Neuronal-glial plasticity in gonadotropin-releasing hormone release in adult female rats: role of the polysialylated form of the neural cell adhesion molecule

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Abstract

The gonadotropin-releasing hormone (GnRH) neurosecretory system undergoes marked structural and functional changes during the ovarian cycle. The aim of this study was to examine the neuroanatomical relationship between GnRH neurons and a polysialylated form of neural cell adhesion molecule (PSA-NCAM), a known marker of neuronal plasticity. Using immunohistofluorescent dual labeling, we determined that axon terminals of GnRH in the median arcuate nucleus (ME-ARC) region of the hypothalamus in the proestrous phase of the estrous cycle were intimately associated with PSA-NCAM. To further examine whether PSA-NCAM expression associated with GnRH neuron terminals varies in conjugation with cyclic changes in ovarian steroid hormone levels, we examined GnRH and PSA-NCAM dual expression in ovariectomized (OVX) and estrogen–progesterone-primed OVX (EBP-OVX) rats. The expression of PSA-NCAM immunoreactivity associated with the GnRH neurons in the

proestrous phase and EBP-OVX rats was significantly higher than during the diestrous phase and in OVX rats where GnRH secretion declines. We further examined whether the structural changes in GnRH axon terminals in the ME-ARC region are also associated with glial plasticity. By extension and retraction of the glial processes, the GnRH neuron terminals in the ME-ARC region could undergo dynamic plastic changes that control GnRH release during the proestrous phase. PSA-NCAM expression was also seen on glial cells in the ME-ARC region. The close association between PSA-NCAM on GnRH and glial cells in the ME-ARC region of the hypothalamus in the rat showed dynamic structural changes in GnRH neuron terminals during the estrous cycle. These observations suggested that PSA-NCAM may act as a molecular substrate to promote neuroplastic changes in the GnRH neurosecretory system.

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Introduction

The gonadotropin-releasing hormone (GnRH) system in the brain constitutes the final common pathway for the central regulation of pituitary gonadotropin release (Sarkar *et al.* 1976). GnRH is synthesized in neuronal cell bodies distributed diffusely in the hypothalamic preoptic area and is secreted from the external zone of the median eminence-arcuate (ME-ARC) region by an increase in the length of the axons (King *et al.* 1995, Kaur *et al.* 2002). In females, the preovulatory surge of luteinizing hormone (LH) is initiated by an abrupt increase in GnRH release, which is preceded by GnRH axon terminal contact with the perivascular space directly (Prevot *et al.* 1999). A rise in circulating estradiol precedes the preovulatory LH surge and the ovarian cycle is also accompanied by a fall in the number of axosomatic synapses on randomly selected arcuate nucleus neurons by the morning of estrus, with a return to the usual levels by the morning of the next day (Naftolin *et al.* 1996). Studies of morphological and

physiological plasticity in the hypothalamus are changing our earlier concepts about the structure–function relationship in the adult mammalian brain. It has recently become clear that fluctuating physiological conditions have the power to reversibly alter the structural relationships among the neurons and glial cells in the hypothalamus, as well as the functional pathways over which information is transmitted to the neuroendocrine neurons.

The GnRH-secreting neurons in mammals project principally to the median eminence (ME) where they abut in the pericapillary space of the primary hypophyseal portal plexus. Prevot *et al.* (1999) demonstrated for the first time the existence of cellular conformational changes in GnRH neuron terminals and the cells of the basal lamina in the external zone of the ME, and their quantified data revealed a statistically significant increase and the presence of contacts between GnRH neuroendocrine terminals and the parenchymatous basal lamina in the proestrous phase and an absence of these contacts in the diestrous phase. The dynamic plasticity changes, which occur in the

external zone of the ME-ARC region on the day of proestrus, could be induced directly by gonadal steroids, since there is a dramatic increase in plasma estrogen levels from diestrus to the afternoon of proestrus prior to LH surge release.

The hypothalamus arcuate nucleus expresses high levels of polysialic acid (PSA) characterizing a highly sialylated isoform of the neural cell adhesion molecule (PSA-NCAM) and maintains a high capacity for neuroplastic changes in the adult brain. The large quantities of PSA-NCAM greatly reduce cell adhesion and render this particular cell surface molecule a key candidate to intervene in dynamic neuronal phenomena, such as synaptic plasticity. The modulation of adhesion arises from the length of the PSA, as this molecule has been shown to decrease NCAM homophilic binding and thereby attenuate cell adhesion. It has been proposed that this negative modulatory role of PSA helps the cells to detach from their neighbors, thus allowing them to respond to guidance or targeting cues, or to exhibit the plastic interactions required for motility (for review see Rutishauser & Landmesser 1996). PSA also appears to intervene in the activation of second messenger pathways and, therefore, in intracellular signaling (Doherty *et al.* 1991). During neuronal development, PSA-NCAM expression is closely correlated with dynamic cellular phenomena underlying neuronal migration, axon path finding, targeting and fasciculation (for reviews see Fryer & Hockfield 1996, Rutishauser & Landmesser 1996, Yoshida *et al.* 1999). In the adult brain, PSA-NCAM continues to be expressed in neurons and glia capable of functional and morphological plasticity (Theodosis *et al.* 1991, Bonfanti *et al.* 1992, Bonfanti & Theodosis 1994). In addition, PSA-NCAM re-expression has been noted in both neurons and glia in response to lesioning (Olsen *et al.* 1995, Bonfanti *et al.* 1996).

