Section: Anatomy

Original Research Article

EVALUATION OF ONTOGENESIS OF ESTROGEN RECEPTORS AMONG WISTAR RAT HIPPOCAMPUS

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Abstract

Background: Brain function and cognition are affected by estrogens, especially 17β-estradiol (E2). Age, E2 levels, and therapy length affect efficacy in rats, same as in women. Later ovariectomized rats demonstrate little effects of E2 treatment on spatial memory. The complicated dose-response pattern of E2 comprises several transcription mechanisms. The oestrogen receptors ERα, ERβ, and GPR30 mediate these actions with varying concentrations and distributions. **Aim and objectives:** To explain ontogenic alterations and study Wistar rat hippocampal estrogen receptor development. **Material and Methods:** 18 male and female pregnant Wistar ratswere studies at All India Institute of Medical Sciences, NewDelhi. Rats had free food and water in a controlled environment. Cresyl Violet staining and immunohistochemistry required anaesthesia, perfusion, fixation, cryoprotection, and slicing. Cresyl Violet staining analysed hippocampus cytoarchitecture, while immunohistochemical staining detected ER-α and ER-β. The study used 6 animals at each stage/days (P15, P40, P60) to mimic newborn, prepubertal, and pubertal human brain development. **Results:** CV staining showed Wistar rats hippocampus cellular architecture, distinguishing Dentate Gyrus(DG) and Cornua Ammonis(CA) with granule and pyramidal cells. Subfields CA1, CA2, and CA3 had separate strata in CA's U-shaped fold. The dentate hilus showed age-related alterations at P15, P40, and P60. ER-βlocalization in CA and DG varied with age, with IR at P60. ER-β, localization showed age-related alterations and subtype-specific irritation. The paper details hippocampus structure and ER expression dynamics during development. **Conclusion:** In adult hippocampus, ER-α and ER-β are often expressed in CA3 and less so in CA1. Steroid hormone-ER interactions may occur throughout prepubertal and pubertal development.

INTRODUCTION

Steroid hormones known as estrogens are produced in the gonads and several other tissues, including the brain. The most potent and common type of estrogen, 17β-estradiol (E2), has several impacts on brain function and cognition. The amount of E2 affects several cognitive functions, and either or both of an E2 concentration causes cognitive impairment in the ideal range.[1]

Studies of memory and learning in rat models, the findings of which are consistent with observations in women, have provided us with a large amount of current knowledge on the processes behind estrogen's impact on cognitive function. For instance, the animal's age, the amount of E2, and the duration of the estrogen decrease before starting E2 treatments all significantly impact the efficacy.[2] Using E2 to improve ovariectomized patients' performance in memory & spatial learning tasks rats. In these experiments, it was shown that E2 therapy, when started in middle age, improved early (3 months) ovariectomized rats' spatial memory but not when started at later ages. However, therapies failed to increase spatial working memory at 17 to 21 months in rats with ovariectomies between 12 and 13 months.[3] The reduced ability of E2 to improve memory in these rat's experiments is consistent with the clinical observations of ERT and indicates that the root cause or mechanisms responsible for the efficaciousness of ERT in enhancing the ability to remember in women are probably complex and contingent on an individual's age and the length of their hormone deprivation. Through fast membrane communication and gene transcription, E2 affects brain function.[4]

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Furthermore, the complicated dose-response pattern is partly explained by the several transcription or processes of post-translational feedback that control E2 activity. Age-related declines in E2 impact several biological processes, including blood-brain barrier maintenance, are well-documented, shield the brain from stroke and neuroinflammation, encourage the growth of the glutamatergic synapses, and activate rapid signalling cascades. The mechanism(s) underlying the decrease in the capacity of ERT to maintain cognitive safety as people age is unclear.[5]

The two main estrogen receptors through which E2 is anticipated to carry out most of its biological effects are estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ). Moreover, a second messenger route modulator has been discovered: GPR30, sometimes called GPER1, is an estrogen receptor coupled with G proteins.[6] Different brain areas have different relative concentrations and subcellular distributions of the nervous system's ERα, ERβ, and GPER1 molecules. ERα is less common than ERβ in the hippocampus areas of both humans and rats; ERα is more likely to be located in the nucleus. It has been suggested that variations in subcellular distribution and structure may account for the distinct biological functions of estrogen receptors and their varying effects on cognition.[7]

