Steroid Hormones Regulate Post-Translational Modification of Neural Cell Adhesion Molecule: Implication For The Neuroendocrine Control of GnRH

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Summary

In rodents, the cell bodies of gonadotrophin-releasing hormone (GnRH) neurones are diffusely distributed in the preoptic region of the hypothalamus. The aim of this study was to examine the effect of estradiol on the neuroanatomical relationship between GnRH neurons and polysialylated form of neural cell adhesion molecule (PSA-NCAM) a known marker of neuronal plasticity. In the present study, we have demonstrated changes in the GnRH cell bodies and their terminals in estradiol and progesterone treated ovariectomised rats and their correlation with expression of some neuronal plasticity markers such as neural cell adhesion molecule (NCAM), and PSA-NCAM). By immunohistofluorescent dual labeling, we have also determined that GnRH cell bodies in the medial preoptic area (mPOA) as well as their axon terminals in external zone of ME region of hypothalamus are intimately associated with PSA-NCAM in the proestrous phase of estrous cycle. To further examine whether PSA-NCAM expression associated with GnRH neuron terminals varies in conjugation with cyclic changes in the ovarian-steroid hormone level, we examined GnRH and PSA-NCAM dual expression in ovariectomized (OVX) and estrogen-progesterone primed OVX (EBP-OVX) rats. Dynamic changes were observed in astrocytes around GnRH perikarya in the mPOA as well as ME regions of the hypothalamus and also increase in the expression of NCAM and PSA-NCAM was observed after EBP induced gonadotropin surge in ovariectomised rat model. These results support that steroid hormones regulates formation and extrusion of hydrophilic PSA-NCAM into POA as well as ME as a part of the mechanism of steroids induced synaptic plasticity.

Key words: GnRH, Neuroplasticity, Ovariectomy, Estrogen, Astrocytes, PSA-NCAM and GFAP

Anahtar Kelimeler: GnRH; Nöroplastisite; Ovarektomi; Östrojen; Astrosit; PSA-NCAM; GFAP

INTRODUCTION
Gonadotrophin-releasing hormone (GnRH) is the neurohormone controlling sexual maturation and adult reproductive function\(^\textsuperscript{29,35,48}\). In rodents, the cell bodies of GnRH neurones are diffusely distributed in the preoptic region which sends their axons to the median eminence region of the hypothalamus to release their neurohormone into the pituitary portal vasculature. On reaching the anterior, pituitary GnRH elicits the secretion of the gonadotrophins luteinising hormone (LH) and follicle-stimulating hormone (FSH) that stimulate gametogenesis and gonadal steroids secretion, and thus support reproductive physiology. Because GnRH neurones are the final common pathway for the central control of reproduction, their activity is regulated by a complex array of excitatory and inhibitory transsynaptic inputs\(^\textsuperscript{47,31}\). Noticeably, both GnRH neurones and the multiple neuronal networks involved in the control of GnRH secretion can be subjected to the direct modulatory influence of gonadal steroids\(^\textsuperscript{15}\).

In females, the preovulatory surge of LH is initiated by an abrupt increase of GnRH release, which is preceded by GnRH axon terminals contact with the perivascular space directly\(^\textsuperscript{3,22,31,32,33,37}\). Recently, it has 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\(^\textsuperscript{30,51}\). The reproductive cycle in rat reflects a functional remodeling within the GnRH neuron in hypothalamus. Specifically, changes in total synaptic input and association with PSA-NCAM have been observed in our previous studies\(^\textsuperscript{19,32,33}\).

Neural cell adhesion molecule (NCAM) is one of the cell surface macromolecules involved in synaptogenesis, regeneration and cell-cell adhesion. The addition of an unusual linear homopolymer, \{alpha\}-2,8-linked polysialic acid (PSA) moiety to the fifth immunoglobulin-like domain of the NCAM facilitates events such as cell migration, neurite growth, and synaptic plasticity\(^\textsuperscript{1,8,27,28,40,42,44,46}\). PSA appears to participate in the estradiol-induced shape changes because treatment with endoneuraminidase, an enzyme that specifically removes PSA from the cell surface, abolished PSA immunostaining and prevented the estradiol-induced morphological changes of astroglia.

