ROLE OF ESTROGEN IN REGULATION OF MORPHOLOGY AND SYNAPTIC CONNECTIVITY IN FEMALE RAT SUBICULUM

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Abstract
Estrogen is reported to exert neuroprotective influences on the cornu ammonis as well as the dentate gyrus, two of the three components of the hippocampal memory system. Subiculum, the third and the output component of this system, plays a vital role in retrieval of previously learnt information (recall tasks). We undertook these studies to understand estrogen's role in regulating the synaptic connectivity, neurotransmission and neural degeneration in subiculum. For this, we used brains of aged (16-18 months) post-estropausal female Wistar rats, and compared them with those of adult (4-5 months) ovary-intact, ovariectomized, ovariectomized-vehicle treated and ovariectomized-estrogen treated rats. The vehicle (sesame oil) and estrogen therapy (17β estradiol, E2) were administered as daily subcutaneous injections (0.1 mg/kg of E2 in 0.1 ml vehicle) for 30 days. Serum estradiol assay was done to determine the levels of circulating serum estradiol levels. Brains were processed for Cresyl Violet, Golgi staining and Immunohistochemical studies [using anti-synaptophysin antibody].

Our results indicate that estrogen deficiency (induced by normal ageing or by ovariectomy) results in subicular neuronal degenerative changes, decreased synaptic connectivity and reduced dendritic arborization, most of which are reasonably reversed with estrogen replenishment. These studies suggest a unique role of estrogen in preventing and even reversing the senile neurodegenerative changes in female subiculum.

Key words: 17β-Estradiol, Ovariectomy, Synaptophysin

INTRODUCTION

The 'hippocampal complex' consists of cornu ammonis (hippocampus proper), dentate gyrus and subiculum. In contrast to the number of research studies available, focusing on hippocampus proper and dentate gyrus, subiculum has been far less studied. However, few recent studies have focused on subiculum, and shown that it is involved in memory 'retrieval' 1 and spatial 'encoding'[2,3].

Subiculum provides an anatomical link between the three-layered hippocampus and the six-layered entorhinal cortex 4, and forms the output gateway of hippocampal learning and memory system. Also, it has been implicated in cognitive disorders, Alzheimer's disease being the most common amongst them[5,6]. Alzheimer's disease is a senile neurodegenerative cognitive disorder, presenting with difficulties in learning and recalling. Various studies have implicated estrogen deprivation as one of the risk factors associated with Alzheimer's disease[7]. Epidemiological data suggest that the disease is more common amongst postmenopausal women, and that estrogen replacement therapy confers protection against the onset and progression of the disease[8].

Since subiculum is reported to regulate recall tasks, and retrieval of previously learnt information is said to be particularly impaired in post-menopausal women suffering from AD, we attempted to find out if subiculum is vulnerable to estrogen deprivation. Also, an important action of estrogen is its synaptogenic potential 21-23. Though this effect has been studied extensively on several cortical regions of the brain, there are scanty reports documenting these synaptic-regulatory effects of estrogen on the subiculum of rat.

The ovariectomized (OVX) rat is a useful model to elucidate the effects of estrogen depletion, which may to some extent, represent the postmenopausal stage of women. Thus the present study was undertaken to investigate the effects of estrogen deprivation (with ovariectomy and by normal ageing) and of estradiol (E2) therapy on neuronal cytoarchitecture and synaptic connectivity in rat subiculum.

MATERIALS AND METHODS

Experimental animals

The present investigations were carried out on adult

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(4-6 months) and aged (post-estropausal, 16-18 months) female Wistar rats (Rattus norvergicus). The animals were procured from the AIIMS Experimental Animal Facility with the approval of the Institute Animal Ethics Committee (IAEC), for the usage of animals in research. The rats were housed in pairs in Experimental Animal Facility under controlled environmental conditions, maintained on a 12:12 light/dark cycle with ad libitum access to food and water. All the animals were treated in accordance with the principles and procedures as laid down by the Ethics Committee. The estrous cycle of the adult animals was monitored by vaginal cytology[9]. Daily vaginal smears were obtained for over 3 weeks, and only those adult animals which exhibited three consecutive and regular 4-5 day cycles, were included in the studies.