It is now well accepted that astrocytes play a fundamental role in the modulation of neuronal activity and metabolism (Araque *et al.* 1998) and local changes in neuronal activity are linked to modification of the expression of glial fibrillary acidic protein (GFAP). The GnRH neurons are extensively associated with non-neuronal elements of specifically glial cells (Fernandez-Galaz *et al.* 1999). GFAP is a component of the intermediary filaments and increase in its expression is associated with the growth of astrocytic processes that may result in structural remodeling in the central nervous system (CNS) (Kohama *et al.* 1995, Stone *et al.* 1998). Glial processes seem to provide a permissive substrate for GnRH axonal extension, and the presence of a chemotropic factor, i.e. basic fibroblast growth factor (bFGF), provides specificity in the ME, which underlies the accurate navigation of the growing axon (Silverman *et al.* 1991). The fact that the GnRH axon displays a remarkable degree of outgrowth during a time of extensive glial hypertrophy and hyperplasia suggests that glial cells may play a facilitatory or permissive role in GnRH axonal

extension (Silverman *et al.* 1991). Glial cells have been shown to regulate GnRH secretion via two related mechanisms, plastic rearrangements and direct cell-cell communication and, both mechanisms require the participation of growth factors (Ojeda *et al.* 2003). The pubertal activation of pulsatile GnRH secretion is not initiated within the GnRH neuronal network itself, but instead is brought about by the molecular and cellular events that take place in the neuronal and astro-glial network functionally connected to GnRH neurons. The present study was designed to explore the potential for PSA-NCAM-mediated plasticity in the GnRH neuron in the adult rat, which shows cyclic changes in the structure and function of the GnRH system (Kaur *et al.* 2002). To further elucidate the role of astro-glial cells in the cyclic changes in GnRH neuron structure and function, we studied the expression of GnRH and GFAP in the proestrous and diestrous phase of the rat estrous cycle. As a first step, we co-localized GnRH and PSA-NCAM and GFAP and PSA-NCAM in the ME-ARC region of proestrous and diestrous phase rats by immunohistochemical dual staining. Using dual immunostaining of GnRH and GFAP, we further determined whether astro-glial cell processes associated with the GnRH neurons vary in conjunction with cyclic changes in GnRH neuronal terminals. To further test the role of steroid hormones in the cyclic neuronal plasticity of the GnRH neuron, we repeated these experiments in ovariectomized (OVX) and estrogen-progesterone-primed OVX (EBP-OVX) rats.

Materials and Methods

Wistar strain female albino rats of 3–5 months of age and weighing 200–250 g were procured from the National Institute of Nutrition, Hyderabad, India. Animals were housed three per cage in a temperature and humidity controlled environment on a 12 h light:12 h darkness cycle with free access to food and water. The estrous cycle was monitored by daily inspection of vaginal cytology. After at least three to four complete 4-day cycles, the animals were killed between 1500 and 1600 h on diestrous ($n=12$) and proestrous ($n=12$) days. Another group of rats was ovariectomized after surgically removing both the ovaries under aseptic conditions and also a sham control group in which only lateral cuts were given and ovaries were not removed in adult cycling female rats of same age group was defined. Experiments were carried out 4–6 weeks after ovariectomy. Half of the rats were given 7.5 mg estradiol benzoate (EB) (Sigma) subcutaneously in corn oil at 1000 h. Progesterone (5 mg) (Sigma) was administered subcutaneously in corn oil 48 h after the EB treatment (EBP-treated rats). All studies were performed between 1330 and 1430 h on the progesterone treatment day when the steroid-induced LH surge release occurs (Berglund *et al.* 1988). Animal care and procedures were followed in

accordance with the guidelines of the Institutional Animal Ethical Committee of Guru Dev University.

Procurement of antibodies for immunofluorescence

The primary antibodies used for the immunohistofluorescence analysis were monoclonal anti-GFAP (clone CA-5; Sigma) and monoclonal antibody specific for PSA-NCAM which was a generous gift from Dr Tatsunori Seki (Juntendo University School of Medicine, Japan).

For immunohistofluorescent localization of GnRH, LR1, an anti-GnRH antibody (a generous gift from G Tramu, CNRS, University of Bordeaux, Bordeaux, France) was used. The secondary antibodies used were Texas Red-conjugated (TRITC) goat anti-rabbit IgG at a dilution of 1:200 for GnRH, fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG at a dilution of 1:200 for GFAP and goat anti-mouse (FITC) IgM μ -chain specific at a dilution of 1:128 from Sigma for PSA.

RIAs for LH and follicle-stimulating hormone (FSH) levels

Serum LH and FSH levels were determined in duplicate using a double-antibody RIA technique. Blood samples from the trunk were clotted at 4 °C, centrifuged for 10 min at 2500 r.p.m., and the sera separated and stored frozen until hormone assay. Serum LH and FSH levels were determined by the RIA method of Rao & Mahesh (1986). EBP-OVX rats showing a serum LH level of 8.5 ng/ml and an FSH level of 10.7 ng/ml were selected for immunohistofluorescent experiments. OVX rats showing a serum LH level of 1.7 ng/ml and an FSH level of 3.5 ng/ml were further considered for experimentation.

