Immunohistochemical colocalization of estrogen receptor-α and GABA in adult female rat hippocampus

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ABSTRACT

Background: Hippocampus is an important target for estrogen action. It is severely affected in patients of Alzheimer's disease. Much of the current research related to estrogen and brain function is focused in two directions. **Purpose:** By attempting to lacalize localizing ERs in GABAergic neurons of the hippocampus we tried to test the hypothesis that the action of estrogen in maintaining the neuronal plasticity and more specially the spine density of pyramidal neurons is through GABAergic neurons. **Methods:** The present study was planned to demonstrate the detailed immunoreactive (IR) distribution pattern of estrogen receptors (ER) in GABAergic neurons of hippocampus. The study was conducted in adult female Wistar rats in estrous phase. 30 μm thick cryostat sections of hippocampal region were obtained from perfusion fixed (with 4% buffered Para formaldehyde) adult female rats (n = 15). The sections were processed free- floating for immunolocalization using the PAP protocol. First they were immunostained for ER using, mouse monoclonal anti-ER-α antibody with DAB as chromogen. Subsequently the same sections were immunostained for GABA using rabbit monoclonal anti-GABA antibody respectively with 9 amino 3 ethyl carbazole (AEC) as chromogen. **Results:** The results showed ER were colocalised in GABAergic neurons in all the subfields of hippocampus with obvious variations. In Cornua ammonis (CA) maximum co-localised neurons were seen in CA3 region. **Conclusions:** The view is strengthened by our results as it was established through previous studies that the immediate target neurons of estrogen in hippocampus is the GABAergic neurons.

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Introduction

Hippocampus is an important target for estrogen action. It is severely affected in patients of Alzheimer's disease. The early clinical studies have shown that estrogen therapy given after menopause may prevent or at least delay the onset of Alzheimer's disease in older women. Much of the current research related to estrogen and brain function is focused in two directions. One involves clinical studies that examine the neuroprotective role of estrogen in protecting against cognitive decline during normal aging and against Alzheimer's disease. The other direction that is also the primary focus of this review involves laboratory studies that examine the mechanisms by which estrogen can affect neuroplasticity.

Estrogen mediates its effects through alpha subtype of ER (ER-α) or through β subtype (ER-β).[1] Within the hippocampus it has been localized on Pyramidal neurons [2] and on interneurons. The main neurotransmitter in the interneurons of hippocampus is gamma-amino-butyric acid (GABA).[3–6] Further, the local interneurons have been shown to contact multiple pyramidal cells suggesting their extensive effects on the hippocampus.[5] There are reports providing direct evidence for the expression of ER subtypes within GABAergic neurons in hippocampal cell cultures. Demonstration of the presence of ER subtypes in GABAergic neurons of neonatal rats were reported.[7] It was also reported that ER-α can mediate the effects of estrogen primarily in GABAergic neurons in the dorsal hippocampus and in both GABAergic and non-GABAergic neurons in the ventral hippocampus.[8] A prominent role for 17 beta-estradiol in maturation of the GABAergic interneurons was documented and suggested that estrogen's effect on the hippocampus may be mediated at least in part by its ER containing GABAergic neurons.[9]

Several mechanisms of neuroplasticity have been postulated and one of them is alteration in dendritic spine density. Studies have shown that the density of dendritic spines in the intact rat hippocampus undergoes marked variations during estrous cycle. A higher density of dendritic spines coincides with high levels of estrogen.[10] Ovariectomy resulted in decrease in Cornua Ammonis (CA1) cell dendritic spines and it was prevented with estradiol treatment.[11–13] One study showed that estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons[14], thereby suggesting that the spine producing effects of estradiol in hippocampal pyramidal cells are possibly mediated by changes in inhibitory interneuronal synaptic efficacy that is lowering of GAD and thus GABA production.

Keeping in view the above mentioned reports, the present study was planned to do co-localization immunohistochemical studies to ascertain whether the two IRs are expressed in the same neurons. By attempting to lacalize ERs in GABA ergic neurons of the hippocampus we tried to test the hypothesis that the action of estrogen in maintaining the neuronal plasticity and more specially the spine density of pyramidal neurons is through GABAergic neurons.

The review of literature has revealed that till date no *in-vivo* studies have been done or reported on the detailed distribution pattern of ER-α in GABAergic neurons of hippocampus.