The experimental animals were divided into the following study groups: Group A: Aged rats (16-18 months, n=10) and Group B: Adult rats (4-6 months, n=40). Group B was further subdivided into: B-I: Normal ovary-intact controls (n=10); B-II: Ovariectomized controls (OVX, n=10); B-III: Ovariectomized vehicle-treated controls (OVX+Vh, n=10) and B-IV: Ovariectomized estrogen treated adults (OVX+E2, n=10).

While the animals of group B-I served as ovary intact normal controls, the animals of groups B-II, B-III and B-IV were subjected to bilateral ovariectomy in their estrous phase. Surgery was performed under aseptic conditions, effective anaesthesia and preoperative antibiotic coverage. The rats were allowed to recover for 2 weeks following the surgery. During their recovery period, vaginal smears of the ovariectomized rats were prepared and examined for 5 consecutive days for the confirmation of complete bilateral ovariectomy. The animals of group B-III and IV were administered a daily subcutaneous injection of sesame oil (0.1 ml) and 17β-estradiol (0.1 mg/kg body weight in 0.1 ml sesame oil) for 30 days, from the 15th post-operative day respectively. The non-treated rats were kept as ovariectomized-controls (group B-II).

Serum Estradiol assay

The serum levels of circulating estradiol were determined by radioimmunoassay (RIA) using commercially available kits (M/S Abbot AXSYM).

Tissue processing

Twenty four hours after the last injection, one set of animals from each group was anesthetized and sacrificed by transcardial perfusion fixation. Once the perfusion was complete, the brains were dissected out, post-fixed and cryoprotected. Each brain was cut into two sagittal halves. The region of the brain bearing the subicular complex (in relation to bregma) was taken according to the stereotaxic co-ordinates by Paxinos and Watson[10]. The tissue blocks were mounted in OCT compound in a Leica CM 1900 cryostat for cryosectioning. Each half of the brain was cut into coronal or horizontal cryostat sections, respectively. For Cresyl Violet staining, 10μ thick sections were taken on clean glass slides. For immunohistochemical staining, sequential 30μ free floating sections were collected in clean culture plates with wells, filled with 0.1 M phosphate buffer. Another set of animals was sacrificed without perfusion fixation, and fresh brains were quickly dissected out and used for Golgi impregnation studies.

Cresyl Violet staining

Cresyl Violet (CV) staining was carried out to study the light microscopic cytoarchitecture of the subiculum in control animals, and to appreciate alterations in cytoarchitecture, if any, by the changing levels of the circulating estrogen, in experimental animals. In brief, the 10μ thick cryostat sections were hydrated by immersion of slides in distilled water for 30 seconds. The slides were then transferred to 0.5% aqueous solution of CV stain for 5-6 minutes, followed by two changes of distilled water to remove the excess stain. Finally, the sections were dehydrated by passing the slides through increasing grades of alcohol for 1-2 minutes each, and differentiated in 95% alcohol containing 10% acetic acid for 10 seconds. Sections were then cleared in xylene, mounted in DPX and coverslipped. The slides were examined under the light microscope (Microphot-FX Nikon Microscope) and photographed. The Nissl substance was visualized as bright purple finely granular material in the perikaryal cytoplasm.