Estrogen is one of the principal determinants of GnRH neuron functioning and acting as a classic homeostatic feedback molecule between gonad and brain, is critical in enabling GnRH neurons to exhibit pulsatile pattern of biosynthetic and secretory activity\(^\textsuperscript{15,16}\). Estradiol promotion of changes in the morphology of astroglia growing in culture depends on the expression of polysialic acid of neural membranes\(^\textsuperscript{11}\). The extrusion of PSA into the intercellular space between axons and glial processes that undergo significant remodeling offers strong correlative evidence that is important to their capacity for glial plasticity. Moreover, enzymatic...
removal of PSA from its complex with NCAM furnished direct evidence for this\textsuperscript{(25)}. Observations in the hypothalamus also demonstrated that PSA is necessary for neuronal and glial plasticity\textsuperscript{(17,50)}. The present study was designed to determine the temporal changes in the markers of synaptic plasticity, viz., high molecular weight isoforms of NCAM and PSA-NCAM in POA and ME-ARC region during estrogen+progesterone induced GnRH surge. Our results show enhanced expression of PSA-NCAM and NCAM-180 and 140 isoforms in POA under the same set of experimental conditions. These observations suggest that these molecular markers of neuronal plasticity promote the outgrowth of GnRH axon terminals to facilitate the release of GnRH in the pituitary portal blood during EBP-induced GnRH surge in OVX rats.

Despite extensive evidence for neuroplasticity within the adult GnRH system, the underlying molecular basis remains unknown. Of interest, NCAM and PSA-NCAM, which promotes plasticity by modifying the stability of cell-cell contacts, are expressed along the prenatal migratory path of GnRH neurons. Both NCAM and PSA-NCAM continue to be expressed in the adult brain and, of particular interest to the present study, are found in regions that contain GnRH perikarya, fibers and terminals. All of these findings are consistent with a role for NCAM and PSA-NCAM in remodeling of GnRH system. The aim of this study was to determine whether PSA-NCAM expression associated with GnRH neurons and their surrounding glial cell levels varies in conjunction with steroid hormone and changes in the activity of the GnRH neurosecretory system in OVX and steroid primed OVX rats using dual immunofluorescence staining and western blotting. Nevertheless, by offering a rare glimpse of the communication between astroglia and neurons, the use of the mPOA as well as external zone of ME region of the hypothalamus as a model system should shed new light on the role of astro-glial cells in the GnRH regulation.

**MATERIAL AND METHODS**

Wistar strain female albino rats in the age group of 3-5 months and weighing 200-250 gm were procured from National Institute of Nutrition (NIN) Hyderabad. Animals were housed three per cage in a temperature humidity controlled environment on a 12-h light –12-h dark cycle with free access to food and water. The estrous cycle was monitored by daily inspection of vaginal cytology. After at least 3-4 complete four-day cycles, the animals were killed between 15.00 and 16.00 h on diestrous (n = 12) and proestrous (n =12) days. Another group of rats were ovariectomized after surgically removing both the ovaries under aseptic conditions. Experiments were carried out 4-6 weeks after ovariectomy. Rats were given 7.5mg estradiol benzoate (EB) subcutaneously in corn oil at 10.00 h. 5 mg of progesterone (P) was administrated subcutaneously in corn oil, 48h after the EB treatment. All studies were performed during 13.30-14.30h on Progesterone treatment day when the steroid induced LH surge release takes place (Berglund et al. 1988) Animal care and procedures were followed in accordance with the guidelines of Institutional Animal Ethical Committee.

**Procurement of antibodies for Immunofluorescence**

The primary antibodies used for the Immunohisto-fluorescence analysis were monoclonal anti-GFAP (Clone CA-5, Sigma) and monoclonal antibody specific for PSA-NCAM was a generous gift from Dr. Tatsunori Seki (Juntendo University School of Medicine, Japan). For immunohisto-fluorescent localization of GnRH, the LR1, an anti-GnRH antibody (a generous gift from G. Tramu, Bordeaux France) was used. The secondary antibodies used were Texas Red conjugated (TRITC) goat anti-rabbit IgG at the dilution of 1:200 for GnRH, fluorescein isothiocyanate conjugated (FITC) goat antimouse IgG at the dilution of 1:200 for
GFAP and for PSA Goat antimouse (FITC) IgM μ-chain specific at a dilution of 1:128 from Sigma, USA.

