Immunohistochemical Localization of Estrogen Receptors in the Hippocampus of the Wistar Albino Rats.

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Abstract

Background:

Estrogen plays an important role in changes taking place in the brain through the regulation of growth and differentiation of axons and dendrites, influence on plasticity, support of survival as well cognitive and behavioral functions. The classical mode of estrogen action is through the activation of its receptors, ER alpha and beta. ERα is more abundant in the arcuate nucleus, and ERβ is more prevalent in the hippocampus. Both receptors are located with the cell (genomic). Most of the literature are also supports the genomic ER importance and its role in cell signaling

Purpose:

To find out the genomic ER role and its cell signaling in hippocampus

Methods and Materials:

In this study extracellular neuronal ERs were identified in control as well as experimental group wistar albino rats by immunohistochemistry. Its importance in cell signaling was explained. In immunohistochemistry, indirect Avidin-Biotin Complex (ABC) formation method was used.

Results:

presence of genomic estrogen beta receptors in the hippocampus of wistar albino rats was measured. Presence of more number of in ER-IR pattern in (80.85±6.53) different subfields of CA as well as in DG by morphometric study. It suggest that presence of ERs through its estrogenic activity. These highly localized shifts in ER regulation and distribution may contribute to stress related decreases in synaptic plasticity While in this study confirms that more number of ER-IR was present within the presynaptic terminal and spines of CA1 pyramidal neurons of hippocampus.

Conclusion:

Genomic location of estrogen receptor in the hippocampus influence the hippocampal neuronal plasticity and cognition

Keywords: Estrogen receptors, hippocampus, genome neuronal plasticity and cognition

INTRODUCTION:

Estrogen plays an important role in changes taking place in the brain through the regulation of growth and differentiation of axons and dendrites, influence on plasticity, support of survival as well cognitive and behavioral functions. The classical mode of estrogen action is through the activation of its receptors, transcription factors. ERs are a group of proteins mostly found inside cells. They are receptors that are activated by the hormone estrogen (17β-estradiol)[1]. Two such estrogen receptors have been cloned, ER alpha & beta. Several isoforms of ER beta have also been characterized, which may be a significant factor in the regulation of estrogen response in the brain. ER is the predominant receptor subtype in the basal forebrain cholinergic neurons of the adult rat brain [2] where it is thought to enhance cognitive functions by modulating the production of acetylcholine. Although both receptors are expressed by neurons in the arcuate nucleus and hippocampus, ERα is more abundant in the arcuate nucleus, and ERβ is more prevalent in the hippocampus[3]. When comparing these receptors, more number of ER was present in the hippocampus.

The hippocampus resemblance to the seahorse is a major component of the brain of humans. It belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Humans and other mammals have two hippocampi, one in each side of the brain. The hippocampus is located under the cerebral cortex; and in primates it is located in the medial temporal lobe, underneath the cortical surface [4]. In addition to genomic signaling, there is increasing evidence that rapid nongenomic signaling via membrane localized extranuclear ER may also play a role in mediating E2 neuroprotective effects in the brain [5,6]. Along these lines, several laboratories have shown that the rapid activation of extracellular signal-regulated kinases 1, 2 (ERKs) by E2 is critical for its neuroprotective effects, as administration of a MEK inhibitor blocks E2 neuroprotection in neurons in vitro [7, 8]. Furthermore, E2-induces ERK activation in the CA1 region after GCI, which is critical for its neuroprotective effects as treatment with a MEK inhibitor blocked E2-induced ERK activation and E2 neuroprotection in the hippocampus [9]. Likewise, a role for the prosurvival serine kinase Akt in E2 neuroprotection has been implicated, as E2 rapidly up-regulates Akt activation in cortical neurons *in vitro*[10], and in the hippocampus CA1 *in vivo* following GCI[11], while treatment with a PI3K inhibitor attenuates the neuroprotective effects of E2 both *in vitro* and *in vivo* [10,11]. In addition, we recently demonstrated that E2 attenuates the rapid activation of the proapoptotic signaling kinase, JNK in the hippocampal CA1 region after GCI. As a whole, these findings suggest that E2-induced rapid nongenomic signaling may play a critical role in E2 neuroprotection [11]

The aim of this study

1. To find out the immunohistochemical location of estrogen receptors in the hippocampus.
2. To study the importance of ER and its cell signaling

Materials and methods

The study was conducted on 30 female healthy adult wistar albino rats. (220±20g) with regular 4-day estrus cycles. The approval of the Institutional Animal Ethical Committee (IAEC) of Saveetha University (IAEC No.Anat.002/2009) was taken prior to the experiments. All the protocols and the but time consuming and complex to perform). ABC system being used will form large complexes of avidin-biotin- label which will bind to the molecules of biotin on the secondary antibodies. Avidin-Biotin Complex (ABC) formation method requires less primary antibody than direct methods of detection.

