Horm Mol Biol Clin Invest 2011;7(2):361–375 © 2011 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/HMBCI.2011.118

Regulation of synaptic plasticity by hippocampus synthesized estradiol

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Abstract

Estradiol is synthesized from cholesterol in hippocampal neurons of adult rats by cytochrome P450 and hydroxysteroid dehydrogenase enzymes. These enzymes are expressed in the glutamatergic neurons of the hippocampus. Surprisingly, the concentration of estradiol and androgen in the hippocampus is significantly higher than that in circulation. Locally synthesized estradiol rapidly and potently modulates synaptic plasticity within the hippocampus. E2 rapidly potentiates long-term depression and induces spinogenesis through synaptic estrogen receptors and kinases. The rapid effects of estradiol are followed by slow genomic effects mediated by both estrogen receptors located at the synapse and nucleus, modulating long-term potentiation and promoting the formation of new functional synaptic contacts. Age-related changes in hippocampally derived estradiol synthesis and distribution of estrogen receptors may alter synaptic plasticity, and could potentially contribute to age-related cognitive decline. Understanding factors which regulate hippocampal estradiol synthesis could lead to the identification of alternatives to conventional hormone therapy to protect against age-related cognitive decline.

Keywords: aging; estradiol; estrogen receptor; hippocampus; hormone therapy; long-term depression; long-term potentiation; neurosteroid; selective estrogen receptor modulator; spine; synaptic plasticity.

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Received June 17, 2011; accepted July 21, 2011; previously published online August 29, 2011

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	androgen receptor
CREB	cyclic-AMP response element binding
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DPN	diarylpropionitrile
E2	estradiol
ER	estrogen receptor
EPSP	excitatory postsynaptic potential
ERK	extracellular signal regulated kinase
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
HSD	hydroxysteroid dehydrogenase
LTP	long-term potentiation
LTD	long-term depression
MAP kinase	mitogen-activated protein kinase
NMDA	N-methyl-D-aspartic acid
OVX	ovariectomy
P45017α	P450 17 α-hydroxylase
P450 arom	P450 aromatase
P450scc	P450 side chain cleavage
PI3K	phosphoinositol-3-kinase
PD	post-natal day
PREG	pregnenolone
PKC	protein kinase C
PPT	pyrazole-trinyl-phenol
SERM	selective estrogen receptor modulator
StAR	steroidogenic acute regulatory protein
T	testosterone
TSPO	translocator protein receptor

Introduction

Estradiol (E2), an important modulator of synaptic plasticity, is synthesized in the hippocampus, a center of learning and memory [1–5]. Hippocampal sex steroids had long been thought to be synthesized in the gonads, reaching the brain via the blood circulation [6], since key steroidogenic enzymes had not been successfully demonstrated in the mammalian brain over many decades, due to extremely low levels of expression [7–9]. However, the expression and activity of the complete steroidogenic machinery necessary for de novo E2 synthesis has now been demonstrated in the hippocampus. Although the P450 and hydroxysteroid dehydrogenase (HSD) steroidogenic enzymes are expressed at very low levels in the hippocampus compared to classic endocrine organs such as the testes or ovary, unlike the classic endocrine actions of E2, where high concentrations of E2 are synthesized by the ovary and diluted in the circulation to be transported to distal target organs, small quantities of hip-

pocampally synthesised E2 can result in large effects on brain function through synaptocrine, autocrine and paracrine actions.

Hippocampal synthesis of estradiol

Technical limitations in the purification of neurosteroid hormones from fatty brain tissue have hampered efforts to quantify E2 levels in the hippocampus. However, recent advancements in the development and validation of sensitive and specific measurement of neurosteroid levels, combining pre-purification with high performance liquid chromatography and mass spectrometry with picolinoyl derivatization, have opened doors for these questions to be addressed in specific brain regions including the hippocampus (Figure 1) [10]. Surprisingly, contrary to the widely held belief that brain E2 levels are very low compared to the periphery, in male young adult rats, E2 concentrations were found to be much higher in the hippocampus (~8 nM) compared with