Golgi staining

4-5 mm thick slices of fresh rat brains were immersed in the Golgi impregnation fixative mixture (Golgi Cox method) for one week. The fixative solution was changed every week for a month. After one month, the slices were taken out, gently brushed free of loose surface crystals and the tissue was dehydrated in ascending grades of ethanol (for 1 hour in each

grade), following which the slices were immersed overnight in a mixture of ether and alcohol (2:1). The specimens were then infiltrated with ascending grades of celloidin (2% for 7 days, 5% for 7 days and 8% for 3 days) dissolved in ether-alcohol mixture (2:1). Finally, the tissue was embedded in 8% celloidin in a paper boat. The block was hardened in chloroform vapour, and the paper was gently peeled off. The block was mounted on a wooden chuck, and cut into 100-120 μm thick sections on a sliding microtome. The sections were collected serially in 70% alcohol, passed through 2 changes of 95% ethanol (20 mins each), followed by N-butanol for 10 mins, and finally cleared in toluene for 30 mins. The sections were mounted in Permount (Fisher Scientific). The subicular neurons along with their dendritic processes were visualized and analyzed under the light microscope.

Immunohistochemical staining

Immunohistochemical staining was carried out to detect and localize the expression of presynaptic protein synaptophysin (SYP) in the subiculum of control and experimental animals. Staining for these proteins was carried out on 30μ thick cryosections using immunohistochemical staining procedure[11] modified and documented by Mehra and coworkers[12] earlier. The free floating sections were incubated in SYP specific rabbit polyclonal (1:500, Santa Cruz Biotechnology) antibody for 48 72 hours. Further incubation was carried out in respective biotinylated secondary antibody (Labvision, Thermoscientific). The immunoreaction was visualized with DAB (Sigma Chemicals, USA). The slides were observed under Microphot-FX Nikon Microscope, analyzed and photographed.

OBSERVATIONS AND RESULTS

Serum estradiol levels

The serum levels of circulating E2 detected in ovary intact and E2-administered groups were 42.3 ± 5.5 and 58.4 ± 6.6 (pg/ml ± S.E.M) respectively. This was in contrast to the low hormone levels (<5 pg/ml) of OVX and aged controls.

Cytoarchitectural studies (Cresyl violet staining)

In the ovary-intact adult animals (group B-I), subiculum was observed as a continuation of CA1, the distinct narrow layer of CA1 abruptly widening out, positioned in between the hippocampus and entorhinal cortex. The Nissl material in the individual subicular neurons was stained bright purple with the CV stain, and appeared as fine granular material in the neuronal cytoplasm, also extending into the dendritic processes (figure 1a). In the cell nuclei, the nucleoplasm was minimally stained, but prominent nucleoli were observed in most of the neurons. In the ovariectomized-control adult animals (group B-II) the neurons appeared to be smaller, with darkly stained Nissl material clumped preferentially in the periphery of the neuronal perikarya, in majority of the neurons (figure 1b). Changes in the vehicle-treated (group B-III) subiculum were similar to, but less pronounced than those seen in the ovariectomized control group. Estrogen treatment (group B-IV) seemed to have reversed the cytoarchitectural changes induced by ovariectomy. However, occasional neurons still continued to show some Nissl clumping. The subiculum of aged animals (Group A) displayed profound Nissl clumping, quite like the ovariectomized control group.

Golgi staining

The subicular neurons as well as their dendritic processes were observed (figure 2a-c). In the ovary intact adult controls (group B-I), the pyramidal neurons displayed a distinct apical dendritic tree (with primary, secondary and tertiary branches) and basal dendrites, with abundant arborization with the surrounding neuronal processes. The dendritic branching and arborization appeared to be drastically reduced in the estrogen deficient groups (ovariectomized controls: group B-II, vehicle treated controls: group B-III and also in the aged animals: group A). However, E2 therapy for 4 weeks (in the estrogen treated animals: group B-IV) seemed to have enhanced the dendritic branching processes and the arborization significantly.