Immunofluorescent staining

For immunofluorescent staining of GnRH+PSA-NCAM, GnRH+GFAP and GFAP+PSA-NCAM in the ME-ARC region, brains were perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.1 M). Brains were kept in the fixative solution overnight at 4 °C and then cryopreserved in 20% and 30% sucrose in phosphate buffer each for 24 h at 4 °C. Coronal sections (20 μ m thick) were cut using a cryostat microtome and sections were mounted on gelatin-coated glass slides and treated in the following manner: three 15-min washes in 0.1 M phosphate-buffered saline (PBS), pH 7.4 and 30 min in 0.3% Triton X-100 in 0.1 M PBS (PBST) for permeabilization. The sections were then washed with 0.1% PBST for 15 min. After washing, sections were preincubated for 1 h at room temperature in a blocking solution (5% normal goat serum in PBS with 0.3% Triton X-100 and 0.01% sodium azide). The sections were incubated with a mixture of primary antibodies, GnRH+PSA-NCAM, GnRH+GFAP or GFAP+PSA-NCAM with appropriate dilutions of anti-GnRH (1:10 000), anti-PSA-NCAM

(1:500), anti-GFAP (1:500) in 0.1% Triton X-100 and 1% bovine serum albumin-PBS for 60 h at 4 °C. Specificity of staining for GnRH, PSA and GFAP was determined by negative staining control procedures without adding primary antibodies to the incubation buffer. Mouse monoclonal PSA-NCAM antibody 12E3 (IgM) was a gift of a sample raised in the laboratory of Dr T Seki. The antibody was characterized by Seki and Arai using anti-PSA antibodies as well as by neuroamnidas (endo-N) treatment (Seki & Arai 1991); the antibodies have strict specificity for PSA residues. The sections were then washed for 15 min with four changes of 0.1% PBST at room temperature. The sections were incubated with specified secondary antibodies: anti-rabbit IgG for GnRH (TRITC with a dilution of 1:200), anti-mouse IgG for GFAP (FITC with a dilution of 1:200), anti-mouse IgM (FITC with a dilution of 1:128) for PSA-NCAM and anti-mouse IgG for GFAP (TRITC with a dilution of 1:200) in 0.32% Triton-PBS for 2 h. Sections were then washed with 0.01% PBST for 15 min. Tissue sections were then cover slipped using the appropriate anti-fading mounting medium (500 mg propyl-gallate, 20 ml ethanol/PBS (pH 7.4) (1:1) and 90 ml glycerol) for fluorescence detection.

Data analysis

The dual-stained sections were examined using a Nikon fluorescent microscope with dual as well as single band filters. Images were captured using a Cool Snap CCD camera (Media Cybernetics, Silver Spring, MD, USA) and the pictures were analyzed using Image Pro-Plus software version 4.5.1 from Media Cybernetics. The extent of PSA-NCAM immunoreactivity associated with GnRH and GFAP immunoreactivity was quantified using two separate methods. The first analysis determined the overall density of GnRH, PSA-NCAM and GFAP immunoreactivity in 58 randomly selected fields in the ME-ARC region in each section using the count/size command of the Image Pro-Plus software. Five consecutive sections each from four to five animals in all groups were used for data analysis. An area of interest was selected at five to eight locations within the ME-ARC region on each section and density measurements were made for dual as well as for single staining of GnRH+PSA-NCAM, GnRH+GFAP or GFAP+PSA-NCAM.

The second method of analysis estimated the co-localization of GnRH and PSA-NCAM as well as of GFAP and PSA-NCAM using the co-localization command of the Image Pro-Plus software. This command measures the co-localization coefficient of two or more molecules (tagged with different fluorescent dyes) in precisely the same space. The percent difference in co-localization of GnRH+PSA-NCAM or GFAP+PSA-NCAM in proestrous and OVX-EBP rats as compared with the diestrous and OVX rats respectively was calculated and are presented as histograms.

Statistical analysis

The values are expressed as means $\pm$ S.E.M. The data were analysed using Student's *t*-test to determine the statistical significance. In the present study, different brain markers were studied in proestrous and diestrous rats in one group and OVX-EBP, OVX and sham controls in the second group. The statistically significant change in OVX-EBP and OVX rats in comparison with the sham control rats is shown in the figures by a, b, c or d. The statistically significant changes in OVX-EBP in comparison with OVX rats is also shown in the figures by a', c' or d'. For each parameter, the mean $\pm$ S.E.M. was determined in each group. Differences were considered statistically significant if $P < 0.05$.

Results

All data reported in the present study are restricted to adult female rat forebrain ME-ARC regions of the hypothalamus. Using double immunostaining microscopy, co-expression of PSA-NCAM with GnRH or GFAP and co-distribution of GnRH and GFAP were examined in the diestrous and proestrous phases of cycling rats as well as in the sham control, OVX and EBP-OVX rats. Brain sections incubated in the absence of primary antibodies mixture showed no immunostaining.