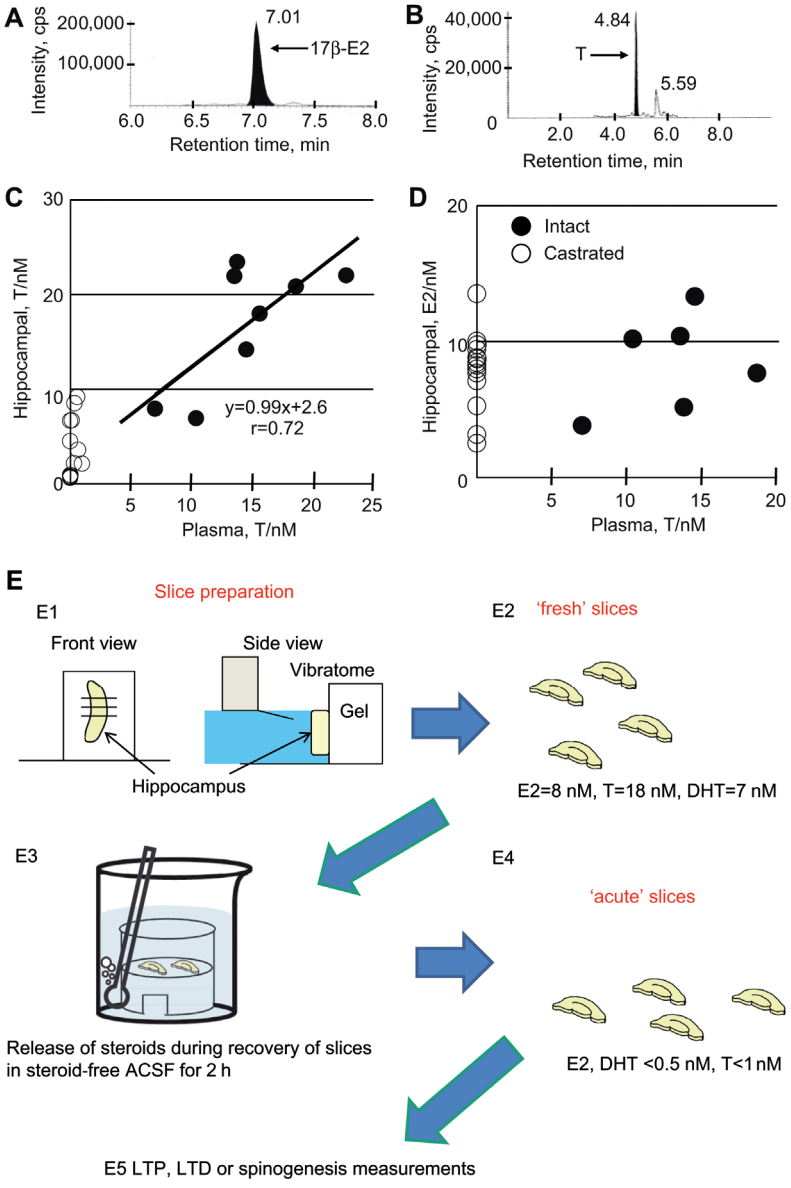

Figure 1 Mass-spectrometric determination of hippocampal sex-steroids. LC-MS/MS chromatograms and steroid derivatives of (A) E2 and (B) T. Shaded portions indicate the intensity of the fragmented ions of 17β-E2-pentafluorobenzoxy-picolinoyl (m/z=39, A) and T-picolinoyl (m/z=253, B), respectively. The vertical axis indicates the intensity of the fragmented ions. The horizontal axis indicates the retention time of the fragmented ions, t=7.01 min for E2 and t=4.84 min for T. The time of injection to the LC system was defined as t=0 min. (C) Reduced plasma and hippocampal T levels following castration. (D) Despite depleted hippocampal T, no effect of castration on hippocampal E2 in young-adult male rats. (E) Schematic illustration of the preparation of 'acute' hippocampal slices resulting in significant depletion of endogenous sex steroid concentrations. 'Acute' hippocampal preparations are widely used for measurement of LTP, LTD and spinogenesis (modified from [10]).

plasma (~0.014 nM) [1, 3, 10]. Hippocampal E2 levels in male rats were even higher than hippocampal E2 concentrations in young adult female rats (~1.7 nM at proestrus) [10]. In the female rat, hippocampal E2 concentrations fluctuated throughout the estrus cycle, ranging from peak concentrations of ~1.7 nM at proestrus to concentrations as low as ~0.2 nM E2 at diestrus [10]. Castration had little effect on male hippocampal E2 levels, despite negligible plasma and brain testosterone (T) levels, suggesting that hippocampal E2 is primarily synthesized locally rather than from gonadal T (Figure 1) [10].