Membrane receptors, such as the GPER1 G-protein linked receptor and the connection between receptors and ERα and ERβ that are metabotropic, promote rapid signalling. These membrane receptors trigger many kinases, including B-RAF, IP3K, Src, ERK, AKT, PKA, & PKC, which are activated as well as Ca2+, phospholipase C, as well as adenylyl cyclase signalling.[8] These kinases can then quickly affect neurological function and phosphorylate transcription variables, such as CREB or ERα, which causes the transcription of genes. Therefore, E2 signaling connects physiological processes associated with growth factor signalling, learning, and memory.[9]

MATERIALS AND METHODS

Study Design

This study was conducted among the hippocampus of 18 Wistar rats, and it was selected for both male and female prenatal ages. In addition, this study was carried out in the Central Animal Facility of the All India Institute of Medical Sciences, New Delhi and maintained in a standard light and temperature-controlled environment. Rats hadad libetum access to food and water. On the other hand, the gestation period in rats was 22 days and birth occurs at embryonic (E) day was 22, also known as Postnatal day 0 (PND0). Similarly, this study followed the P15, P40, and P60 hippocampal specimen stages. Stage P15 was the developmental stage equivalent to the human brain of newborns, P40 was the Prepubertal stage, and P60 was the Pubertal stage. 6 animals were included in each stage or group.

Tissue Processing

Sodium Pentobarbital (50mg/kg.wt. i.p.) anaesthetized timed-pregnant Wistar rats of various ages. Transcardially (with descending aorta clamped), 30-40 ml of normal saline was perfused, followed by 100-250 ml of cold 4% paraformaldehyde in 0.1M PB at pH 7.4. Removed brains were postfixed overnight in the same fixative at 4°C. After cutting these into 4-5 mm coronal tissue blocks, they were cryoprotected in 10% and 30% sucrose. The tissue specimens (middle blocks) were cryostat-blocked in an OCT compound. The current investigation used brain blocks from the middle region (including the hippocampus), with the surplus preserved in a -70° deep freezer. The tissue blocks in this investigation were sliced into 10 and 30 µm cryostat slices. Cresyl violet (CV) staining was performed on ten µm thick sections, whereas immunohistochemistry was performed on 30 µm thick sections.

Cresyl Violet (Cv) Staining

Researchers examined the hippocampal cytoarchitecture under a light microscope using CV staining. Cryostat sections with a thickness of 10 µm were placed on gelatin-coated slides and allowed to dry at room temperature. After that, we followed this process to stain them using an aqueous CV stain. Two changes of distilled water passed each slide; even slides were examined by Microphot-FX Nikon Microscope, analyzed, photographed and transferred to 0.5 % aqueous solution of cresyl violet for 15 min.

Immunohistochemical Staining

The sections were stained using indirect immunoperoxidase as a processing step. To identify ER-α and ER-β, 30 µm thick cryostat sections were immersed in 0.1M phosphate buffer saline (PBS) and subsequently stained with specific monoclonal antibodies for mice and rabbits, respectively, using an immunohistochemical (Imht.) procedure. A free-floating processing method was used for the sections during the Imht technique. This study followed different procedures, such as washingthe sections in PBS (0.1M, pH 7.4). Incubated in methanol-hydrogen peroxide for 10 min to quench endogenous peroxidase. For 1 hour at RT, incubated with blocking solution containing 1% normal goat serum and 0.2% Triton X 100 in PBS (0.1M). Removed blocking solution. The primary antibodies ER-α (1:50) and ER-β (1:100) were incubated. Washing with PBS after primary antibody removal. Incubated with secondary antibody, goat-anti-mouse or goat-anti-rabbit IgG for ER-α or ERβ for 8 hours at 4°C. Wash twice with PBS. Incubated with monoclonal mouse or rabbit PAP (1:100) for 4 hours at 4°C for ER∝ or ERβ, respectively. Tape off PAP and 2X PBS wash. For ten min., incubated with substrate-chromogen reaction mixture (3,3' diaminobenzidine) at RT in the dark to create a final colour product. Wash with tap water, then distilled.

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Placed dyed sections on gelatin-coated slides and dried overnight. Dehydrated mounted portions with increasing ethyl alcohol. Xylene-cleared. DPX-mounted and microscoped.