Cryopreservation

It was performed at sub-zero temperatures

(-21˚C). Sudden freezing of hippocampal tissue damage prevented by cryoprotectant solution containing combination of 15% and 30% sucrose. The hippocampus was placed in 15% sucrose overnight till it sank and then in 30% sucrose overnight till it sank.

experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purchase of Control and Supervision of Experiments on Animals (CPCSEA).

Immunohistochemical staining using ABC technique

It can vary from one step procedure where the label was conjugated directly to the primary antibody [12] (quick and easy but low sensitivity) to multiple step procedure where the label was conjugated to a secondary antibody or an Avidin-Biotin Complex (ABC)[13] (very high sensitivity

Cryosection for immunohistochmistry

The hippocampus was placed in the chuck. It embedded with optimum cutting temperature (OCT) medium and block was made. Then the tissue was sectioned using cryostat of 30 µm thickness. For each tissue the sections were collected separately in the multivial culture plates and labelled. For free floating immunohistochemical localization the antibodies for estrogen receptor was obtained from Sigma Laboratories (USA). The procedure was standardized for these antibodies at different dilutions ratios like 1:500, 1:1000, 1:2000 and 1:4000 were used and the perfect ratio 1:1000 where the staining optimal was determined and noted. For those standardization at various dilution ratios the tissue sections of rat cerebrum with hippocampus was obtained.

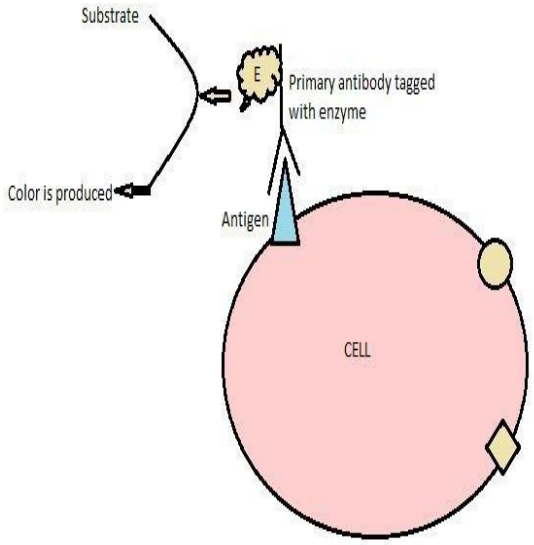
Protocol for immunohistochemitry

Hippocampal sections were transferred to a multivial plate containing 0.1 M PBS -Tx and washed for three times 5 min each in PBS-Tx. Incubate the sections in methanol+H_2O_2 solution for 30 min in dark to prevent the high endogenous peroxidase activity which causes high back ground staining. The sections were wahsed with PBS-Tx for three changes each for 10 min at room to permeabilize the cell membranes and also to reduce the surface tension of aqueous solutions used in immunostaining. Non-specific binding sites were blocked with 10% Normal Goat Serum (NGS) in 0.1 M PBS + 0.25%Triton X 100 for 2 hours at room temperature.The sections were incubated in primary antibody Anti-ER (1:1000), from Sigma Laboratories (USA) for 48- 60 h at 4˚C. The sections were washed with 0.1M PBS-Tx three changes 5 mi. The sections were incubated in goat anti-rabbit biotin conjugated secondary antibody 10ml PBS-Tx in mixing bottle + 1 drop of biotinylated secondary antibody for 2 hours at room temperature.

The sections were washed with 0.1 M PBS-Tx for three times. Subsequent to buffer wash, Avidin Biotin Complex (ABC - 2 drops of reagent A in 5 ml of buffer + 2 drops of reagent B in 5 ml of buffer) – dilutions were made according to ectastain Elite ABC kit in PBS-Tx (Vector Laboratories Inc., Burlingame, CA). Then 3 washes with 0.1 M PBS was done 0.1.