Local hippocampal E2 synthesis is catalyzed by the P450 and HSD enzymes, requiring the sequential conversion of cholesterol → [P450 side chain cleavage (P450scc)] → pregnenolone (PREG) → [P450 17 α-hydroxylase (P45017α)] → dehydroepiandrosterone (DHEA) → [17β-HSD] → androstenediol → [3β-HSD] → T → [P450 aromatase (P450 arom)] → E2 (Figure 2) [3, 10]. T may also be synthesized from DHEA through an alternative pathway: DHEA → [3β-HSD] → androstenedione → [17β-HSD] → T. T not only forms a substrate for E2 synthesis, but is also converted to dihydrotestosterone (DHT) by 5α-reductase. The complete complement of steroidogenic enzymes necessary for E2 synthesis are expressed in hippocampal gultamatergic neurons in the CA1-3 regions and the dentate gyrus, as demonstrated by PCR, in situ hybridization, immunohistochemistry and Western blot analyses.

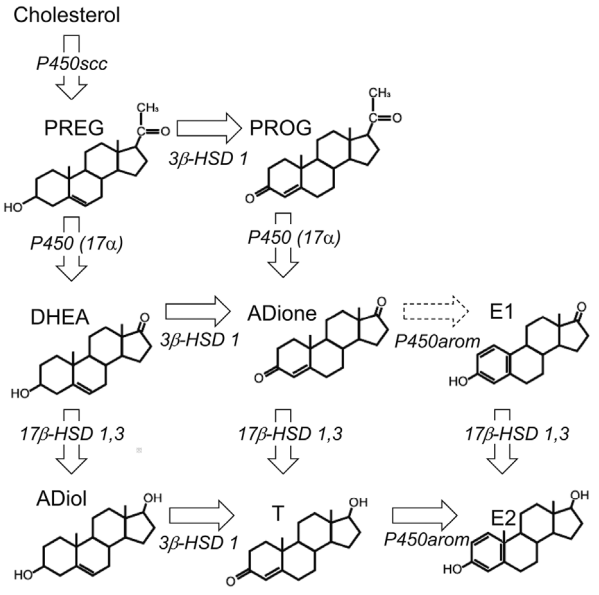

Figure 2 Hippocampal E2 biosynthesis pathways. Pathway of sex-steroid synthesis in the rat hippocampus, thus far identified. Cytochrome P450scc is expressed at extremely low levels in the adult hippocampus, therefore PREG synthesis from cholesterol may be very slow. In addition to endogenously synthesized PREG, approximately half of hippocampal PREG is incorporated from the circulation. Only 3β-HSD type 1 is present, other types of 3β-HSD (types 2–4) are absent in the hippocampus. Androstenediol (ADiol) is the major product of DHEA. Although sex differences in hippocampal neurosteroid synthesis require further investigation, in the male rat, adione conversion to estrone (E1) is very weak (indicated by the dashed arrow). However, in the female rat adione may be converted to E1 as efficiently as adione to testosterone (T). Once hippocampal E2 is produced, it is not readily inactivated. PROG, T and E2 are also significantly incorporated into the hippocampus from the circulation, since gonadectomy decreases hippocampal concentrations of these hormones between 50% and 80% (modified from [11]).

Transport of cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone, is the rate limiting step in neurosteroid production; this process is regulated by the translocator protein receptor (TSPO) and steroidogenic acute regulatory protein (StAR) [12]. TSPO is expressed in steroid synthesizing tissue including the brain [13], found predominantly in microglia, reactive astrocytes [14–16] and some neuronal cells [17–21]. While hippocampal TSPO expression in the hippocampus of rat [22, 23] and humans [24] has been demonstrated with radioligand binding studies, cellular localization of TSPO in the hippocampus is yet to be clarified. StAR is expressed in the hippocampal principal neurons of CA1–3 and the dentate gyrus of rats [1–3, 5, 23, 25, 26] and mice [27]. However, little hippocampal colocalization was observed between astrocyte marker glial fibrillary acidic protein (GFAP) and StAR immunoreactivity [1, 5]. StAR mRNA has also been identified in the human hippocampus by RT-PCR [28].

Once localized at the inner mitochondrial membrane, cholesterol is converted to PREG by the mitochondrial specific enzyme, P450scc. PREG is the precursor to all other neurosteroids and the most abundant neurosteroid hormone in the brain. Early studies indicated that glial cells may contribute significantly to neurosteroidogenesis, with extensive P450scc immunolabeling of glia observed throughout the adult rat brain with anti-P450scc antisera [29]. However, while subsequent studies have demonstrated that cultured glial cells synthesize PREG from cholesterol [30], in the adult male rat hippocampus, little P450scc immunolabeling of GFAP positive astrocytes is observed with purified P450scc antibodies [1]. This suggests that neurons may be the primary site of hippocampal PREG synthesis in the healthy adult rat brain [1]. P450scc expression is colocalized with StAR immunoreactivity in pyramidal and granule neurons of the hippocampus of adult male rats [1, 3, 31]. In the human brain, P450scc mRNA has also been observed in the amygdala and hippocampus [28, 32, 33], with significantly higher levels observed in women compared to men [32, 34].