**Immunofluorescent**
For immunofluorescent staining of GnRH+PSA-NCAM and GnRH+GFAP in the mPOA and ME region of hypothalamus, brains were perfused transcardially with 4% Paraformaldehyde in PB (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 hours at 4°C. 20μm thick coronal sections were cut using cryostat microtome and sections were mounted on gelatin coated glass slides and treated in the following manner: three 15 min washes in 0.1M PBS, pH 7.4; 30 min in 0.3% TritonX-100 in 0.1M PBS for Permeabilization. Then sections were washed with 0.1% PBST for 15 min. After washing sections were preincubated for 1h at room temperature in a blocking solution (5% NGS in PBS with 0.3% TritonX-100 and 0.01% (sodium azide). The sections were incubated with mixture of primary antibodies GnRH+PSA-NCAM, GnRH+GFAP or GFAP+PSA-NCAM with appropriate dilution of anti-GnRH, (1:10,000), anti-PSA-NCAM, (1:500), anti-GFAP (1:500) in 0.1% Triton X-100 and 1% BSA-PBS for 60h at 4°C. The specificity of PSA-NCAM staining was checked by alternatively omitting one or another primary antibodies in the presence of both secondary antibodies. In addition, the specificity of the PSA-NCAM was verified by pre-treating a set of sections with the endoneuroaminidase enzyme, endo-N (provided by Dr. Urs Rutishauser), which specifically cleaves sialic acid polymers in chains of eight or longer (43). The sections were then washed for 15min 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 dilution of 1:200) and anti mouse IgG for GFAP (FITC with dilution of 1:200) and anti mouse IgM (FITC with dilution of 1:128) for PSA-NCAM in 0.32% Triton-PBS for 2 hours. After that sections were washed with .01% PBST for 15 Minutes. Tissue sections were then cover slipped using the appropriate anti-fading mounting medium (500mg propyl-gallate 20ml ethanol/PBS (pH 7.4) (1:1) and 90ml glycerol) for fluorescence detection.

**Western blotting**

**Protein sample preparation and quantification:**
The brains were sliced into 200-μm thick slices using cryostat microtome. Relevant brain regions (olfactory cortex (OC), POA, ME-ARC and dentate gyrus (DG)) were micropunched (Fig. 1A and B). For each EBP-primed OVX and OVX group (n=48), micropunched tissue from the brains of six rats were pooled as one sample to yield sufficient protein content for analysis by Western blot in both the groups. Ten percent homogenates were prepared in 20 mM HEPES; pH 6.8 (Sigma) buffer containing 0.4 mM EDTA (Sigma), 0.5 mM DTT (Amersham), 100 μM Na3Vo4 (Sigma), 1μg/ml Pepstatin A (Amersham), 10 μg/ml Aprotinin (Amersham) and 0.2% IGEPAL- 630 (Sigma) using a potter elvehjam type of homogenizer fitted with a teflon plunger. The homogenate was spun for 30 min.at 10,000 x g. The Supernatant was collected and its protein content was estimated using Bradford method. Each homogenate was then diluted in homogenization buffer so as to give a final concentration of 2mg protein/ml (2 μg/μl). The samples were mixed 1:1 with sample buffer (0.25 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 1 mg bromophenolblue) and stored at -20°C.

**Immunoblotting**
An aliquot of each samples (25-μl for PSA-NCAM, 15-μl for NCAM were fractionated electrophoretically on SDS/6.5% polyacrylamide gel for NCAM and PSA-NCAM and then transfer was done at constant current of 1.1 mA/cm2 for 6 hrs. The nonspecific binding sites were blocked with 5% (wt/vol) nonfat milk in
Tris-buffered saline (TBS, pH 7.4) containing 0.2% Tween 20 (Sigma), and the membranes were incubated at 4°C overnight with the primary antibody (anti-PSA-NCAM; 1:500, anti-NCAM; 1:500, anti-GAP-43; 1:1000 and anti-GFAP; 1:1000 diluted in TBS/Tween). The blots were washed in TBS/Tween and incubated for 2h at room temperature with anti-mouse peroxidase conjugate and antimouse IgM peroxidase conjugate (in PSA-NCAM blotting) for 2h at room temperature. Membranes were again washed in TBS-T buffer 3x15 min. The blots were developed using ECL Plus western blot detection system (Amersham Biosciences) and exposed to Hyperfilm ECL. The films were then developed and the antibody-labeling intensity (relative optical density) was analyzed using Gel documentation system (AlpaEase™, Alpha Innotch Corporation). Values are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used to compare the levels of expression of different markers in OVX and EBP-primed OVX groups of rats. When ANOVA detected a difference, these sets of OVX and EBP-primed OVX rats were compared using student's t-test to determine the statistical significance, which was assumed to be different when comparison showed a significance level of P<0.05.

**Statistical Analysis:**
The values are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used to compare the results in different groups of rats. When ANOVA detected a difference, these sets of proestrous, diestrous as well as EBP-OVX and OVX rats were subjected to post hoc comparison using the Bonferroni’s test (Sigma-Star 2.03) for pair-wise multiple comparisons to determine the statistical significance, which was assumed to be different when comparison showed a significance level of p<0.05.

**RESULTS**
All data reported in the present study are restricted to adult female rat forebrain mPOA and ME region of hypothalamus. Using double immunostaining microscopy, co-expression of PSA-NCAM with GnRH and also GFAP with GnRH were examined in diestrous and proestrous phase of cycling rats as well as in OVX and EBP-OVX rats.