RESULTS

Cellular Architecture (Cv Staining)

Tissue slices stained with CV were examined here. In coronal sections of the hippocampus (Figure 1 and 2), their compactly arranged granule cell layers can easily distinguish the DG and CA and pyramidal cells that extend from dentate hilus (DH) to subiculum.

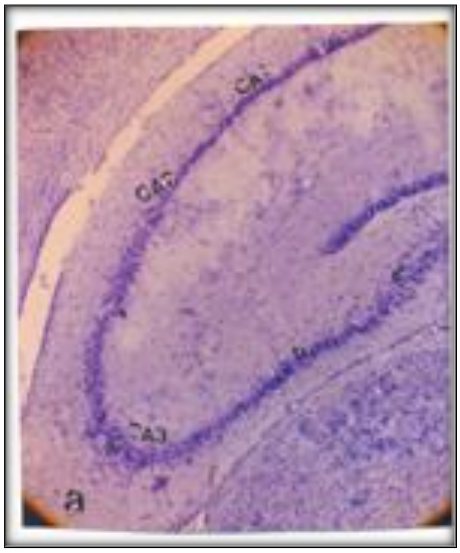

Figure 1: Low magnification photomicrograph of CV stained coronal section of hippocampus showing CA region

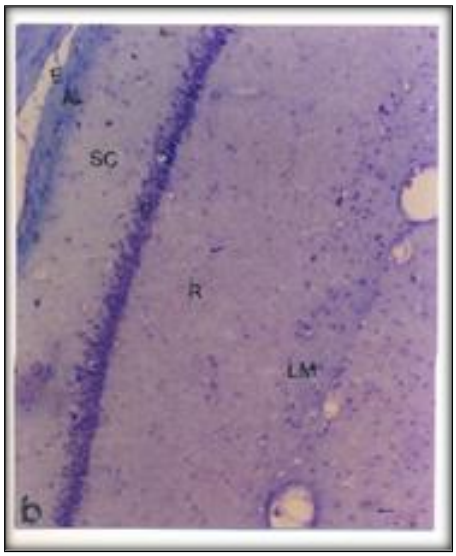

Figure 2: High magnification photomicrograph of CV stained coronal section of hippocampus showing CA1 region (with a narrow pyramidal layer. Note: Various layers: E (Ependyma), AL (Alveus), SO (Stratum Oriens), P (Stratum pyramidale), R (Stratum radiatum), LM (Stratum lacunosum moleculare). Scale Bar - 50 □m

Cornua Ammonis (CA)

The Ammon's Horn, or CA, is characterised by a unique U-shaped fold of cortex that includes three subfields: CA1, CA2, and CA3. Layers within the structure include the ependyma that lines the ventricle. This alveus corresponds to white matter; the stratum oriens contains interneurons and basal dendrites, the stratum pyramidale houses major pyramidal neurons, the stratum radiatum has sparse apical dendrites, and the stratum lacunosum moleculare that has terminal tufts (Figure 3). Staining with CV reveals cytoarchitectural changes and different interneurons. Compared to CA3, which has a broad pyramidal layer, bigger principal neuron bodies, and separate zones (a, b, and c), CA1 has a small layer of densely packed, medium-sized cells.

CA2 occupies a middle ground between CA1 and CA3.

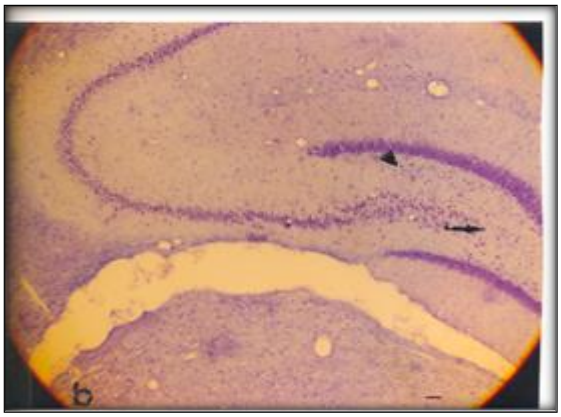

Figure 3: Cornua Ammonis with stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum

Dentate Gyrus (DG)

The dentate gyrus (DG) splits into three distinct areas: the crest, ectal, and endal limbs. Its structure is highly folded. The thalamus and CA1 are opposite sides of the crest, which joins the limbs. DG is made up of a three-layered cortex: one with a molecular layer that forms a cell zone towards the convexity, one with a granule cell layer that is mostly small-bodied granule cells densely organised, and one with a polymorphic layer that faces the concavity and houses medium to big neurons in different shapes. The Dentate Hilus (DH), formerly known as CA4, is located under the polymorphic layer and is composed of modified pyramidal cells, fusiform cells, and polymorph cells.