Although DHEA concentrations are much higher in the brain than in plasma [35–37], P45017α, the microsomal enzyme which converts PREG to DHEA, was thought to be absent from the mammalian brain, since early studies failed to detect P45017 mRNA [9], protein [8] or activity [38]. With improved RT-PCR sensitivity, subsequent studies detected P450c17 mRNA in the embryonic rodent brain [39], neonatal astrocytes and neuronal cultures [40, 41] and adult rat hippocampus [3]. Immunohistochemistry studies demonstrated P45017α localization in the pyramidal cells of CA1–3 and dentate neurons of the hippocampus [3]. Immunoelectron microscope studies, using postembedding immu-

nogold, demonstrated that P45017α was localized in not only the endoplasmic reticulum of hippocampal neurons, but also presynaptic and postsynaptic terminals [3]. In the axon terminals, P45017α immunolabeling was associated with synaptic vesicles, while in dendritic spines, labeling was localized within the head of the spine [3].

The conversion of DHEA to T is catalyzed via two pathways; the majority of hippocampal T is produced via the conversion of DHEA → [17β-HSD] → androstenediol → 3β-HSD → T; however T may also be synthesized through the conversion of DHEA → [3β-HSD] → androstenedione → [17β-HSD] → T [3, 10]. RNA expression of 17β-HSD types 1, 3 and 4 have been demonstrated in human hippocampus [34], while types 1–4 have been shown in rat hippocampus [3] by RT-PCR. Immunohistochemical studies have shown 17β-HSD type-1 immunolabeling of principal neurons in rat hippocampus [42]. Likewise, 3β-HSD transcripts have been demonstrated in hippocampal principal by in situ hybridization [25].

P450arom, typically located in the endoplasmic reticulum, catalyzes the conversion of T to E2. In the rodent brain, expression of P450arom has been demonstrated in hippocampus [43], localized in the pyramidal and granule neurons of the hippocampus via in situ hybridization and immunohistochemistry [3, 44, 45]. In the healthy rodent brain, P450arom is expressed primarily in neurons [3, 46–49], although P450arom expression is observed in cultured glia [50–52], glioma cell lines [53] and in astrocytes following injury [54–56]. Therefore, it is possible that aromatase expression is induced in astrocytes following injury or under pathological conditions as a neuroprotective mechanism [56, 57]. P450arom mRNA [58, 59] and protein [60, 61] has also been observed in human hippocampus. Unlike rats, P450arom is also expressed in a subpopulation of astroglial cells in the normal human brain [62]. Despite this, immunohistochemical studies have demonstrated that humans [60] and monkeys [61] have a similar pattern of hippocampal P450arom localization to the rat, with P450arom immunolabeling observed in the pyramidal neurons of CA1–3 and granule neurons of the dentate gyrus.

In situ and immunohistochemical studies indicate that in the rodent hippocampus, CA1–3 pyramidal neurons and granule neurons of the dentate gyrus are the predominant steroidogenic cells, expressing the complete steroidogenic machinery for E2 synthesis. To establish the functional activity of the hippocampal steroidogenic enzymes, de novo synthesis of DHT, T and E2 from radiolabeled precursors in adult male rat hippocampal slices was demonstrated [1, 3, 10]. Neurosteroid synthesis was abolished with specific pharmacological inhibitors of the P450 enzymes [1, 3]. Likewise, de novo E2 synthesis has been demonstrated in dissociated neuron [63] and hipppocampal slice cultures [4]. In these systems, E2 synthesis could be inhibited with the P450arom inhibitor, letrozole [64]. In acute hippocampal slice preparations, synthesis of E2 from T was slow, however, E2 was not readily converted to other metabolites or degraded, indicating that upon synthesis, E2 may be relatively stable [3, 10].