**Co-localization of GnRH+PSA-NCAM expression in proestrous, diestrous as well as EBP-OVX and OVX rats:** GnRH cell bodies in mPOA were seen to distinctly express PSA-NCAM on the cell surface from proestrous phase (Fig. 1A) as compared to diestrous phase (Fig. 1B) rats as well as from EBP-OVX (Fig. 1C) and OVX (Fig. 1D) rats. At higher magnification, GnRH cell bodies in mPOA were seen to distinctly express PSA-NCAM on the cell surface as shown from proestrous phase (Fig. 1E) and EBP-OVX (Fig. 1G) as compared to diestrous phase (Fig. 1F) and OVX (Fig. 1H) rats. PSA-NCAM immunoreactivity was associated primarily with the periphery of the GnRH cell soma. PSA-NCAM immunoreactivity was associated primarily with the periphery of the GnRH cell soma and axon terminals. This pattern of PSA-NCAM labeling represented immunoreactivity associated with non-GnRH neurons or glial
cells. The results of single staining of GnRH cell bodies are shown in Fig. 1I (Proestrous) and 1J (diestrous) phase rats as well as Fig. 1K (EBP-OVX) and L (OVX) rats. The results of co-localization are shown in Fig. 1M and N for proestrous and diestrous rats and Fig. 1O and P for EBP-OVX and OVX rat respectively. In the present study, we observed dynamic temporal and spatial upregulation of PSA-NCAM expression on GnRH cell bodies in proestrous phase and EBP-OVX rats as compared to diestrous phase and OVX rats.

In the ME region from proestrous phase as well as 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 staining for GnRH and PSA-NCAM in this phase revealed that heavy PSA-NCAM expression was colocalized with GnRH immunoreactivity as is evident from yellow colour staining appearing in co-localized area for proestrous and EBP-OVX rats. PSA-NCAM positive structures that appeared as rings of immunoreactivity but did not exhibit GnRH immunoreactivity were seen in proximity to some GnRH neurons. On the other hand, GnRH and PSA-NCAM expression was much lower in diestrous phase (Fig. 2B) and OVX (Fig. 2D) rats. The results of single staining of PSA-NCAM and GnRH immunoreactivities are shown in Fig. 2E, I (Proestrous phase) and 2F and J (diestrous phase) rats. The results of single staining of PSA-NCAM and GnRH immunoreactivities are shown in Fig. 2G, K (EBP-OVX) and 2H and L (OVX) rats. Results of co-localization analysis shown in Fig. 1G and H further illustrate that GnRH and PSA-NCAM are being expressed together.

By quantitative immunofluorescence analysis of staining intensity measurements, we showed statistically significant higher expression (P<0.001) of PSA-NCAM and GnRH dual staining in the ME-ARC region from the proestrous and EBP-OVX rats as compared to the diestrous and OVX rats respectively (Fig. 2Q). Similarly an analysis of single staining for GnRH and PSA-NCAM showed significantly higher expression in proestrous and EBP-OVX as compared to diestrous and OVX rats respectively (Fig. 2Q).

**GnRH and GFAP expression in proestrous, diestrous as well as EBP-OVX and OVX rats:**

Using double immunostaining, GnRH neurons and GFAP-immunoreactive astrocytes in the mPOA of the hypothalamus were examined for possible close appositions. Astro-glial cells directly apposed to GnRH cell bodies in the mPOA were seen to undergo changes in surface contact area in proestrous phase and EBP-OVX rats. Our data clearly showed that astro-glial cell surface area in contact with GnRH cell bodies was decreased significantly in the afternoon of the proestrous and EBP-OVX rats (Fig. 3A and C) but in the diestrous phase (Fig. 3B) and OVX (Fig. 3D) rats, both the number as well as the contact area of astro-glial cells with GnRH cell bodies was higher and GnRH cell bodies were enwrapped in GFAP-ir processes emanating from cells that exhibit small and round nuclei. The morphometric changes seen in astro-glial cells in the mPOA of young adult female rats were unique to astrocytes apposed to GnRH neurons. Astrocytes that were farther away from GnRH cell bodies but still in the mPOA did not undergo changes in morphology. The results of single staining of GFAP and GnRH cell bodies are shown in Fig. 3E, I (Proestrous phase) and Fig. 3F, J (diestrous phase) rats. In second group results of single staining of GFAP and GnRH cell bodies are shown in Fig. 3G, K (EBP-OVX) and Fig. 3H, L (OVX) rats.
In the ME region of hypothalamus in the proestrous phase GnRH axons were co-distributed with the glial elements in the internal zone of ME in the proestrous phase and EBP-OVX rats but in the external zone there were very less number of processes of glial cells, whereas, GFAP staining was observed both in internal and external zones of ME in diestrous phase and OVX rats, thus indicating reduced glial apposition with GnRH axon terminals in the parenchymatous space to facilitate GnRH release in proestrous phase and EBP-OVX rats. The result of GnRH and GFAP dual immunostaining are presented for proestrous phase (Fig. 4A) and diestrous phase (Fig. 4B) rats and as well as for EBP-OVX (Fig. 4C) and OVX (Fig. 4D) rats. The results of single staining of GFAP and GnRH cell bodies are shown for Proestrous phase (Fig. 4C, E) and diestrous phase (Fig. 4D, F) rats. In second group results of single staining of GFAP and GnRH cell bodies are shown in Fig. 3G, K (EBP-OVX) and Fig. 3H, L (OVX) rats. The results of single staining for GnRH showed higher expression in proestrous and EBP-OVX rats as compared to diestrous phase and OVX rats but reverse was observed for GFAP expression.