GnRH immunoreactivity in the ME-ARC region of the hypothalamus

The immunofluorescent staining for GnRH using anti-GnRH LR1 antibody showed a marked increase in the length and branching of GnRH axon terminals in the ME-ARC region in the proestrous phase as well as in EBP-OVX and sham control rats. On qualitative analysis, many GnRH terminals were seen to emit very thin processes in the direction of the perivascular space in the

proestrous and EBP-OVX rats. GnRH terminals in the ME-ARC region were heavily stained in the proestrous phase and EBP-OVX rats, indicating that these are the sites of active GnRH secretion. In the ME-ARC region from proestrous and EBP-OVX rats, GnRH nerve endings were also seen to make close contact with the parenchymatous space. Results are presented in Figs 1A and 3A and C (proestrous, EBP-OVX and sham control respectively) and Figs 1B and 3B (diestrous and OVX respectively).

Co-localization of GnRH+PSA-NCAM expression in proestrous and diestrous rats

In the ME-ARC region from proestrous rats, dense PSA-NCAM immunoreactivity was identified along the lateral border of the external zone of the ME, which was co-localized with a region of intense GnRH immunoreactive axon terminals. Double staining for GnRH and PSA-NCAM in this phase revealed that heavy PSA-NCAM expression was co-localized with GnRH immunoreactivity as is evident from the yellow staining appearing in co-localized areas. Results are presented in Fig. 1A. On the other hand, GnRH and PSA-NCAM expression was much lower in diestrous phase rats (Fig. 1B). Results of co-localization analysis shown in Fig. 1C and D further illustrate that GnRH and PSA-NCAM were being expressed together. Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase ($P < 0.001$) in the PSA-NCAM and GnRH dual staining in the ME-ARC region from the proestrous rats as compared with the diestrous phase rats (Fig. 2A). Similarly, an analysis of single staining for GnRH and PSA-NCAM showed significantly higher expression in proestrous as compared with diestrous phase rats (Fig. 2A). Comparison of co-localization of GnRH and PSA-NCAM in the ME-ARC region showed a

Figure 1 Photomicrographs of 20 μ m thick coronal section through the ME-ARC region of the hypothalamus; double immunostaining of the section for GnRH and PSA-NCAM is shown for the (A) proestrous and (B) diestrous phases. The intense PSA-NCAM and GnRH dual immunostaining was located along the external zone of the lateral portion of the ME in sections from proestrous as compared with diestrous phase rats. Note that the area where PSA-NCAM and GnRH immunoreactivities overlap appears as heavy (yellow) staining as shown in (A) and (B). Both immunoreactivities appear to be co-localized in the ventral arcuate nucleus and in the internal and external zone of the ME. Co-localization of GnRH and PSA-NCAM expression was analyzed using the co-localization function of Image Pro-Plus software and results of co-localization are shown in (C) proestrous and (D) diestrous stages. Photomicrographs of 20 μ m thick coronal section through the ME-ARC region of the hypothalamus; double immunostaining of the section for GFAP and PSA-NCAM is shown for (E) proestrous and (F) diestrous phases. The intense PSA-NCAM immunostaining was localized along the external zone of the ME in sections from proestrous phase rats whereas PSA-NCAM-ir was much lower in diestrous rats. The expression of GFAP immunostaining was localized along the external and internal zone of the ME in sections from (F) diestrous rats but in (E) proestrous phase rats GFAP expression was restricted to internal zone of the ME. Note that the area where PSA-NCAM and GFAP immunoreactivities overlap appears yellow as shown in (E) and (F). Co-localization of GFAP and PSA-NCAM expression was analyzed using the co-localization function of Image Pro-Plus software and results of co-localization are shown in (G) proestrous and (H) diestrous rats. Photomicrographs of 20 μ m thick coronal section through the median eminence arcuate region; double immunostaining of the section for GnRH and GFAP is shown for (I) proestrous and (J) diestrous phase rats. Immunostaining in both appeared to be co-distributed in the same area of ventral arcuate nucleus and of the internal and external zone of the ME. The expression of GFAP was observed in both the external and internal zone of the ME in diestrous rats but its expression was restricted to the internal zone of the ME in the proestrous rats. The expression of GnRH increased in proestrous as compared with diestrous phase rats. 3v, third ventricle. Magnification $\times 50$. This figure appears in colour at <http://joe.endocrinology-journals.org/content/vol186/issue2/>.

Figure 2 Intensity measurement data of the PSA-NCAM immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of proestrous (dotted bars) and diestrous (lined bars) phase rats; (A) depicts the staining intensity of GnRH and PSA-NCAM in proestrous and diestrous rats. GnRH+PSA depicts the intensity data of dual-stained sections, PSA depicts PSA-NCAM immunoreactivity and GnRH depicts the GnRH immunoreactivity in proestrous and diestrous rats. (B) Quantitative data of percent change in co-localization of GnRH and PSA-NCAM in proestrous and diestrous rats was analyzed from Fig. 1C and D using the co-localization function of Image Pro-Plus software. Intensity measurement data of the PSA-NCAM immunoreactivity associated with the glial cell in the ME-ARC region of proestrous and diestrous phase rats; (C) depicts the staining intensity of GFAP and PSA-NCAM in proestrous and diestrous rats. GFAP+PSA depicts the intensity data of dual-stained sections, PSA depicts PSA-NCAM immunoreactivity and GFAP depicts the GFAP immunoreactivity in proestrous and diestrous rats in the IZ and EZ. (D) Quantitative data of percent change in co-localization of GFAP and PSA-NCAM in proestrous and diestrous rats was analyzed from Fig. 1G and H using the co-localization function of Image Pro-Plus software. Intensity measurement data of the GFAP immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of proestrous and diestrous phase rats; (E) depicts the staining intensity of GnRH and GFAP in proestrous and diestrous rats. GnRH+GFAP depicts intensity data of dual-stained sections, GFAP depicts GFAP-ir in the IZ and the EZ and GnRH depicts the GnRH-ir in proestrous and diestrous rats. For each type of data analysis in (A), (C) and (E) measurements were performed on five to eight sections each from four to five proestrous and diestrous phase rats. All statistical analyses were performed using Student's *t*-test. Values are means $\pm$ S.E.M.; a= P <0.05, b= P <0.02, d= P <0.001. IZ, internal and EZ, external zones of the ME.