Developmental changes in the expression of steroidogenic enzymes and sex steroid receptors

Neurosteroidogenesis has been believed to be only transiently active during fetal and neonatal development, becoming inactive in the adult [65]. While expression of P450scc is markedly downregulated in the adult compared to postnatal rat hippocampus [66], despite the low levels of necessary steroidogenic enzyme, PREG is abundant in adult rat hippocampus [39]. On the other hand, only gradual decreases in expression of downstream steroidogenic enzymes, including 3β-HSD, 17β-HSD, P45017α and P450arom, are observed in adult the hippocampus, with adult hippocampal mRNA levels decreasing by 30%–50% of levels observed in rat hippocampus at postnatal day 1 (PD1) (Figure 3) [66, 67]. This gradual decrease in expression of steroidogenic enzymes is accompanied by a moderate decrease in the rate of sex-steroid metabolism in the adult hippocampus, with

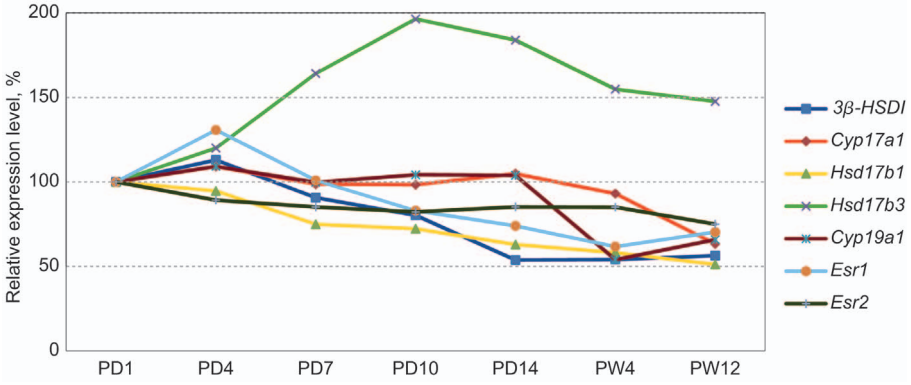

Figure 3 Developmental changes in the hippocampal mRNA expression of ER and steroidogenic enzymes. Hippocampal expression of 3βHSD1, 17βHSD type1 (gene: Hsd17b1), P45017α (gene: Cyp17a1) and P450arom (gene: Cyp19a1) gradually declined between postnatal day (PD) 1 and postnatal week (PW) 12. Expression of 17βHSD type 3 (gene: Hsd17b3) increased moderately in PW12 rat hippocampus compared to PD1. Expression of ERα (gene: Esr1) and ERβ (gene: Esr2) also gradually declines to approximately 30% of PD1 levels in the adult rat (PW12) (modified from [66]).

neurosteroidogenesis in the mature adult between 2- and 7-fold lower than PD10 rat hippocampus [67].

Concomitantly, estrogen receptors (ER) also gradually decreases during development by approximately 30% of PD1 levels in the adult rat hippocampus [66]. In contrast, androgen receptor (AR) levels gradually increase over three-fold PD1 levels in the adult rat hippocampus [66]. The abundance of ER in the postnatal rat hippocampus may reflect the important organizational effects of E2 during development, while in the adult male rat hippocampus, androgens may play a more important role in activational hormone effects.

Regulation of hippocampal E2 synthesis

Although it is clear that the regulation of plasma and brain E2 concentrations is not necessarily synergistic, the relationship between the two is not well understood and factors regulating hippocampal neurosteroidogenesis remain largely unknown. The regulation of brain neurosteroid synthesis has been investigated more extensively in bird and amphibian models, since higher neurosteroid production allows easier quantification [68]. In rodents, synaptic activity and excitation may induce transient increases in hippocampal E2 synthesis through N-methyl-D-aspartic acid (NMDA) activation [1]. In isolated hippocampal mitochondria, NMDA increases levels of the active form of StAR [1], while in hippocampal slices, NMDA application increases PREG and E2 levels two-fold [1, 3, 31]. Changes in hippocampal E2 synthesis may also contribute to the regulation of complex hippocampal-dependent behaviors, for example, increased hippocampal E2 levels and mRNA expression of StAR, P45017α 17βHSD and P450arom are observed in rats following social isolation [69]. Further, some factors regulating gonadal E2 synthesis may also play a role in hippocampal E2 synthesis, for example, retinol not only stimulates increases in testicular P45017α and T and E2 production, but also dose dependently increased E2 levels in the hippocampus [70].

Hippocampal E2 neurosteroidogenesis may also be upregulated in response to injury and disease. In astrocytes, neurosteroidogenic machinery including TSPO [71] and P450arom [56] are upregulated following injury. In Alzheimer's disease sufferers, increased brain mRNA levels of neurosteroidogenic enzymes are observed compared to age-matched cognitively normal controls, perhaps indicating a compensatory increase in synthesis of neuroprotective hormones [72]. However, hippocampal E2 metabolism may also be disrupted by neuropathology, with impaired neurosteroidogenesis observed in cell lines treated with the toxic Alzheimer's beta amyloid peptide and oxidative stress [73, 74]. Abnormal expression of steroidogenic enzymes, including StAR, is also observed in malignant glial tumors [75], however, whether increased neurosteroidogenesis contributes to pathology in these cell lines, is unclear.