**Fig 1:** High power immunofluorescent images of 40µm thick coronal section through the medial preoptic area (mPOA) of hypothalamus. PSA-NCAM (green) and GnRH (red) immunoreactivity within mPOA of proestrous (A), diestrous (B) as well as EBP-OVX (C) and OVX (D) rats. Yellow colour indicates the area of co-localization of PSA-NCAM and GnRH immunoreactivities. PSA-NCAM-ir is observed around the periphery of most GnRH cell bodies. PSA-NCAM immunostaining was more in proestrous phase and EBP-OVX rats shown in (E) and (G) as compared to diestrous (F) and OVX (H) rats. Single GnRH-ir in proestrous phase and EBP-OVX rats shown in (I) and (K) as compared to diestrous (J) and OVX (L) rats. The results of co-localization are shown in for proestrous (M) and EBP primed OVX (O) rats and for diestrous phase (N) and OVX (P) rats. At higher magnification these GnRH single cell often displayed a dotted pattern of PSA-NCAM expression as shown by arrows for proestrous phase (E) and EBP-OVX (G) rats. Scale 100X (A-P) Scale bars = 5 µm
Fig 2: Low power immunofluorescent images of 20µm thick coronal section through the median eminence arcuate region of hypothalamus. Double immunostaining of the section for GnRH and PSA-NCAM is shown for proestrous (A), diestrous (B) as well as EBP-OVX (C) and OVX (D) rats. Yellow colour indicates the area of co-localization of PSA-NCAM and GnRH immunoreactivities. The intense PSA-NCAM and GnRH dual immunostaining was located along the external zone of the lateral portion of the ME in section from proestrous and EBP-OVX as compared to diestrous phase and OVX rats. Note that area where PSA-NCAM and GnRH immunoreactivities overlap appears yellow as shown in A and B. The intense PSA-NCAM and GnRH immunostaining was located along the external zone of the lateral portion of the ME in section from OVX-EBP as compared to OVX rats. Both immunoreactivities appear co-localized in the ventral arcuate nucleus and in the internal and external zone of the median eminence. PSA-NCAM immunostaining was more in proestrous phase and EBP-OVX rats shown in (E) and (G) as compared to diestrous (F) and OVX rats. Single GnRH-ir in the external zone of ME in proestrous phase and EBP-OVX rats shown in (I) and (K) as compared to diestrous (J) and OVX (L) rats. Co-localization of GnRH and PSA-NCAM expression was analyzed using co-localization function of Image Pro-Plus software and results of co-localization are shown for proestrous (M) and EBP primed OVX (O) rats and for diestrous phase (N) and OVX (P) rats. Intensity measurement data of the PSA-NCAM immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of proestrous, diestrous as well as EBP-OVX and OVX rats. (Q) GnRH+PSA-NCAM depicts intensity data of dual stained sections, PSA depicts PSA-NCAM immunoreactivity and GnRH depicts the GnRH-ir in proestrous, diestrous as well as EBP-OVX and OVX rats. Statistical analysis was performed using Bonferroni test after ANOVA. Bar values are means ±S.E.M *p<0.05 proestrous versus diestrous rats, #p<0.05 EBP-OVX versus OVX rats, †p<0.05 Proestrous versus OVX rats, ‡p<0.05 EBP-OVX versus diestrous rats. Scale 20X (A-P). Scale bars = 20 µm (A-P). IZ – Internal zone of ME and EZ – External Zone of ME.
Quantitative immunofluorescence analysis of staining intensity measurements, showed statistically significant increase in the GFAP staining in the ME-ARC region in the diestrous and OVX rats as compared to the proestrous and EBP-OVX rats respectively. Results are presented in Fig. 4M. Similarly an analysis of single staining for GnRH showed higher expression in proestrous and EBP-OVX rats as compared to diestrous and OVX rats but reverse was observed for GFAP expression (Fig. 4M).