Immunohistochemical studies

(i) Synaptophysin (presynaptic protein)

Synaptophysin immunoreactivity (figure 3a and b) seen as fine brown punctate staining, showed a marked reduction in ovariectomized control rats (group BII), sesame oil-administered ovariectomized rats (group B-III) and the aged rats (group A), whereas ovariectomized adult rats treated with 17β-estradiol (group B-IV) displayed an upregulation in the intensity of SYP immunoreactivity in their subicular region. The results were further confirmed by IOD analysis and protein estimation (data not shown).

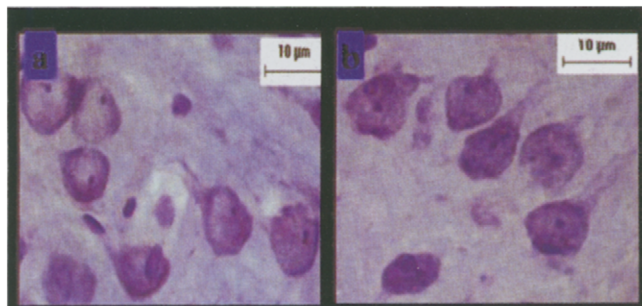

Figure 1: High magnification photomicrographs of CV stained sections of subiculum from the (a) ovary intact group and (b) ovariectomized control group. Note: the fine Nissl granularity in a, and the coarse clumping of the Nissl granules in b. Prominent nucleoli can be observed in all the neurons of both the sections. Scale bar 10μ

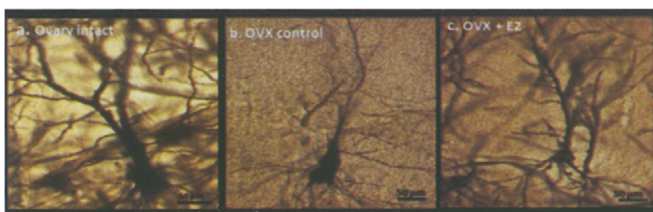

Figure 2: Golgi stained sections of subiculum from (a) ovary intact, (b) OVX control and (c) estrogen treated rats. Note the reduction in the dendritic branching in (b) and near-normal dendritic branching in (c).

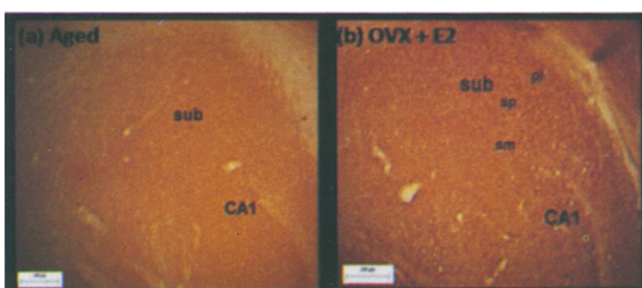

Figure 3: Photomicrographs of synaptophysin immunostained sections of subiculum from (a) aged group showing mild immunoreactivity, and (b) estrogen-treated group showing enhanced synaptophysin expression. sp-stratum pyramidale, sm-stratum moleculare, pl-polymorphic layer. Scale bar 200µ.

DISCUSSION

The present studies have demonstrated that the hormone estrogen plays a role in modulation of neuronal morphology, synaptic plasticity, neurotransmission and dendritic arborization in the adult and aged female rat subiculum. The cytoarchitecture of subiculum has not been studied in great details, and whatever reports are available in literature are either very primitive[13] or are quite variable in description[14-17]. The subiculum, like the hippocampus, presents three principal layers, namely: a cell sparse molecular layer, an enlarged pyramidal cell layer and a polymorphic cell layer. In the Cresyl Violet stained sections of rat subiculum, in the present studies, the cell packing in the pyramidal layer of subiculum is observed to be 'looser' than that seen in the hippocampal subfields, as also previously described [18].