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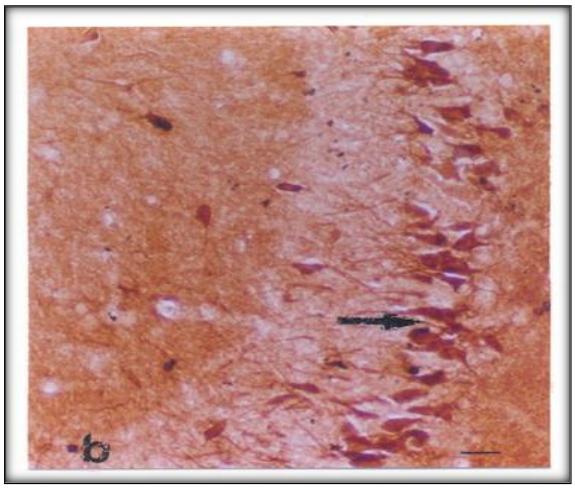

Figure 4: dentate gyrus (DG) with its three distinct areas

P15, P40 and P60

The CA and DG areas of the hippocampus were evident at all three postnatal ages. However, the two zones grew more prominent with age. However, CA cytoarchitectural changes were not significant between ages. P15 dentate hilus was extremely cellular. This zone was less cellular at P40 and P60 as age increased. The latter stage resembled the adult hippocampus.

ER-LOCALIZATION

ER-immunoreactivity (IR) was found in hippocampal neuronal perikarya and dendritic processes. CA3 had more ER-positive neurons than CA2-CA1, especially in stratum pyramidal and stratum oriens. In CA2-CA1, only interneurons showed ER-IR; however, in CA3, both principal (pyramidal) and interneurons did. Notably, CA3a has the most immunoreactive perikarya. The DG's granule cell layer included immunopositive perikarya, more prevalent in the limbs than the crest. IR neurons in DG limbs resembled interneurons, making it impossible to identify ER expression localised in interneurons or crest granule cells.

P15, P40 &P60

The immunoreactivity (Ir) pattern in developing rat hippocampus matched adult levels. Neuronal perikarya and processes showed irritability, peaking at P60 and decreasing at P15. Positive neurons were most prevalent in CA3a's stratum pyramidale at P60, while faintly stained Ir neurons were in CA3's stratum radiatum. Pyramidal neurons were irritable only in CA3, not CA2-CA1, although positive interneurons were detected across subfields. At P15, a few weakly stained neurons developed in the ectal limb's polymorphic layer in the dentate gyrus (DG), extending to all locations by P40 except the crest. DG limb irrity exhibited widespread granule cells and interneurons. In particular, P60 enhanced granule cell layer Irty. P60 had more ER-Ir expression than adults, and whereas prepubertal and pubertal phases had ER-Ir in granule cells, adults had it in the crest.

ER--βLocalization

Ir neurons were found in all hippocampus areas, including CA and DG, and ER- distribution was identical to that of rats. ER- immunoreactivity was found in neuronal perikarya, axons, and dendrites. Immunopositive neurons stain cytonuclearly. Ir neurons are most abundant in CA3 in all CA subregions, followed by CA2-CA1. In interneurons and principal cells, stratum pyramidale had the most positive neurons, followed by stratum oriens. Irty was found in CA3 pyramidal neurons but not CA2-CA1. Positive interneurons were found in all subfields. +ve perikarya and puncta were more common in CA3c than CA3a. All DG regions had some Irty. Ectal limb immunopositive neurons outnumbered endal limb neurons. Although we saw interneurons in both limbs, the granule cell layer has the most neurons. Few Ir neurons were seen in the crest and hilar areas.