highly significant increase in percent co-localization in the proestrous phase rats as compared with the diestrous group (Fig. 2B).

Co-localization of GFAP+PSA-NCAM expression in proestrous and diestrous rats

In the ME-ARC region, double immunofluorescence staining in the proestrous phase of the estrous cycle showed that GFAP protein expression was restricted only to the internal zone of the ME-ARC but, in the case of diestrous rats, GFAP expression was observed in both the external and internal zone of the ME-ARC. PSA-NCAM immunoreactivity was identified along the internal and external zone of the ME (with more pronounced expression in the external zone) in proestrous rats whereas immunoreactive PSA-NCAM (PSA-NCAM-ir) was much lower in diestrous rats. Results are shown in Fig. 1E for proestrous rats and Fig. 1F for diestrous phase rats. Quantitative immunofluorescence analysis of staining intensity measurements showed an increase in the dual PSA-NCAM and GFAP staining in the ME-ARC region in the diestrous rats as compared with the proestrous phase rats. But an analysis of single staining for PSA-NCAM showed significantly higher expression in proestrous as compared with diestrous phase rats, whereas GFAP expression was much higher in diestrous as compared with proestrous rats. Results are presented in Fig. 2C. Comparison of co-localization of dual GFAP and PSA-NCAM in the internal zone of the ME-ARC region showed an insignificant change in percent co-localization in the diestrous and proestrous rats (Fig. 2D).

GnRH and GFAP expression in proestrous and diestrous rats

Immunofluorescence data indicated that GnRH expression increased significantly in the ME-ARC region of the hypothalamus in the proestrous phase as compared with the diestrous rats. GnRH axons were co-distributed with the glial elements in the internal zone of the ME in the proestrous phase, whereas GFAP staining was observed in both internal and external zones of the ME-ARC in diestrous phase rats, thus indicating reduced glial apposition with GnRH axon terminals in the parenchymatous space to facilitate GnRH release in the proestrous phase. The results of GnRH and GFAP dual immunostaining are presented in Fig. 1I for proestrous and Fig. 1J for diestrous rats. Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase in the GFAP staining in the ME-ARC region in the diestrous rats as compared with the proestrous phase rats. Results are presented in Fig. 2E. Similarly, an analysis of single staining for GnRH showed higher expression in proestrous as compared with diestrous phase rats but the reverse was observed for GFAP expression (Fig. 2E).

Co-localization of GnRH and PSA-NCAM expression in OVX, steroid-primed OVX and sham control rats

In the ME-ARC region from EBP-OVX rats, dense PSA-NCAM immunoreactivity was identified along the lateral border of the external zone of ME, which was co-localized with a region of intense GnRH immunoreactive axons terminals. Double immunostaining for GnRH and PSA-NCAM in these rats revealed that heavy PSA-NCAM expression was co-localized with GnRH immunoreactivity as is evident from the yellow staining appearing in the co-localized area. Results are presented in Fig. 3A. On the other hand, GnRH and PSA-NCAM expression was much lower in OVX rats as well as in sham control rats (Fig. 3B and C). Results of co-localization analysis shown in Fig. 3D, E and F further illustrate that GnRH and PSA-NCAM were being expressed together. Results of co-localization analysis shown in Fig. 3D, E and F for OVX-EBP, OVX and sham control rats respectively further illustrate that GnRH and PSA-NCAM were being expressed in the same cell. Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase ($P < 0.001$) in the PSA-NCAM and GnRH dual staining in the ME-ARC region from EBP-OVX rats as compared with OVX as well as with sham control rats (Fig. 4A). Similarly, an analysis of single staining for GnRH and PSA-NCAM showed significantly higher expression of these markers in EBP-OVX rats as compared with OVX and sham control rats (Fig. 4A). Comparison of co-localization of GnRH and PSA-NCAM in the ME-ARC region showed a highly significant increase in their percent co-localization in the EBP-OVX rats as compared with OVX and sham control rats (Fig. 4B).