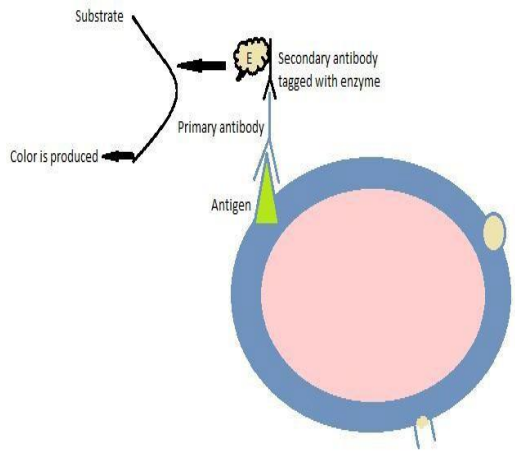
Visualization of immune complex-3,3 diaminobenzidine complex - 0.025% DAB, Sigma Laboratories) was done, where this immune complex acts as a chromogen. Sections were incubated for 10 minutes. DAB-0.025% (25mg) M PBS - 100 ml; H_20_2- 0.05%(50µl); H_2O_2 was added to this mixture just before using it. Following buffer wash, sections were mounted on gelatin coated slides, dehydrated with alcohol and then the slides were cover slipped with DP.

Direct immunohistochemical staining method



Direct immunohistochemical method where primary antibody was tagged with enzyme(E) reacts with the substrate producing colour reaction.

Indirect immunohistochemical staining method



Indirect immunohistochemical staining method, primary antibody specific to antigen was added, followed by adding secondary antibody specific to Fc region of primary antibody tagged with enzyme(E). Staining was produced on adding substrate.

antibody tagged with enzyme (E). Staining was produced on adding substrate

Results

These results indicate that estradiol decreases GABAergic inhibition in the hippocampus, which appears to effectively increase the excitatory drive on pyramidal cells, and thus may provide a mechanism for formation of new dendritic spines. Dendritic spines are the primary loci of excitatory synapses in central neurons and had long been associated with neuronal plasticity.

The results of the current experiment demonstrate for the first time that increasing levels of ER in the hippocampus with increases activation an ER regulated kinase and enhances cognitive function. In addition to impacting cognition, increasing hippocampal levels of ER resulted in increased level of phosphorylated ERK/MAPK, regulation of which was mediated by ER signaling. These data provide support for the hypothesis that treatments that increase or maintain levels of ER in the hippocampus in aging people and stressed population are beneficial to the hippocampus and hippocampus-dependent behavior.

Collectively results suggest that repeated estradiol influences hippocampal neurogenesis. White arrows showing more number ER-IR neurons in dentate hilus (DH)(Fig 1) and CA1-CA4(Fig 2)

Immunohistochemical localization of ER.

FIG 1

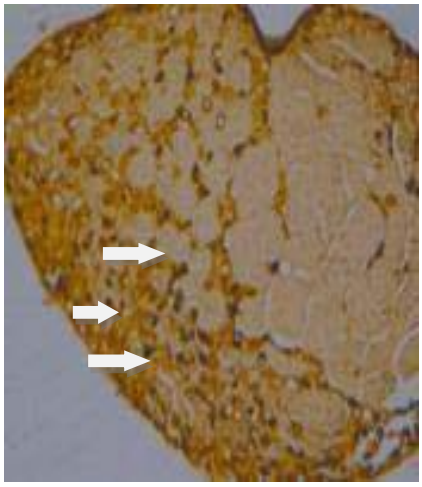
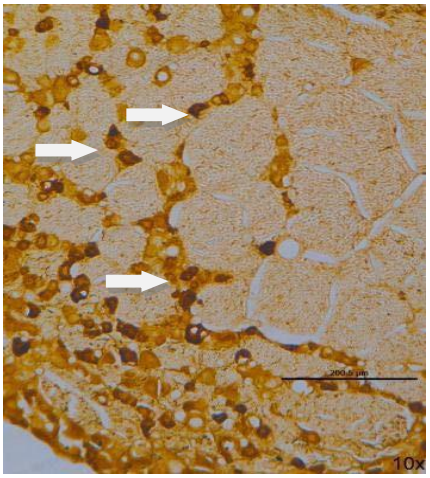


FIG 2



Photonic of coronal section of 5X and 10X of rat hippocampus. White arrows showing more number ER-IR neurons in dentate hilus (DH) (Fig 1) and CA1-CA4 (Fig 2). ERs were darkly stained. They are present at the junction and in the membrane (White arrow). Here the staining intensity of ERs neurons were very high. This shows the activity of neurons