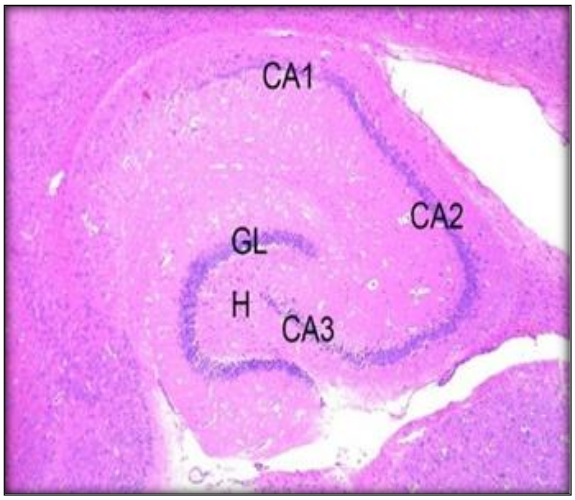

Figure 5: Hippocampus section of the specimen. CA1, CA1 pyramidal layer; CA2, CA2 pyramidal layer; CA3, CA3 pyramidal layer; H, hilus; GL, granular layer. (HE stain)

P 15, P40, & P60

ER- subtype irritability was prevalent at all ages. Irritation was higher at P15 than at P40 and P60. The expression decreased from P15 to P40 and P60. At P15, the stratum pyramidale of CA3 had the highest +ve perikarya, most of which were pyramidal neurons. Some immunopositive neurons were found in stratum oriens. CA2-CA1 had a few +ve interneurons in stratum pyramidale and oriens. DH had 1 or 2 weakly marked Ir neurons at P15. We found no immunopositive neurons elsewhere. DG did not show ER-Irty at P40 or P60. ER-Irty was low at above ages compared to adults, especially prepubertal and pubertal.

DISCUSSION

The nuclear ER, or estrogen receptor, mediates the physiological effects of estrogen on the pituitary, including hormone regulation and cellular

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proliferation production. The goal of this research was to use particular antibodies—a polyclonal antibody produced against ERβ and a monoclonal antibody called (1D5) for ERα—to evaluate the pituitary expresses both ERα and ERβ in an ontogenetic manner. Initially, we verified the identification of 66- & 55-kDa bands as ERα & ERβ, respectively, using Western blotting in adult rat pituitary extract. Subsequently, prenatal when adult Wistar rat tissues were immunostained with these antibodies and coupled with either PRL or LHβ immunohistochemical.[10] Strong On day 12 of the experiment, the pituitary showed an ERβ signal pregnancy. On the other hand, ERα staining was only noticeable starting on day 17 of pregnancy. ERβ-positive cells were confined to the adult rat's anterior lobe, but ERα-positive nuclei found extensively dispersed in the same region, in contrast to the fetal stage. ERβ-positive cells showed colocalization of LHβ but not PRL. According to our findings, most ER isoforms inside the rat pituitaries have undergone changes since birth. ERβ Expression could be involved in pituitary cell differentiation functions to produce a specific hormone.[11]

Immunohistochemistry was used to look at the rat hypothalamus's estrogen receptor (ER) developmental expression. ER-immunoreactivity was seen in the ventromedial and medial preoptic nuclei as early as E17. However, ER expression of proteins was not seen until E19 for the arcuate nucleus and the periventricular preoptic nuclei. These findings demonstrate that sex variations in ER levels begin to be noticeable during the perinatal stage after an ER protein expression unique to a certain location.[12]

Using immunocytochemistry (ICC) and a polyclonal antibody (PG-21), researchers looked at the ontogeny of an androgen receptor (AR) in Sprague-Dawley rats' hippocampal tissues. At each age encompassing the newborn (PND 2, 5, 7), junior (PND 14), prepubertal (PND 25), or pubertal (PND 40) periods, the brains of a total of five male and female siblings were investigated, with one male and female tested at PND 60. To search for a potential ligand-dependent increase in staining, two sets of PND 7 & PND 25 male and female littermates had injections with dihydrotestosterone ester (DHTB) 30 minutes before to perfusion.[13] As DHTB therapy does not cause a nuclear translocation as seen in adult animals, and AR density is not correlated with blood testosterone (T) levels; it is inferred that mechanisms other than circulating androgen may govern neonatal and prepubertal hippocampus AR. Instead, the AR is uniformly distributed throughout the cell independent of androgen therapy. As a result, the roles and regulations of neonatal and adult AR may differ. -- Key words: hippocampal ontogeny; androgen receptor (AR); antibodies (ICC); PG-21; dihydrotestosterone benzoate (DHTB).[14]