Methods

15 adult female Wistar rats (body wt. 200-210 g) in estrous phase of the estrous cycle (vaginal smears tested positive for cornified epithelial cells), housed in the Central Animal Facility of the All India Institute of Medical Sciences with 12 hrs light/dark photoperiod and ad libitum access to food and water, were anesthetized with Sodium Pentobarbital (50 mg/kg.wt.i.p) and perfused transcardially with 4% Paraformaldehyde in 0.1 M Phosphate buffer (pH 7.4). Brains

were removed and the tissue specimens (middle blocks) were sectioned on a cryostat. 30μm sections were processed for immunhistochemistry (Imht) using Peroxidase anti Peroxidase (PAP) technique. Ethical approval was obtained for the study.

Co-localization

Colocalization was carried out for demonstration of ER and GABA immunoreactivity (IRty) in same neurons by PAP technique using two different chromogens. The sections were first immunostained for ER using the PAP protocol mentioned below with 3, 3′ diaminobzidine tetrahydrochloride (DAB) as the chromogen and subsequently the same sections were immunostained for GABA again using the PAP protocol with 9 amino 3 ethyl carbazole (AEC) as chromogen. The colocalized sections were mounted on clean glass slides and cover slipped with glycerin.

Immunohistochemistry

Sections were incubated in a methanol-hydrogen peroxidase mixture for 10 minutes (to quench the endogenous peroxidase activity) followed by blocking solution (1% normal goat serum and 0.2% Triton × 100 in PBS; 0.1 M) for 1 hr at room temperature (RT). After draining out the blocking solution sections were incubated with primary antibody i.e. mouse monoclonal anti-ER-α antibody (1:100, France, Marseille) or rabbit monoclonal anti-GABA antibody (1:1000, Sigma, USA) for 72 hrs at 4°C followed by incubation with secondary antibody i.e. goat-anti-mouse IgG for ER and goat-anti-rabbit IgG for GABA for 8 hrs at 4°C followed by monoclonal mouse PAP for ER(1:100) and rabbit PAP (1:100) for GABA for 4 hrs at 4°C. After each incubation sections were washed with PBS and incubated with substrate chromogen DAB for ER & AEC for GABA at RT in dark for 10 minutes. Washing with distilled water was carried out. Stained sections were mounted on gelatin coated (subbed slides) and left for drying overnight. Dehydration of mounted sections was done by passing them through graded alcohol series and cleared in xylene. Mounted with DPX and observed under the microscope.

End reaction product of staining showed brownish black stain when done with DAB and red with AEC. Colocalized neurons showed both these reactions.

Immunohistochemical Controls:

The immunohistocheical controls included elimination of the primary antiserum and replacing species specific antiserum with normal serum of the appropriate species (normal goat serum). Some positive control sections were always from the rat hypothalamus.

Results

Immunohistochemical Studies

CA

Immunostaining for both ER and GABA was seen to co-exist in various neurons of CA. This was evident in PAP stained sections. The co-localized neurons were unevenly distributed in various sub regions of CA. Thus the number of co-localized neurons was prounounced in CA3-CA2 than CA1. Consistent with findings on individual immunoreactivities of ER and GABA the co-localized neurons were also of different shapes and sizes.

Dentate Gyrus (DG)

Co-localized neurons were seen mainly in polymorphic layers of the two limbs of hippocampus (ectal & endal) and were predominantly located in the ectal limb. Only a few positive neurons were seen in other layers. However, no neurons were seen in the crest region.

CA3

Maximum numbers of IR neurons were seen in stratum pyramidale followed by stratum radiatum (Fig. 1). In Stratum oriens only occasional IR neurons could be seen. IR neurons were of various morphological types described below:

- Bipolar – medium to small sized neurons both vertically or horizontally oriented.
- Spindle shaped-medium sized, seen in CA3c and CA3b region mainly. The CA3a region had small sized multipolar GABAerigic neurons in it.

CA1

Density of IR neurons was less compared to CA3-CA2. Maximum number of neurons were seen in stratum pyramidale followed by stratum oriens. No IR neurons were seen in stratum radiatum and stratum lacunosum moleculare. Significantly enough number of GABA+ve neurons in this subfield chiefly consisted of vertically oriented bipolar, multipolar or spindle shaped neurons.