FIG 3

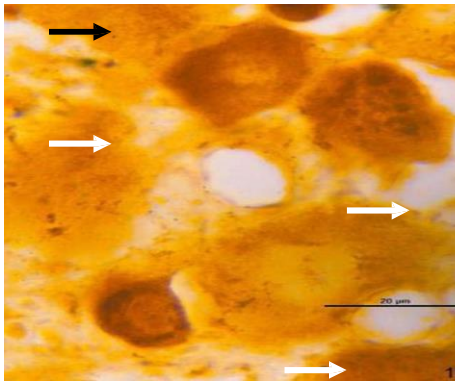
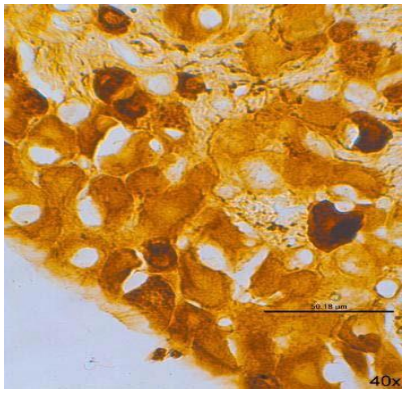


FIG 4



Photonic. Of 40X and 100X showing a few ER +ve neurons. Arrows indicating ER neuron in the hippocampus. They were less densely stained ER neurons which shows the activity of neuron.

Discussion

Harris and Kater, (1994)[14] demonstrated the estradiol exposure for 40 days in middle-age begun at the time of ovariectomy and resulted in increased levels of hippocampal ER as well as

enhanced cognitive function up to eight months following the termination of the estradiol treatment.

One proposal was that the continuous replacement of neurons in the dentate gyrus provides a refreshable repository for temporary processing of new information that was later moved elsewhere for long term storage [15]. Neuron addition in the hippocampal formation does appear to be accelerated under conditions that require or enhance learning [16, 17]. Hence this study was an important step toward understanding the detailed and region-wise ER IRty of hippocampal region. The present study results support the possibility that the persistent increase in hippocampal levels of ER by which short-term estradiol in midlife could permanently enhance cognition.

Since previous studies principally used E2, which can activate both extranuclear and nuclear estrogen receptors [14], it has been difficult to distinguish the importance and contribution of extranuclear receptor-mediated signaling in E2 neuroprotective effects. To address this issue, the current study employed two E2 conjugates, E2-BSA conjugate[15,16,17] and the newer E2 dendrimer conjugate (EDC) [17], which due to their size and charge cannot enter the cell nucleus. EDC and E2-BSA retain their ability to induce rapid extranuclear-mediated nongenomic signaling, but lack significant nuclear ER-mediated genomic signaling ability due to their inability to enter the cell nucleus and interact with nuclear ER[16, 17]. Thus, their use has the potential to provide important insight into the role and importance of extranuclear estrogen receptors in E2 neuroprotective effects in cerebral ischemia. The results of our study reveal that EDC and E2-BSA administered intracerebroventrically (icv) rapidly activates ERK, Akt and CREB signaling pathways in the hippocampus, enhances levels of the CREB transcriptional target, brain-derived neurotrophic factor (BDNF), strongly protects the hippocampal CA1 region against neuronal cell death, and significantly improves hippocampal-dependent cognitive function in the Morris water maze following GCI.

The study thus provides important new evidence of a critical role for extranuclear estrogen receptor activation in estrogen-induced neuroprotection and improved functional cognitive outcome following GCI, and suggests that ERK-Akt-CREB-BDNF signaling is an important component mediating extranuclear estrogen receptor beneficial neural effects.

There are two principal signal transduction pathways involving the G-protein coupled receptors: cAMP signal pathway and Phosphatidylinositol signal pathway Gilman *et al*., (1987)[18]. Both activate a G protein ligand binding. G-protein is a trimeric protein. The 3 subunits are called α、β and γ. The α subunit can bind with guanosine diphosphate, GDP. This causes phosphorylation of the GDP to guanosine triphosphate, GTP, and activates the α subunit, which then dissociates from the β and γ subunits. The activated α subunit can further affect intracellular signaling proteins or target

functional proteins directly.

Conclusion: The present study results support the possibility that the persistent increase in hippocampal levels of ER by which short-term estradiol in midlife could permanently enhance cognition.These results indicate that estradiol decreases GABAergic inhibition in the hippocampus, which appears to effectively increase the excitatory drive on pyramidal cells, and thus may provide a mechanism for formation of new dendritic spines. Dendritic spines are the primary loci of excitatory synapses in central neurons and had long been associated with neuronal plasticity.

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