Hippocampal ER expression

The classic ER subtypes, ERα and ERβ, are thought to mediate hippocampal E2 effects on synaptic plasticity, although receptor-independent mechanisms have also been proposed. Cell membrane ER elicits rapid, non-genomic, signalling cascades, including activation of ERK/mitogen-activated protein kinase (MAPK), phosphoinositol-3-kinase (PI3K), protein kinase C (PKC) and Src/extracellular signal regulated kinase (ERK) pathways. Activation of MAPK signalling can also contribute to slow-genomic effects through the phosphorylation of cyclic-AMP response element binding (CREB). Nuclear ER induces slow-genomic effects, altering transcription of target genes. In the hippocampus, nuclear and synaptic ERα has been demonstrated in pyramidal neurons of CA1 and CA3, and granule cells of the dentate gyrus, using post-embedding immunogold electron microscopic analysis [76]. Western blot analysis has demonstrated that synaptic ERα is tightly associated with the postsynaptic density (Figure 4) [76]. In this study, non-purified ERα antisera was found to react significantly with an unknown 62 kDa protein in the brain, therefore affinity-column purified anti-ERα antibody RC-19 was used (Figure 4A). The specificity of the purified antibody to the 67 kDa ERα protein was confirmed by Western blot and MALDI-TOF mass spectrometry analysis [76]. Previous studies using unpurified ERα antisera reported ERα-immunoreactivity in the nuclei of inhibitory gamma-aminobutyric acid (GABA)-ergic interneurons, therefore it had been thought that E2 targeted interneurons [77–79]. However, the use of purified antibody confirms synaptic and nuclear ERα in principle glutamatergic neurons of the hippocampus. Several investigations post-embedding immunogold electron microscope analysis, also suggest extranuclear expression of ERβ, localized at dendritic spines in CA1 principal neurons of rat [80, 81] and mouse hippocampus [82].

AR has also been demonstrated in the hippocampus. Immunoelectron microscopic analysis demonstrates that AR is localized not only in cytoplasm and nuclei, but also in axon terminals and spines [83]. AR mRNA and protein are predominantly expressed in CA1 hippocampal neurons, with lower levels of expression observed in CA3 and DG [84, 85]. Synaptic localization of AR suggests androgens, like E2, may also play an important role in the rapid modulation of synaptic plasticity.

Regulation of synaptic plasticity

E2 modulates hippocampal LTP and LTD

Electrophysiological investigations demonstrate that E2 modulates long-term potentiation (LTP) and long-term depression (LTD), both of which play a critical role in the cellular mechanisms underlying memory formation and erasure, respectively. E2 has been implicated in slow enhancement of LTP through genomic effects [86, 87]. Rapid modulation of LTP also occurs following E2 administration [88–90]. It is possible that the rapid effects of E2 on LTP may be age-dependent. In the hippocampus from 4 to 6 weeks old or 200–350 g (approx. 6–8 weeks old) Sprague-Dawley rats, perfusion of 1–10 nM E2 rapidly increased basal excitatory postsynaptic potential (EPSP) (thereby enhancing LTP) at CA1 synapses. In 4-week-old Wistar rats, rapid basal EPSP