Estrogens enhance dendrite arborization, permanent potentiation, and neuroprotection and are involved in various processes linked to memory, learning, and emotional responses in the hippocampus. It has been shown that the hippocampus has both the alpha (ERα) as well as beta (ERβ) versions of the estrogen receptor and that these receptors have distinct physiological functions there.[15] This work aimed to examine the expression pattern of ERα and ERβ in the rat hippocampal tissues during the oestrous cycle utilizing immunohistochemistry and Western blot. The reactivity of the antibodies utilized against ERα and ERβ, as well as their relative presence in the hippocampal region, were verified by Western blot analysis.[16]

Immunohistochemical study results show that over the whole oestrous cycle, ERβ expression in the CA1 and CA3 areas rose higher than ERα. The majority of ERβ immunoreactivity was expressed in CA1 during metestrus and oestrous and CA3 during diestrus. It was primarily found in the nucleus. In the CA1 region, ERα was more prevalent during estrus than other periods of the reproductive cycle, but in the CA3 region, it was more prevalent during metestrus.[17] It's interesting to note that the ERα subtype had both nuclear and cytoplasmic immunolocalization. The total data show that both ER subtypes have distinctive expression patterns, cellular locations, and distributions in CA1 and CA3 areas, indicating that these two receptors have distinct functions in the hippocampus during the estrous cycle.[18]

An increase in spines in the apical dendrites of CA1 pyramidal cells following systemic administration for estradiol to ovariectomized rats was a standard indicator of estrogen-induced synaptic plasticity. Recent research doubts estradiol's direct endocrine control of synaptogenesis.[19] For the first time, we have demonstrated that rat hippocampus neurons undergo de novo synthesis of estrogens. An aromatase inhibitor called letrozole was utilized. Drastically reduce the amount of estrogen in both hippocampus dispersion cultures and slice cultures. The number of sensorimotor boutons and the abundance of spines & spine synapses significantly decreased in response to letrozole therapy.[12] The hippocampal region's dose-dependent downregulation of the spine and presynaptic markers, spinophilin and synaptophysin, was shown by quantitative immunohistochemistry. Surprisingly, the cells showed no reaction when estrogen was applied exogenously.[21] This phenomenon may be explained by subcortical nuclei acting as intermediaries for the indirect effects of estrogens. After seven days of therapy, the insertion of estrogen-filled cannulae within the hippocampal projection, the median raphe, resulted in an impressive rise in hippocampal spine density.[22]

The number of neurons of serotonergic input in the CA1 region's stratum lacunosum molecular & radiatum decreased in tandem with this rise. In addition to being controlled endogenously, our

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results indicate that indirect mechanisms and direct endocrine processes mediate estradiol-induced spinogenesis within the hippocampus.[23]

CONCLUSION

This study has concluded that ER-α -and ER-β, subtypes are distributed similarly in the adult hippocampus, with the greatest +ve perikarya in CA3 and minimum in CA1. Both receptors are found in pyramidal and interneurons in CA3, although only in CA2-CA1. DG granule cells lack Irty. However, certain interneurons are positively labelled in both DG and DH. ER- and ER- subtype Ir expression in younger hippocampus differs greatly. ER-Irty increased steadily from P15 to P60, peaking even compared to adults. ER-Irty decreased from P15 to P60, being nearly negligible at the later age. These findings imply that steroid hormone-ER-subtype interactions may affect hippocampal neuronal activity during prepubertal and pubertal alterations. While early growth and adult activities may involve interactions with both groups. Estrogen receptor (ER) dynamics in the adult hippocampus are well understood, but prepubertal and pubertal developmental changes are not. The study suggests age-related ER expression changes but does not explain the causes or functional implications. The complicated dose-response pattern of 17β-estradiol (E2) in hippocampus development is still unclear. Future study should investigate the complex molecular mechanisms that regulate hippocampal oestrogen receptor ontogenic changes. Investigating the transcriptional pathways involved in E2's dose-response pattern during different developmental stages may provide greater insight. Exploring the functional effects of age-related ER expression variations on hippocampal-dependent behaviours and cognitive functioning would help fill the information gap. Integrating molecular, cellular, and behavioural research will help explain the dynamic relationship between steroid hormones and hippocampus development.

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