Discussion

Our investigations showed co-localized ER and GABA in the same neurons. To our knowledge this is the first study demonstrating coexistence of ER and GABA immunoreactivities *in-vivo* in hippocampal neurons. As mentioned earlier the hippocampus it is associated with the higher cognitive pro-

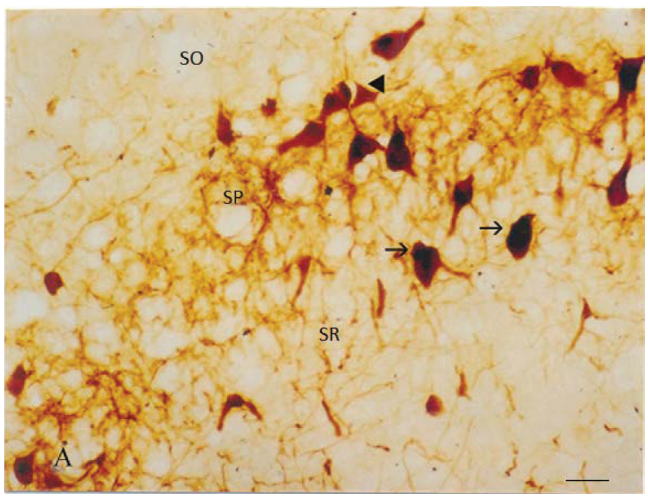

Fig. 1: Photomicrographs of coronal section of rat hippocampus showing three layers; Stratum Oriens (SO), Stratum Pyramidale (SP), Stratum Radiatum (SR). Maximum co-localization of ER (red color) and GABA (brownish) in CA3. PAP staining with two chromogens (Scale bar -25μm)
Note: (1) Coexistence of two immunoreactivities (arrow) in most of the neurons in SP. (2) Some neuron showed only ER immunorectivity (arrow head).

cesses of learning and memory. Hence the importance of understanding the neurochemical nature of its neurons cannot be overestimated.

The previous study by us provided detailed information on distribution of ER-α immunoreactivity in rat hippocampus.[15] In the present study we examined the neurochemical nature of the neurons in which the receptors are present.

In the present study GABA IR neurons were seen to be distributed unevenly in hippocampus, the density being highest in CA3 and DG. Further, the location and the morphological characters of IR neurons showed these to be nonpyramidal neurons (interneurons). An earlier study[3] that used GABA synthesizing enzyme glutamic-acid-decarboxylase (GAD) immunostaining has demonstrated GABAergic neurons in hippocampus to be interneurons. Later studies of several other investigators have also supported the same view.[3,16] GABA has been shown to be the only inhibitory neurotrabsmitter in hippocampus.[17] Another study[4] found 7-10% of neurons in hippocampus to be GABAergic in nature.

Earlier investigations[18] have reported maximum incidence of GABA+ve neurons in CA1 which is contrary to the present findings. The discrepancy in results is difficult to explain, except that these investigators have earlier conducted their experiments on male rats whereas in the present investigations focus on female rats in estrous phase have been used.

Colocalization or ER and GABA immunoreactivities

Our results on co-localization have demonstrated ER immunoreactivity close to GABAergic neurons in rat hippocampus. These results are the first reported study to show co-existence of two immunoreactivities *in-vivo* in rat hippocampus.

Number of studies has linked estradiol to GABAergic activity in various regions of brain. GABA concentration in the hypothalamus has been shown change during estrous cycle.[19]

Further steroid induced changes in GABA neurotransmission are postulated to be responsible for sexual differentiation during development.[20] In medial preoptic area increased estrogen levels were shown to increase basal as well as extracellular levels of GABA.[21] Estradiol has also been shown to regulate the levels of mRNA for both the forms of GAD (GAD 65, GAD 67) in various regions of rat brain.[22] Within the hippocampus ER alpha was expressed in only a subset of GAD-positive cells.[23] Estrogen increases dendritic spine and excitatory synapse numbers in CA1.[11,12,24] The density of these dendritic spines as well as synapses associated with them has been shown to fluctuate naturally during 5 day estrous cycle in rats.[12] *In-vitro* studies[25] have shown that estrogen decreases GABA levels in cultured hippocampal interneurons, which effectively increases the excitatory drive on pyramidal neurons and thus provide a mechanism for formation of new dendritic spines.

This view is strengthened by our results as it was established through previous studies that the immediate target neurons of estrogen in hippocampus is the GABAergic neurons. Thus it is possible that decreases in inhibitory activity and increase in spine density could contribute towards profound functional consequences in hippocampus.

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