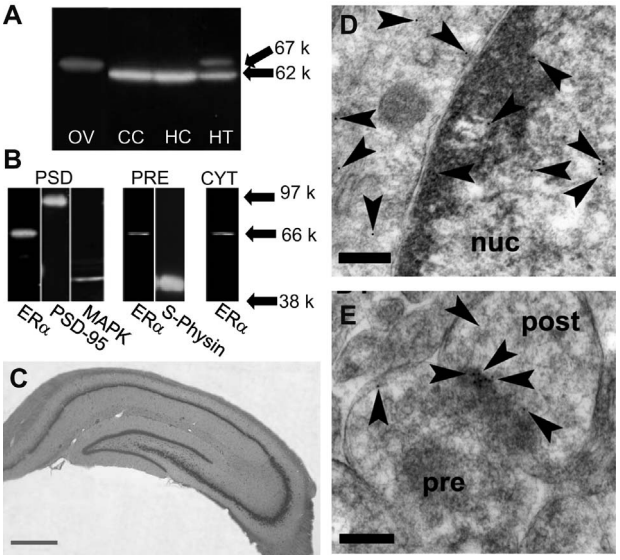

Figure 4 Synaptic and nuclear localization of ERα in hippocampus. (A) Western immunoblot with non-purified MC-20 antisera revealed a single 67 kDa band in the ovary (OV), but a second, with an unknown 62 kDa protein, was observed in the cerebral cortex (CC), hippocampus (HC) and hypothalamus (HT). (B) Western immunoblotting with column purified RC-19 antibody single 67 kDa ERα band in postsynaptic density (PSD), presynaptic membrane (PRE) and cytosolic (CYT) fractions of the hippocampus. (C) ER immunoreactivity in CA1-CA3 pyramidal neurons and granule cells of dentate gyrus using RC-19 antibody. (D) Immunoelectron microscope analysis demonstrating extensive ERα (arrowheads) in the nuclei (nuc) of CA1 neurons. (E) Gold particles (arrowheads) indicating ERα at the pre and postsynaptic regions in CA1 neurons (modified from [3]).

elevation upon E2 perfusion was sometimes (<20% probability) observed [91, 92]. On the other hand, 1–10 nM E2 did not affect basal EPSP and LTP in the hippocampus of 3-month-old adult Wistar rats [76, 92, 93]. No rapid basal EPSP elevation upon E2 perfusion was observed in 3–5 or 18–24 months old Sprague-Dawley rats [94]. Therefore, E2 may have significant effects on basal EPSP of younger rats (4–8 weeks old) and may not have an effect on older adult rats (12 or more weeks old). In 3–4 weeks old (early puberty) rats, E2 even suppressed LTP down to the similar level to that of 12 weeks old rats [93, 95]. In these investigations, high-frequency tetanic stimulation is used for LTP-induction, in which the phosphorylation of AMPA receptors by CaM kinase II is a dominant process at the high Ca^{2+} concentration (approx. 10 μM) and ERα may not play an important role in LTP induction.

E2 also rapidly enhances hippocampal LTD. In adult hippocampus, LTD is experimentally induced by the transient application of NMDA, which results in a moderate Ca^{2+} influx and subsequent activation of phosphatase and dephosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [96]. E2 perfusion significantly enhanced LTD in adult rat hippocampal slices, decreasing the plateau EPSP amplitude in CA3-CA1 synapses 60 min after LTD-initiation by 30 μM NMDA application [76]. E2 may enhance LTD through ERα, since the ERα agonist, pyrazole-trinyl-phenol (PPT), also enhances LTD in the CA1 [97]. In contrast, the ERβ agonist, diarylpropionitrile (DPN), elicits the opposite effect, suppressing LTD in the CA1 [97]. E2-bound ERα may activate phosphatase under moderate Ca^{2+} concentrations induced upon transient NMDA application and LTD induction [98], thereby facilitating the dephosphorylation of AMPA receptors and enhancing LTD. However, the role of classic ER in E2-induced rapid modulation of electrophysiological properties has been questioned, since these effects of E2 are not inhibited by the ER antagonist, ICI-182780 (ICI) [99]. ICI inhibits classic ER mediated signalling by preventing receptor dimerization, therefore, it is possible that E2 acts through ER without dimerization.

ERα knock-out mice may be useful in investigating the participation of ERα in the modulation of synaptic plasticity. Electrophysiological investigations in ERα knock-out mice reported no essential contribution of ERα to the E2-induced rapid enhancement of the kainate currents of CA1 neurons [99, 100]. ERα-Neo knock-out mice which have been constructed by the method of Neomycin insertion into exon 1 (the previously named exon 2) [101]. However, in ERα-Neo knock-out mice, N-terminal-modified ERα (61 kDa) is still expressed [101–103], which still binds E2 and drives genomic processes [101–103]. ERα knock-out mice, in which the whole exon 2 of the mouse ERα gene is deleted, may help to resolve the role of ERα in E2 mediated synaptic plasticity [104].

It should be considered that in the 'acute' hippocampal slice, used for in vitro investigations of LTP/LTD, steroid levels are depleted during recovery incubation in steroid-free artificial cerebrospinal fluid (ACSF) following sectioning. In the acute slice, concentrations of E2, T and DHT deplete to <0.5 nM, therefore, in this E2 depleted system, the effects

of very low E2 (1–10 nM) doses can be observed (Figure 1E) [10].

