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Research Report

Estrogen receptor α and β immunoreactive neurons in normal adult and aged female rat hippocampus: A qualitative and quantitative study

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Abstract

We have studied the distribution pattern and levels of expression of two estrogen receptor (ER) subtypes, ERα and ERβ, in the normal adult ($n = 10$) and the aged ($n = 10$) female rat hippocampus with the objective to establish baseline data and the changes that occur during aging. Techniques including immunohistochemical localization, co-localization with double immunofluorescence and confocal microscopy, image analysis including neuronal counts/mm^2 area and measurements of optical density (OD) of immunoreactivity in immunoreactive neurons and Western blot analysis have been used. The results revealed ERα and ERβ positive neurons in all subfields of the hippocampus with maximum presence in the stratum pyramidale of CA3. Some stained neurons in CA3 exhibited pyramidal neuron like morphological characteristics; such neurons were not found in CA1. All other immunoreactive neurons showed non-pyramidal neuron like morphological characteristics. Neuronal counts revealed a significant decrease in the number of immunoreactive neurons in CA3–CA1 of aged hippocampus. The percent decrease in counts of the immunoreactive neurons/mm^2 area in the aged rat (compared to the adult) was 78% for the ERα and 88% for the ERβ ($P < 0.001$) in CA3. In CA1, it was 56% ($P < 0.001$) and 41% ($P < 0.01$) respectively. The OD of immunoreactivity was significantly decreased ($P < 0.01$) in CA3 but increased ($P < 0.01$) in the CA1 immunoreactive neurons. Western blot analysis also showed a significant decline ($P < 0.01$) in the levels of the ERα and ERβ proteins in the aged hippocampus. Co-localization revealed that the two ER subtypes do co-exist in the same hippocampal neurons.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Receptor modulation, up- and down-regulation

Keywords: Estrogen receptors α and β; CA3–CA1; Aging; Immunohistochemistry; Western blot; Co-localization

1. Introduction

Besides regulating the reproductive neuroendocrine axis, a significant number of basic and clinical studies suggest the execution of non-reproductive functions by estrogens from gestation to senescence. A good amount of evidence exists associating ovarian steroid hormone with hippocampal activity [50], an area of brain related to cognition and memory. Presence of estrogen receptors (ER) in the hippocampus gives further evidence to it being one of the target brain regions for the hormone activity [4,21,26,31,34,38,40–42,44,51]. An estradiol-induced increase of dendritic spines has been demonstrated in vitro in dispersed hippocampal neuronal cultures [29]. Fluctuations in hippocampal dendritic spine and synapse density have been related to different phases of estrous cycle in rats, with highest density being present in the proestrous phase when the estrogen levels are high [14,53,54]. Estradiol treatment to ovariectomized rats increased ligand binding to NMDA receptors in the CA1 region of the hippocampus [50], indicating an intimate functional relationship between the hormone and this brain region.

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Functionally, the effects of estrogen in the brain range from rapid actions on membrane excitability and synaptic transmission to long term action on sexual differentiation, development and maturation of the brain, morphological characteristics of neurons, neuroprotection, endocrine regulation and initiation of reproductive behavior [8,14,26,53]. The biological effects of estrogen are known to be mediated by its interactions with specific receptor (ER), even though ER independent mechanisms have also been considered [15]. Views have been put forward on rapid mechanisms of action through membrane bound putative receptors via activation of intracellular signaling pathways as well [25,26].

ER is a transcription factor, which consists of two subtypes, ERα and ERβ [19]. Even though estradiol has similar affinity for both these receptor subtypes [18,47], yet they are seen to have different or opposite effects on certain transcription related cellular activities when bound to estrogen [30,37]. These observations indicate that the α and the β receptors need to be evaluated separately as they may differ in certain other characteristics also. The identification and cloning of ERα [16] and ERβ [18,28] has led to demonstration of their specific mRNAs and immunoreactivities in different regions of brain [17,34,42].

The morphological and functional effects of estrogen have been mostly investigated in young animals. Recent studies of Adams and colleagues [1] on sub-cellular distribution of ERα have reported decreased immunoreactivity in pre-synaptic and post-synaptic compartments of aged female rat hippocampal neurons.

The current studies were planned to investigate the immunohistochemical (IHC) distribution pattern and the levels of expression of two estrogen receptor subtypes, ERα and ERβ, in the hippocampus of the normal adult and the aged female rats. Female aging results in alterations in estrogen levels and release pattern. In women, it is characterized by natural depletion of hormone levels and menopause, whereas in rodents, it results in estropause where the female animals go into either persistent estrous phase or persistent diestrous phase [24]. How and in what quantity the target neurons are expressing their steroid receptors in the course of altered physiological cyclic status remain to be fully understood.

The ER expressing neurons were analyzed qualitatively in two areas of the hippocampus only, i.e., CA3 and CA1. Neurons (pyramidal or interneuron) expressing ERα or ERβ were identified. Subsequently, double immunohistochemistry and confocal microscopy were applied to demonstrate whether the two ER subtypes co-existed in the same hippocampal neuron. The changes in the expression of ERα and ERβ protein levels were further studied by more direct quantitative approaches of Western and immunoblotting coupled with densitometric measurements. In these neurochemical studies, the whole hippocampus was used including *cornu ammonis* and *dentate gyrus*.

2. Materials and methods

2.1. Animals

The present studies were carried out on 10 adult (8–10 months) and 10 aged (22–24 months) female Wistar rats. The animals were 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 Institute Ethics Committee for the Usage of Animals in Research. All the animals used in the present study were monitored on daily basis for their estrous cycle. The adult female rats, which exhibited three to four consecutive and complete 4–5 days estrous cycles, were included in the present study, whereas only those aged female rats which showed persistent diestrous phase were included. Both the adult and the aged rats were divided into two groups; one set of animals (n = 5 in each age) was used for IHC studies and the other (n = 5 in each age) for Western blot procedures. All the animals were sacrificed in their diestrous phase (persistent diestrous in case of the aged).

2.2. Immunohistochemistry

For the IHC studies, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg) and transcardially perfused with cold 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). The brain was then dissected out and post fixed in the same fixative overnight, cryoprotected by first sinking in 10% and then in 30% sucrose (in 0.1 M phosphate buffer) at 4 °C. It was then blocked in OCT compound in a *Leica* CM 1900 cryostat, 30 μm thick coronal sections were cut and collected in phosphate buffer (PB).

The free-floating sections were incubated in the presence of ERα specific mouse monoclonal (Neomarker, USA) and ERβ specific rabbit polyclonal (Affinity Bioreagents, USA) antibodies using the peroxidase–anti-peroxidase (PAP) staining technique [46] as adapted by our laboratory earlier for other neurotransmitters, peptides, proteins, etc. [9,27]. All other immunochemicals and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were obtained from Sigma Chemicals USA. The primary incubation was carried out at 4 °C for 72 h, and both primary antibodies were used at a dilution of 1:100. Further incubation was carried out in goat–anti-mouse IgG secondary antibody (1:30) for the ERα and in goat–anti-rabbit IgG (1:30) for the ERβ for 2 h at room temperature (RT) followed by mouse monoclonal PAP (1:100) and rabbit polyclonal PAP (1:100) respectively for 1 h at RT. Following each incubation, the sections were washed twice with Tris buffer saline (TBS). The dilution fluid in all instances contained 1% normal goat serum (NGS) and 0.2% Triton X-100 in 0.1 M TBS (pH