access of the GnRH peptide to the portal capillary system is different between young and aged rats, and may be related to the decreased pulsatile (Scarborough and Wise, 1990; Wise et al., 1988) and surge (Lloyd et al., 1994; Rubin et al., 1994; Wise, 1982; Rubin and Bridges, 1989) release of GnRH/LH reported in aging rats. Although we could not measure GnRH release, we measured serum LH levels and found that they were significantly higher in the young than the old OVX females, a finding suggesting that GnRH release is correspondingly different, possibly due in part to the ultrastructural changes with age. In contrast to decreased serum LH level of old OVX rats, we have shown a trend of increased GnRH immunopositive terminals containing large amount of dense-core vesicles in direct contact to the portal capillary basal lamina in old rats. We consider a proper coordination between the median eminence GnRH release, portal system transportation and pituitary response is needed to stimulate LH release.

Our 3D analyses demonstrated a complex interaction between GnRH neuroterminals and glia. These results, showing the ensheathment of GnRH terminals by tanycytes, extend previous studies on the GnRH-glia interaction reported using 2D analysis (King and Rubin, 1994; Prevot et al., 1998, 1999). Although we could not do quantitative measures on our 3D reconstructions, we were able to quantify the apposition of GnRH terminal membranes with glial membranes in our 2D images. The results showed a small but highly significant decrease in membrane apposition between GnRH terminals and glia in old compared to young rats. Whether this 13% decrease of membrane interaction can result in a decrease of GnRH release is uncertain, but it suggests that the underlying anatomical framework and neural-glial communication is different with aging. This finding may relate to reports showing that cell-surface molecules involved in structural properties underlying cellular communication including PSA-NCAM, a neural cell adhesion molecule (Gascon et al., 2007); contactin, a neural surface protein (Parent et al., 2007); and SynCAM1, an adhesion molecule for synaptic assembly (Ojeda et al., 2008), are involved in cytoskeletal organization. These cell-surface molecules are involved in interactions between and among GnRH terminals and their surrounding glial cells (Ojeda et al., 2008). It will be interesting to know whether the expression of these neuronal-glial cell surface molecules changes with age.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A: Light microscopy image of a semithin section from a representative young OVX female rat, showing the orientation of the lateral median eminence. The interface between the edge of neural profiles and the portal capillaries can be identified. After several steps of trimming, a 50 μm by 2 mm cutting zone (marked by parallel lines) is saved for serial electron microscopy sectioning. B: Electron microscopy image shows serial ultrathin sections on a formvar-coated gold slot grid. Serial images are taken from section to section as shown by a trace of the electron beam (arrows). Scale bar (A and B) = 100 μm . C and D: Electron microscopy low-magnification images of the pericapillary area of the lateral median eminence from a representative young (C) and old (D) OVX female rat. The glial organization (glia) in the pericapillary area can be

seen. In the young OVX rat (C) the glial organization is linear and oriented perpendicularly from the basal lamina (BL with arrowhead) towards the third ventricle (not shown). The basal lamina can be seen covered by glial endfeet. In the old OVX rat (D) the median eminence is less linearly structured and the glial cells are wider and not clearly organized along the basal lamina. The basal lamina is also very convoluted and its edges hard to define. Scale bar (C and D) = 5 μm . Abbreviations: BL = Basal lamina, Cap = capillary, Glia = glial elements; 3V = 3rd ventricle.

Figure 2.

Electron microscopic image and 3D projections of a GnRH immunopositive terminal and its surrounding glial environment in a representative young OVX rat. A: A GnRH immunopositive terminal in the median eminence (framed in box; immunolabels can be seen at higher magnification in serial sections E and F) is shown about 2 μ m from the portal capillary basal lamina (BL with arrows). B-D: This GnRH immunopositive terminal and its environment are reconstructed with elements added in with each succeeding panel. B: The glial processes (green) show a linear pattern and perpendicular orientation to the capillary bed (pink). C and D: The relationship among the GnRH immunopositive terminal (orange), GnRH immunonegative terminals (yellow, corresponding to “T” in Panel A) and the capillary bed (pink) is shown. Scale bar (A-D) = 2 μ m. Panels E and F show the GnRH terminal in adjacent sections of the same rat at higher magnification. Large dense-core vesicles (arrowhead), small vesicles (small arrow) and mitochondria (Mit) are the major contents in GnRH terminals. Scale bar (E-F) = 200 nm. Abbreviations: BL = basal lamina; Glia = glial processes; Mit =

mitocontria, T = terminals (GnRH immunonegative). A 3D animation of panels B-D is presented in Supplemental Animation 1.

Figure 3.