Co-localization of GFAP and PSA-NCAM expression in OVX, steroid-primed OVX and sham control rats

In the ME-ARC region, double immunofluorescence staining in EBP-OVX rats during the estrous cycle showed that GFAP protein expression was restricted only to the internal zone of the ME-ARC but, in the case of OVX and sham control rats, GFAP expression was observed in both the external and internal zone of ME-ARC. PSA-NCAM immunoreactivity was identified along the internal and external zone of the ME (with more pronounced expression in the external zone) in EBP-OVX rats whereas PSA-NCAM-ir was much lower in OVX and sham control rats. The results of GFAP and PSA-NCAM immunostaining are presented in Fig. 3G for EBP-OVX and Fig. 3H for OVX and Fig. 3I for sham control rats. Results of co-localization analysis shown in Fig. 3J, K and L for OVX-EBP, OVX and sham control rats respectively further illustrate that GFAP and PSA-NCAM were being expressed in the same cell. Using quantitative immunofluorescence analysis of staining intensity measurements,

we showed a statistically insignificant increase in the dual PSA-NCAM and GFAP staining in the ME-ARC region in the OVX and sham control rats as compared with the EBP-OVX rats. Similarly, an analysis of single staining for PSA-NCAM showed a significantly higher expression in EBP-OVX as compared with OVX and sham control rats, whereas GFAP showed that expression was higher in the OVX and sham controls as compared with EBP-OVX rats. Results are presented in Fig. 4C. Comparison of co-localization of dual GFAP and PSA-NCAM in the internal zone of the ME-ARC region showed an insignificant change in percent co-localization in the OVX and EBP-OVX rats (Fig. 4D).

GnRH and GFAP expression in OVX, steroid-primed OVX and sham control rats

Immunofluorescence data indicated that GnRH expression significantly increased in the ME-ARC region of the hypothalamus in the EBP-OVX as compared with the OVX and sham control rats. GnRH axons were co-distributed with the glial elements in the internal zone of the ME in the EBP-OVX rats, whereas GFAP staining was observed in both internal and external zones of the ME-ARC in OVX and sham control rats, thus indicating reduced glial apposition with GnRH in the parenchymatous space to facilitate GnRH release in EBP-OVX rats. The results of GnRH and GFAP immunostaining are presented in Fig. 3 M for EBP-OVX, Fig. 3N for OVX and Fig. 3O for sham control rats. Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a significant increase in GFAP immunoreactivity in the internal as well as the external zone in the ME-ARC region in OVX and sham control rats, whereas GFAP immunoreactivity was restricted to the internal zone of EBP-OVX rats. Results are presented in Fig. 4E. Similarly, an analysis of single staining for

GnRH showed higher expression in EBP-OVX as compared with OVX rats but the reverse was observed for GFAP expression.

Discussion

In the present investigation we have demonstrated that the GnRH neuron terminals in the external zone of the ME undergo dramatic conformational changes resulting in the physical contact between GnRH nerve endings and parenchymatous basal lamina, so as to facilitate the entry of GnRH into the portal circulation during proestrus. Several previous studies using quantitative image analysis have shown dynamic transformation of individual GnRH terminals in the ME-ARC region of the hypothalamus as a function of gonadectomy as well as during different phases of the estrous cycle in rats (Prevot *et al.* 2000). The synaptic restructuring of the ME-ARC region during the proestrous phase of the estrous cycle indicates that the adult pattern of synaptic connections could be altered in the rat hypothalamus in relation to endocrine events, as is also evident from GnRH axon sprouting shown in Fig. 1A. Since neuroendocrine GnRH neurons are diffusely distributed throughout the hypothalamus, the ME-ARC region provides a common point of convergence for the control of GnRH neuronal activity and hence plasticity.

The present results have further shown that the GnRH neuron terminal-containing area, i.e. the ME-ARC region in the rat hypothalamus, retains the capacity to express PSA-NCAM in the adult brain, and also that PSA-NCAM expression shows cyclic changes in the estrous cycle of the rat. PSA-NCAM expression in the ME-ARC region during the proestrous phase may be responsible for the structural reorganization of the GnRH neuron terminals in the proestrous phase. Other compelling evidence has been reported in our earlier study

Figure 3 Photomicrographs of 20µm thick coronal section through the ME-ARC region of the thypothalamus; double immunostaining of the section for GnRH and PSA-NCAM is shown for (A) OVX-EBP, (B) OVX and (C) sham control rats. The intense PSA-NCAM and GnRH immunostaining was located along the external zones of the lateral portion of the ME in sections from OVX-EBP as compared with OVX and sham control rats. Note that the area where PSA-NCAM and GnRH immunoreactivities overlap appears as heavy (yellow) staining as shown in (A) and (B). Both immunoreactivities appear co-localized in the ventral arcuate nucleus and in the internal and external zone of the ME. Co-localization of GnRH and PSA-NCAM expression was analyzed using the co-localization function of Image Pro-Plus software and results of co-localization are shown in (D) OVX-EBP, (E) OVX and (F) sham control. Photomicrographs of 20µm thick coronal section through the ME-ARC region of the hypothalamus; double immunostaining of the same section for GFAP and PSA-NCAM is shown for (G) OVX-EBP, (H) OVX and (I) sham control rats. The intense PSA-NCAM immunostaining was localized along the external zones of the ME in sections from OVX-EBP rats (A) whereas PSA-NCAM-ir was much lower in OVX sham control rats. The expression of GFAP immunostaining was localized along the external and internal zone of the ME in sections from (H) OVX rats and (I) sham control but in OVX-EBP rats GFAP expression was restricted to the internal zone of the ME as shown in (G). Note that the area where PSA-NCAM and GFAP immunoreactivities overlap appears yellow as shown in (G), (H) and (I). Co-localization of GFAP and PSA-NCAM expression was analyzed using the co-localization function of Image Pro-Plus software and results of co-localization are shown in (J) OVX-EBP, (K) OVX and (L) sham control. Photomicrographs of 20µm thick coronal section through the median eminence arcuate region; double immunostaining of the section for GnRH and GFAP is shown for (M) OVX-EBP, (N) OVX and (O) sham control rats. Both immunostainings appeared co-distributed in the same area of ventral arcuate nucleus and of the internal and external zones of the ME. The expression of GFAP was observed both in the external and internal zone of ME in OVX and sham control rats but its expression was restricted to the internal zone of ME in the OVX-EBP rats. The expression of GnRH increased in OVX-EBP as compared with OVX and sham control rats. 3v, third ventricle. Magnification × 50. This figure appears in colour at <http://joe.endocrinology-journals.org/content/vol186/issue2/>.