**Immunoblotting analyses of plasticity markers:NCAM isoforms and PSA-NCAM expression:**

Immunoblot analysis was undertaken to confirm the presence, and to quantify the relative amounts of PSA-NCAM and high molecular weight isoforms of NCAM in OC, POA, ME-ARC and DG regions. Immunoreactive PSA-NCAM from POA and ME-ARC regions appeared as broad band migrating in the 150-280 kDa range, whereas, NCAM appeared as three bands at apparent molecular weights of 180, 140 and 120 kDa as the AF11 antibody is specific for these three isoforms.

The NCAM-180 immunoreactivity from POA and ME-ARC region was significantly enhanced, in EBP-primed OVX rats (54.6±0.88; p<0.01 in POA and 55.4±1.31; p<0.01 in ME-ARC regions) as compared to OVX rats (45.3±0.88; p<0.01 in POA and 44.6±1.31; p<0.01 in ME-ARC regions). Immunoblotting of NCAM-140 revealed marginal but insignificant
increase in the content of this isoform in EBP-primed OVX rats over OVX rats from POA and ME-ARC of hypothalamus. NCAM-120 isoforms did not show any change in all brain regions studied. Further, there was no significant change observed in NCAM-180 and NCAM-140 isoforms level in OC and DG regions from EBP-primed OVX rats as compared to OVX rats. Results are shown in Fig. 5A and B.

Quantitative analysis showed significant increase in the PSA-NCAM content from POA (55.0±1.38; P<0.02) and ME-ARC (56.6±0.88; P<0.01) regions in the EBP-primed OVX rats as compared to the OVX rats (45.0±1.38; p<0.02 in POA and 43.3±0.88; p<0.01 in ME-ARC regions), whereas, the tissue content of PSA-NCAM was below detectable limits in the DG and OC regions (Immunoblot data of DG and OC regions is not shown). Results are shown in Fig. 6A and B.

Fig 4: Photomicrographs of 20µm thick coronal section through the median eminence arcuate region of hypothalamus. Double Immunostaining of the section for GnRH and GFAP is shown for proestrous (A) and diestrous (B) rats as well as EBP-OVX (J) and OVX (D) rats. Both Immunostainings appeared co-distributed in the internal and external zone of the median eminence. The expression of GFAP was observed both in the external and internal zone of ME in diestrous (F) and OVX (H) rats but its expression was restricted to the internal zone of ME in the proestrous (E) and EBP-OVX (G) rats. The expression of GnRH increased in proestrous (I) and EBP-OVX (K) rats as compared to diestrous phase (J) and OVX (L) rats. Intensity measurement data of the GFAP immunoreactivity associated with the GnRH axon terminals in the ME-ARC region of proestrous, diestrous as well as EBP-OVX and OVX rats. (M) depicts the staining intensity of GnRH and GFAP in proestrous, diestrous as well as EBP-OVX and OVX rats. Statistical analysis was performed using Bonferroni test after ANOVA. Bar values are means ±S.E.M *p<0.05 proestrous versus diestrous rats, #p<0.05 EBP-OVX versus diestrous rats, †p<0.05 EBP-OVX versus OVX rats. ‡p<0.05 Proestrous versus OVX rats. §p<0.05 EBP-OVX versus diestrous rats. Scale 20X (A-P). Scale bars = 20 µm (A-P). IZ – Internal zone of ME and EZ – External Zone of ME.
Fig 5: (A) Representative western blot hybridization signals using antibodies specific for NCAM-180, NCAM-140 and NCAM-120 from olfactory cortex (OC), preoptic area (POA), median eminence arcuate (ME-ARC) and dentate gyrus (DG) regions from OVX (○) and EBP-primed OVX (△) rats (B) Mean values of NCAM-180, NCAM-140 and NCAM-120 levels for each of the four regions from OVX (n=4) and EBP-primed OVX rats (n=4) expressed as the percentage change. Statistical analysis performed using Student’s ‘t’ test. c = p<0.01.
DISCUSSION

In the present study, we demonstrated by immunohistochemistry and Western blotting that the greatest amount of hypothalamic PSA-NCAM is present in the POA as well as in the ME regions on the afternoon of proestrus; this is the time of maximum estrogen release and the estrogen-induced gonad-otropin surge is beginning. Proestrus is also the time of the most prominent perineural distribution of ir-PSA-NCAM and maximal astroglial remodeling. 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 estrous cycle in rats\(^{(35,36,49)}\). The synaptic restructuring of the ME 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 and C. Since neuroendocrine GnRH neurons are diffusely distributed throughout hypothalamus, the ME region provides a common point of convergence for the control of GnRH neuronal activity and hence plasticity.