Electron microscopy 2D and 3D images from two representative young OVX rats showing the relationship between immunopositive GnRH terminals and glial elements in the portal capillary region. A: The GnRH immunopositive terminal (framed in box) is approximately 3 μ m in distance from the basal lamina (BL with arrow) of the portal capillary (Cap) zone. A glial element (Glia) in contact with GnRH immunopositive terminal also covers the basal lamina of the capillary. Scale bar = 1 μ m. B: This same immunopositive GnRH terminal is shown at higher magnification, with the immunogold labels located in large dense-core vesicles (arrowheads). Glial elements containing a high density of glycogen granules (white arrows) are in contact with the GnRH terminal. The relationship between this same GnRH terminal

and glial elements (Glia) is reconstructed and shown from two different angles in panels C and D. Here, the GnRH neuroterminal (orange) is shown containing numerous large secretory vesicles (purple) and small vesicles (red). Finger shaped glial elements (green) make direct contact with the GnRH neuroterminal. An animation of the terminal in A-D is shown in Supplemental Animation 2. Panels E – F are adjacent sections from another OVX young rat showing immunogold labeling of GnRH in all the large dense-core vesicles. Although some vesicles (arrowheads) may not appear labeled in one image, the adjacent section clearly shows immunolabels in the same vesicles. The membrane interactions between the GnRH terminal and surrounding glial elements are marked by green in panel F to depict how apposition of GnRH terminals and glia was measured. Scale bar (B, E, F) = 500 nm. BL = basal lamina, Cap = portal capillary, Glia = glial element, Mit = mitochondria.

Figure 4.

Electron microscopy 2D and 3D images taken from two representative old OVX rats showing the relationship between immunopositive GnRH terminals, glial elements and portal capillary basal lamina in the pericapillary region of the median eminence. A: An immunogold labeled GnRH neuroterminal (framed in box) is embedded in the highly convoluted portal capillary basal lamina (BL with arrows). This terminal is in direct contact with the basal lamina as shown in higher magnification in panel B. Glial elements (Glia) are loosely surrounding the GnRH terminal and in contact with a GnRH immunonegative terminal (T). C: A 3D image was reconstructed from 21 serial electron microscopy images of the terminal shown in A. In the GnRH neuroterminal, the distribution of large dense-core vesicles (purple) and mitochondria (blue) is seen. The relationships among the GnRH terminal (orange, from panel A), non-GnRH terminal (yellow), glial elements (green) and portal capillary bed (pink) are shown. An animation of panels B and C is shown in Supplemental Animation 3. D: An immunogold labeled GnRH neuroterminal (framed in box) from another representative old rat is shown at low and high magnification (D and E, respectively). Glial elements separate the GnRH terminal from the convoluted portal capillary basal lamina (BL with arrows) in panel E. Panel F shows a reconstructed 3D image from 20 serial electron microscopy images of the terminal in panel E. The high density of large dense-core vesicles (purple) can be seen. A portion of the terminal branch is ensheathed by glial elements and shown at higher magnification in serial sections (G-I) which show all large dense-core vesicles are labeled with immunogold particles. The immunoreactivity of some vesicles can only be identified by labels shown in adjacent sections

(arrows). Scale bar (A and D) = 1 μ m, Scale bar (B, C, E, F and G-I) = 200 nm. Abbreviations: BL = basal lamina; Glia = glial processes; T = GnRH immunonegative terminals. An animation of panel F is shown in Supplemental Animation 4.

Figure 5.

Serial electron microscopy images and 3D image showing the relationship of neural profiles including extruded membranes. Images are shown from three different rats. Panels A-C show a series of three adjacent electron microscopy images from a representative young OVX rat, with the 3D image (produced from a total of 15 sections) shown in Panel D. GnRH immunogold labeling was not performed in this series so it is unknown if it is GnRH immunopositive or -negative. The extruded membrane with low electron density and no cytoplasmic contents can be seen (arrow in panels A-C), and may represent a region where the vesicle contents have been released. Panel D: The neural profile (tan) is reconstructed to show the relationship to another nearby neural profiles (light yellow) and nearby glial elements (green). The arrow points to the extruded membrane region. Scale bar (A-D) = 200 nm. Panels E and F are from GnRH immunopositive terminals of another young OVX (E) and old OVX (F) rat. Arrows point to the extruded membrane structures in GnRH immunopositive terminals. Scale bar (E and F) = 500 nm. Glia = glial processes.

Table 1

GnRH HU11b Primary antibody

Antigen	Immunogen	Manufacturer, species antibody was raised in, antibody catalog and lot #	Dilution used
Gonadotropin-releasing hormone (GnRH), also called luteinizing hormone releasing hormone (LHRH)	GnRH decapeptide (mammalian sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂) conjugated to bovine thyroglobulin	Produced in Dr. Henryk Urbanski's laboratory, Oregon Primate Center. BALB/c mice were immunized and hybridoma clones were produced. The antibody is a mouse monoclonal, HU11B, Lot # 4 (Urbanski, 1991)	1:100