Figure 4 Intensity measurement data of the PSA-NCAM immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of OVX-EBP (dotted bars) and OVX (lined bars); (A) depicts the staining intensity of GnRH and PSA-NCAM in OVX-EBP and OVX rats. GnRH+PSA depicts the intensity data of dual-stained sections, PSA depicts PSA-NCAM immunoreactivity and GnRH depicts the GnRH immunoreactivity in OVX-EBP and OVX rats. (B) Quantitative data of percent change in co-localization of GnRH and PSA-NCAM in OVX-EBP, OVX and sham control rats was analyzed from Fig. 3D, F and G using the co-localization function of Image Pro-Plus software. Intensity measurement data of the PSA-NCAM immunoreactivity associated with the glial cell in the ME-ARC region of OVX-EBP, OVX and sham control rats; (C) depicts the staining intensity of GFAP and PSA-NCAM in OVX-EBP and OVX rats. GFAP+PSA-NCAM depicts intensity data of dual-stained sections, PSA depicts PSA-NCAM immunoreactivity and GFAP depicts the GFAP immunoreactivity in OVX-EBP, OVX and sham control rats. (D) Quantitative data of percent change in co-localization of GFAP and PSA-NCAM in OVX-EBP, OVX and sham control rats was analyzed from Fig. 3J, K and L using the co-localization function of Image Pro-Plus software. Intensity measurement data of the GFAP immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of OVX-EBP and OVX rats; (E) depicts the staining intensity of GnRH and GFAP in OVX-EBP and OVX and sham control rats. GnRH+GFAP depicts intensity data of dual-stained sections, GFAP depicts GFAP co-distribution in the IZ and the EZ and GnRH depicts the GnRH immunoreactivity in OVX-EBP and OVX rats. For each type of data analysis in (A), (C) and (E), measurements were performed on five to eight sections each from four to five OVX-EBP, OVX and sham control rats. All statistical analyses were performed using Student's *t*-test. Values are means $\pm$ S.E.M.; a, a' = $P < 0.05$, b = $P < 0.02$, c, c' = $P < 0.01$, d, d' = $P < 0.001$. IZ, internal and EZ, external zones of the ME.

(Kaur *et al.* 2002) where we have shown that Western analysis of membrane extract from the ME-ARC region from proestrous phase rats revealed a PSA-immunoreactive band in the 150–250 kDa range. The

present immunohistofluorescent results have further indicated that PSA-NCAM expression within the hypothalamus is confined to the ME-ARC region. Viguie *et al.* (2001) reported that the amount of PSA-NCAM

associated with GnRH neurons also increased in the breeding season of ewes. The preovulatory surge of LH is initiated by an abrupt increase in GnRH release (Levine 1997, Prevot *et al.* 2000). These observations are also supported by previous studies which have demonstrated the existence of dynamic plasticity changes in the external zone of the ME, which allows physical contacts between GnRH nerve endings and the perivascular space on the day of proestrus to facilitate the release of this neurohormone into pituitary portal blood (Alonso *et al.* 1997, Prevot *et al.* 1999, Prevot *et al.* 2000).

PSA-NCAM continues to be expressed in areas of the adult CNS, which shows the capacity for morphological and physiological plasticity (Seki & Arai 1993). The ME-ARC region having GnRH neuron terminals seems to be a potential area of adult expression of PSA-NCAM. The molecular mechanism by which PSA intervenes to bring about dynamic changes in the GnRH neuron terminal remains unknown. In the GnRH neurosecretory system, steroid hormones seem to be an inductive factor to enhance PSA-NCAM expression and GnRH neuron terminals, as is evident from enhanced expression of PSA-NCAM in EBP-OVX rats as compared with OVX animals. We also observed greater expression of PSA-NCAM associated with GnRH terminals in the proestrous phase than during the diestrous phase. These observations clearly suggested that the expression of PSA-NCAM in the hypothalamic regions that regulate reproduction is more prevalent under a stimulatory period preceding LH surge release in the afternoon of proestrus or in EBP-OVX rats. Moreover, PSA-NCAM expression during the proestrous phase and in EBP-OVX rats was more pronounced in the external zone of the ME, i.e. towards the perivascular space (Figs 1A and 3A), which further indicates the permissive role of PSA-NCAM in GnRH release. Viguie *et al.* (2001) reported that, besides influencing synaptic rearrangements, PSA-NCAM might also promote rearrangement of cells in close vicinity to the GnRH neurons and their terminals. PSA-NCAM may serve to regulate the extracellular environment through the molecular interactions, which may facilitate or inhibit neuronal-glial remodeling.