The present results indicate that in the adult cycling female rats, PSA-NCAM immunoreactivity is intimately associated with GnRH neuron perikarya in the mPOA. Recent studies from our laboratory suggested a functional link between PSA-
NCAM expression in the ME-ARC region in the modulation of GnRH release during the proestrous phase of estrous cycle in rat (19,32,33). Steroid hormones seem to be the inductive factors to enhance GnRH axon terminals sprouting and PSA-NCAM expression as evident from higher expression of PSA-NCAM and GnRH-ir axon terminals sprouting near perivascular space in EBP primed OVX rats as compared to OVX rats (4,32). Earlier reports have also implicated PSA-NCAM in the remodeling of both the developing (e.g. prenatal GnRH migration) (26,56) and adult neuroendocrine system (50,51). These finding together with the widespread role of PSA-NCAM in promoting neuroplasticity (33,38,39,41) suggest that PSA-NCAM may provide a molecular substrate for structural remodeling of GnRH system and its surrounding astro-glial cells in adult cycling female rats.

Mammalian neurons express three splice variants of NCAM (180, 140, and 120 kDa). Of these, NCAM-140 and NCAM-180 are believed to localize to synapses (34,45). The 180-kDa isoform has also been suggested to play a role in synapse stabilization and rapidly accumulates at sites of contact between axonal growth cones and postsynaptic cells in hippocampal cultures (54). Of the three NCAM isoforms tested, NCAM-140 and -180 were decreased after the proestrous estrogens peak. However, there was no significant change in the NCAM-120 level in the PVA from metestrous and proestrous mice. This might be due to the differential sialylation of NCAM isoforms. However, further studies are needed to confirm these findings.

In GnRH neurosecretary 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 to OVX animals. We also observed greater expression of PSA-NCAM associated with GnRH terminals in the proestrous phase than during diestrous phase. These observations clearly suggest that the expression of PSA-NCAM in hypothalamic regions that regulate reproduction is more prevalent under a stimulatory period preceding LH surge release in the afternoon of proestrous or in EBP-OVX rats. Moreover PSA-NCAM expression during proestrous phase and in EBP-OVX rats was more pronounced in the external zone of ME i.e. towards the perivascular space (Figs 1E and 1G), which further indicates the permissive role of PSA-NCAM in GnRH release. Viguie et al. (53) reported that besides influencing synaptic rearrangements, PSA-NCAM might also promote rearrangement of cells in the close vicinity of 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. Latest report by Tan et al. (49) clearly indicates that increased PSA-NCAM in the paraventricular area (PVA) is associated with increased expression of the sialylation enzymes PST and STX during the proestrous estrogens peak. The utilization of NCAM isoforms by these enzymes explains the manner by which NCAM's restrictive cell-cell binding can be substituted by molecular prestidigitation in which hydrophilic sugars that allow nondestructive astroglial spreading are added. The glial processes disrupt PVA synapses and allow disinhibition of GnRH and the resultant gonadotrophin surge. After the estrogens peak, the astroglia retract, the PVA synapses are reconstituted, and the sialylated NCAM is replaced by undeco-rated NCAM. These findings confirm with recent reports of PSA-NCAM being a necessary permissive substrate, which facilitates neuroglial plasticity.

The present study represents the first detailed morphological characterization of neuro-glial interactions for GnRH neurones in the adult cycling rats and
steroid primed OVX rats. In keeping with previous observations demonstrating the ensheathment of both perikarya and neuroendocrine terminals of GnRH neurones by astroglia processes in rodents and nonhuman primates\(^{(7,12,14,37,55)}\) our findings show that astrocytes and tanyocytes are morphologically associated with GnRH neurones within the human hypothalamus. Previous work has established patterns of neuronal afferents to GnRH neurones\(^{(6)}\) and documented action sites of oestrogen\(^{(5,20,21)}\) within the human hypothalamus. However, we know remarkably little about how astroglia, which are key signalling components with the potential to modulate the way information is generated and disseminated within the brain interact with GnRH neurones in the adult brain hypothalamus. By using antibodies to intermediate filament proteins such as GFAP, we succeeded in visualising the anatomical relationship between GnRH neurones and astrocytes in the mPOA as well as ME region of hypothalamus. Our fluorescent microscopy results indicate that each individual GnRH cell body is surrounded by several astrocytes that wrap themselves around their soma. We have further shown that ovariectomy lead to increased apposition of glial processes to the axon terminals of GnRH neurones in the external zone of ME region and a corresponding decrease of ensheathment in EBP-OVX rats. These observations suggest that alterations in the circulating gonadal steroid levels influence the morphology and function of glial cells. In a recent article, Galbiati et al.\(^{(10)}\) have reviewed the mechanism through which steroid hormone may influence the GnRH neurosecretory system. Estrogen has been shown to increase both the mRNA and protein levels of bFGF in hypothalamic astrocytes and also mRNA level of bFGF in the hypothalamus of OVX rats was elevated by estrogen treatment\(^{(9,23,24)}\). These neuronal and glial conformational changes in the external zone of ME region are reversible and occur in a cyclic fashion, which are accompanied by changing expression of PSA-NCAM expression on both GnRH neuron terminals and glial cells.