Our finding of Nissl clumping in the subicular neurons of estrogen-deprived groups suggests some form of ongoing neurodegenerative process, occurring in response to the estrogen depletion. The fine granular purple Nissl substance visualized in the ovary-intact neurons seems to be replaced by clumped, darkly stained Nissl granules, deposited mostly at the periphery of the neuronal perikarya, in the estrogen-deficient groups. Estrogen therapy of 4 weeks reversed these changes in most of the neurons, but not in all. We believe that the occasional neurons that continued to show degenerative changes, may have either undergone degenerative process beyond repair, or may be slow recoverers. Our results are supported by the earlier reports documenting the occurrence of neuronal degeneration in the supraoptic nucleus of hypothalamus in prepubertal rats, as a consequence of ovariectomy[19]. No degenerating neurons were observed in estrogen-treated ovariectomized rats or sham-operated rats by these investigators. Similar studies have described estrogen-mediated age-related morphological changes (in the medial preoptic nucleus of ovariectomized Long-Evans rats) like neuron atrophy, shrinkage and high pyknotic ratio, comparable to those seen in the 'ovariectomized controls' and 'aged' ovary-intact females of the present studies [20].

We also investigated the potential of estrogen to regulate synaptic plasticity in the female rat subiculum, in the present study. For this, we analysed the presynaptic protein synaptophysin immunohistochemically, and also studied the

dendritic arborization patterns of subicular neurons through Golgi impregnation technique. The results of our study clearly indicate that in estrogen deficient states (following ovariectomy as well as in normally aged animals) there was an obvious downregulation of synaptophysin immunoreactivity, as well as of the dendritic branching and arborization of subicular neurons. On the other hand, following estrogen therapy the synaptic protein immunoexpression as well as the subicular neuronal dendritic branching seemed to be considerably enhanced. These results not only indicate the potential for plasticity in the adult subicular neurons but also emphasize the positive influence of estrogen in modulating this plasticity. The results of our investigations (an upregulation of synaptophysin in estrogen-treated group) further substantiate earlier reports suggesting that estradiol increases pre- and post-synaptic proteins in the CA1 region of hippocampus in the brains of adult female rhesus macaques[21]. Through radioimmunocytochemistry, they reported that estrogen increased the expression of the proteins syntaxin and synaptophysin (presynaptic proteins) and spiniphilin (postsynaptic protein) in the stratum oriens and radiatum of the CA1 region. Even in vitro studies have suggested that estrogen upregulates synaptophysin in slice cultures of rat hippocampus [22]. In this regard, authors have postulated that during estrogen-mediated synaptogenesis (which they studied across the estrous cycle of rat [23], the immunoreactivity of ribosomes increases in the dendrites of the CA1 stratum radiatum, implying a role of estrogen in the local protein synthesis in the dendrites (for formation of new synapses)[23]. Investigators have demonstrated similar synapto-facilitatory effects of estrogen through their morphological studies using Golgi impregnation and electron microscopy and reported an increase in the dendritic spine density in the CA1 region of monkeys following estrogen therapy[24]. The estrogen mediated upregulation of synaptic connectivity through induction of dendritic spine formation and enhancing dendritic arborization has been reviewed across species[25]. Our studies also clearly show that the subicular neurons are capable of responding to their estrogenic environment, and estrogen possesses the potential to stimulate synaptogenesis even in the adult subicular neurons. This is significant from the point of view of subicular involvement in the memory mechanisms.

Considering subiculum's role in the memory functions, aging subiculum has been suggested to be involved in the very early stages of Alzheimer's disease[6]. Studies have reported that in Alzheimer's disease, subicular thickness and subicular dendritic arborization (quantified as dendritic arborization index) decrease, as the neurofibrillary tangle density in the subicular neurons increases[5]. Estrogen's potential to modulate neuronal structure, viability and synaptic plasticity in the subiculum, as observed in the current studies, may be of vital cognitive significance in this regard.

The results of the present investigation emphasize an immense sensitivity of subicular neurons to the estrogenic environment, which we believe may have potential cognitive implications considering estrogen's capability to enhance cognitive processes through its synaptogenic and neuroprotective properties.

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