Prevot *et al.* (1999) have reported that there is an increased contact of GnRH nerve terminals with the perivascular space in the ME region during proestrus, but it was not clear whether this increased contact was due to GnRH neurite extension or withdrawal of glial ensheathment. Another report (Garcia-Segura *et al.* 1988) has shown that estrogen induces a transient disconnection of axo-somatic inhibitory synapses in the arcuate nucleus during the estrous cycle. In the arcuate nucleus of female rats, changing circulating estrogen levels have been shown to reversibly reduce the number of axo-somatic GABA synapses, along with changing ensheathment of neuronal somata by astrocytes (Hoyk *et al.* 2001). These previous reports and our present results strongly suggest that glial

cells provide an important tool for modulating the number of inputs onto GnRH neurons. Recent reports suggest that astrocytes have important neuroregulatory function in addition to their classic function of support and regeneration of neurons (Dhandapani *et al.* 2003). In addition to releasing neuroactive factors, astrocytes may also be capable of controlling GnRH neuronal function via regulation of hypothalamic synaptic plasticity. Glial ensheathment or extension and withdrawal of astrocytic processes may serve to modulate the synaptic contacts of GnRH neurons and their axon sprouting. The present result of dual staining of GnRH and GFAP in the ME-ARC region from cycling rats revealed reduced glial ensheathment of GnRH neuron terminals in the external zone of the ME region, whereas in diestrous phase animals GFAP staining was seen in both the external and internal zone of the ME (Fig. 1J). These observations suggested that withdrawal of glial ensheathment of GnRH neuron terminals in the external zone of the ME in the proestrous phase may facilitate the GnRH release process.

We have further shown that ovariectomy led to increased apposition of glial processes to the axon terminals of GnRH neurons in the ME-ARC region and a corresponding decrease of ensheathment in EBP-OVX rats. These observations suggested that alterations in the circulating gonadal steroid levels influence the morphology and function of glial cells. In a recent article, Galbiati *et al.* (2003) have reviewed the mechanism through which steroid hormone may influence the GnRH neurosecretory system. They have hypothesized that the two different types of signals regulating GnRH-secreting neurons, i.e. steroid hormones and growth factors such as transforming growth factor (TGF)- β , TGF α and bFGF, might be integrated at the level of hypothalamic astrocytes. Several reports have indicated that glial cells influence the activity of hypothalamic GnRH-secreting neurons via the release of growth factors (Ma *et al.* 1994, Cavarretta *et al.* 1999, Galbiati *et al.* 2001). Estrogen has been shown to increase both the mRNA and protein levels of bFGF in hypothalamic astrocytes, and also the mRNA level of bFGF in the hypothalamus of OVX rats was elevated by estrogen treatment (Melcangi *et al.* 2001, 2002, Galbiati *et al.* 2002).

Additional evidence supporting a role for glia in modulating GnRH function comes from our present studies demonstrating the co-expression of GnRH with GFAP and of GFAP with PSA-NCAM, which varies during the estrous cycle. PSA-NCAM was expressed in both glia and neurons. Removal of PSA from the NCAM molecule *in vivo* in rats by using endo-N enzyme has been shown to block both the glial retraction and the increase in synaptic inputs on the magnocellular neuroendocrine neurons during lactation and dehydration (Theodosis *et al.* 1999, Hoyk *et al.* 2001). An important indirect functional consequence of a modified glial coverage seems to permit the GnRH neuron terminals to undergo rearrangement.

These neuronal and glial conformational changes in the ME-ARC region are reversible and occur in a cyclic fashion and they are accompanied by changing expression of PSA-NCAM expression on both GnRH neuron terminals and glial cells.

The present data clearly showed that the morphological changes observed in the GnRH neuron terminal in the ME-ARC region along with changes in their glial apposition certainly has physiological consequences. These alterations are a part of the GnRH neurosecretory mechanism, which is further supported by the fact that the ME-ARC region was strongly positive for PSA-NCAM in the proestrous phase as well as in OVX-EBP rats. PSA-NCAM expression was downregulated in the diestrous phase and in the OVX rats. These observations suggested that the neuronal-glial cell membrane interaction through a permissive factor, i.e. PSA-NCAM, is actively involved in the structural synaptic remodeling in adult animals. These processes are influenced by changes in gonadal steroid levels. The present results suggested a mechanism whereby large quantities of PSA on extracellular surfaces would attenuate adhesion via physical impedance or charge repulsion, thus allowing dynamic structural modifications in GnRH neurons. In several recent reports, PSA-NCAM has been considered a necessary permissive substrate which facilitates morphological neuronal and glial plasticity (Viguie *et al.* 2001, Jansen *et al.* 2003, Theodosis *et al.* 2004), allowing hypothalamic neurons and their synapses to undergo structural changes whenever the proper stimulus intervenes. Besides acting as a permissive molecular factor, PSA-NCAM, by permitting or inhibiting neuronal glial remodeling, may also be affecting the presence of astrocytic processes in the external zone of the ME. In conclusion, the GnRH neurosecretory system, by its physiological capacity to express PSA-NCAM, can undergo dynamic transformations of its axon terminals and their glial ensheathment. The GnRH system can serve as useful model to illustrate the molecular mechanisms of neuronal plasticity and its functional consequences in the adult brain.

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