Despite intense investigations like, study of morphological changes occurring in the basal lamina\(^{(37)}\) and studies of axosomatic profile of the arcuate region\(^{(27)}\), little effort has been made to characterize the intrinsic signals for growth at GnRH neuron terminals during preovulatory GnRH surge. Prevot et al.\(^{(36)}\) were the first to report the expression of GAP-43 in diagonal band of Brocca (DBB), medial preoptic area and median eminence regions during diestrous and proestrous phase of estrous cycle. The result of the present study of increased expression of PSA-NCAM and high molecular weight isoforms of NCAM (NCAM- 180) in POA and ME-ARC regions of EBP- primed OVX and OVX rats revealed that GnRH perikarya and axon terminals undergo remodeling during steroid induced GnRH surge. The present results are in agreement with the hypothesis that the modulations of the number of GnRH synaptic contacts is dependent on gonadal hormones effects on neuronal membrane components.

In several recent reports, PSA-NCAM has been considered a necessary permissive substrate, which facilitates morphological neuronal and glial plasticity\(^{(2,13,18,38,51,52,53)}\), allowing hypothalamic neurones 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 ME. The decreased available estrogens levels could indirectly affect estrogen-related synaptic changes in areas such as the hypothalamus and the hippocampus, effecting autonomic and cognitive function, respectively. The differential expression of PSA-NCAM in the PVA could also help us learn more
about the pathophysiology of certain clinical conditions such as menopause and hypothalamic causes of secondary amenorrhea.

In conclusion, GnRH neurosecretory system by its physiological capacity to express PSA-NCAM can undergo dynamic transformations of its axon terminals and their glial ensheathment. GnRH system can serve as useful model to illustrate the molecular mechanisms of neuronal plasticity and its functional consequences in the adult brain. Altogether, these results provide the exciting possibility that radial tanyocyte processes constitute glial elements which, in addition to serving as the scaffolding for GnRH neuroendocrine axons, may provide a regulatory role for those neuroendocrine nerve endings in the adult human median eminence. In summary, our findings demonstrate that a close anatomical association exists between PSA-NCAM and GnRH neurons in the adult cycling and steroid primed OVX rats. This holds both for the mPOA and ME-ARC region, where GnRH perikarya, fibers and terminals are juxtaposed to PSA-NCAM immunoreactivity. These findings provide evidence that PSA-NCAM is well positioned anatomically to promote remodeling within the GnRH axis, and they encourage future work to establish the functional significance of this molecule to both the structure and the secretory activity of the GnRH system in adults cycling rats.

Acknowledgements
We thank Dr. Tatsunori Seki (Juntendo University, School of Medicine, Tokyo, Japan) for his generous supply of PSA-NCAM antibody 12E3 and Dr. G. Tramu, (Lab neurocytochimie fonctionnelle, CNRS, UMR, 5807 Bordeaux France) for LR1 anti-GnRH antibody. This work was supported by a grant from Department of Biotechnology, Government of India. One of the author Jyoti Parkash is thankful to DBT, GOI for the research fellowship provided during this research work.

Literature search and computational work help provided by the DBT, GOI sponsored DISC facility in the department is highly acknowledged.

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Received by: 08 February 2010
Revised by: 04 March 2010
Accepted: 07 March 2010

The Online Journal of Neurological Sciences (Turkish) 1984-2010
This e-journal is run by Ege University Faculty of Medicine, Dept. of Neurological Surgery, Bornova, Izmir-35100TR as part of the Ege Neurological Surgery World Wide Web service.
Comments and feedback: E-mail: editor@jns.dergisi.org
URL: http://www.jns.dergisi.org
Journal of Neurological Sciences (Turkish)
Abbr: J. Neurol. Sci.[Turk]
ISSNe 1302-1664

REFERENCES