E2 induces hippocampal spinogenesis

E2 rapidly and potently alters hippocampal spine morphology and promotes spinogenesis, creating potential sites for new neuronal contacts. Slow, genomic modulation (over 1–4 days) of spinogenesis by E2 has been extensively studied through the manipulation of gonadal and exogenous E2 sources [105]. The first evidence that E2 may play a role in hippocampal spinogenesis came from the observations that hippocampal spine density fluctuates with E2 levels throughout the estrous cycle of female rats [106] and that ovariectomy (OVX) decreases spine density on CA1 pyramidal neurons [105]. OVX induced reductions in spine density are reversed by E2 supplementation [105–108], with spinogenesis observed as rapidly as 5 h after E2 injection [107]. In vitro, CA1 spine density increases following several days' treatment of cultured hippocampal slices with E2 [109].

More recently, E2 has been demonstrated to rapidly modulate hippocampal spine density and morphology, acting within 1–2 h of E2 application [76, 91, 110–112]. In acute hippocampal slices, application of a low dose of E2 (1 nM) increases spine density by 50% in the stratum radiatum of the CA1 region within 2 h [85]. In the acute hippocampal slice model, endogenous neurosteroid levels are depleted, with E2 concentrations $<1/10$th normal adult hippocampus E2 concentrations [10], allowing the effects of nanomolar concentrations of E2 to be examined. Rapid effects of E2 on spinogenesis, are also observed in vivo, E2 injection induces synaptic rearrangement in OVX rats as rapidly as 30 min post-injection [107].

E2 not only rapidly promotes increases in total spine density, but also induces distinct morphological changes. Analysis of morphological changes in spines is helpful to discriminate the different effects of steroid treatments. The effects of E2, T and DHT on total spine density are indistinguishable, however, when we assessed spine head diameter distribution, different morphological profiles of T, DHT and E2 treatments were observed. Conventionally, spine morphology is classified into mushroom (distinct head and neck), thin, stubby (no neck) and filopodium (no head). However, in adult hippocampal slices, the majority of spines ($>95\%$) have distinct heads and necks, while the populations of stubby spines ($\sim 5\%$) and filopodium ($\sim 1\%$) are very small. We have applied an alternative spine morphology classification determined by assessment of spine head diameter distribution to differentiate complex morphological changes.

To automatically identify spines and accurately determine their diameter for morphological classification, we developed Spiso-3D software (Figure 5). Spiso-3D identifies spines based on geometric features, rather than using brightness alone to define spine boundaries as employed by other methods such as the ray-bursting method [114, 115]. Instead, identification of a spine head is performed by extraction of points in an isolated closed volume with a closed surface after subtraction of the dendrite. For theoretical analysis, we use Hessian tensor that is obtained as second derivatives from Taylor expansion of the spine brightness function $I(\mathbf{x})$ in each optical slice, in 20–30 z-series optical slices obtained by confocal images.

$$H=\begin{pmatrix} \frac{\partial^2 I}{\partial x^2} & \frac{\partial^2 I}{\partial x \partial y} \\ \frac{\partial^2 I}{\partial y \partial x} & \frac{\partial^2 I}{\partial y^2} \end{pmatrix}, \quad diagH=\begin{pmatrix} \lambda_+ & 0 \\ 0 & \lambda_- \end{pmatrix},$$

where λ_+ and λ_- ($\lambda_+ > \lambda_-$) are the eigenvalues of diagonalized Hessian tensor.

The spine head region points are extracted as points where both λ_+ and λ_- yield negative values. Determination of spine head diameter is performed using the ''distance image'' that is, the digitized ''radius detection image'' which is assembled from the spine head region points. The final spine diameter is obtained by integration of spines 3-dimensionally. Results obtained by Spiso-3D are almost identical to those by Neurolucida (manual software, MicroBrightFeild, Williston, VT, USA), and Spiso-3D considerably reduces human errors and experimental labor of manual software. For quantitative comparison, we classify spines into three subclasses, i.e., small-head spines (0.2–0.4 μm), middle-head spines (0.4–0.5 μm) and large-head spine (0.5–1.0 μm) (Figure 5D).

Using Spiso-3D, we clearly distinguished the different effects of E2, T and DHT, on spine density and morphology of CA1 pyramidal neurons in acute hippocampal slices (Figure 6). While the effects of T, DHT and E2 treatment on the total spine density were indistinguishable, marked differences in the distribution of spine head diameter were observed between the treatments. DHT increased large- and middle-head spines, whereas T increased large- and small-head spines. In contrast, E2 rapidly promoted the formation of small headed spines (0.2–0.4 μm) without affecting the density of large headed spines (0.5–1.0 μm). Differences in spine subpopulations may have functional implications, for example, large-head spines may contain more AMPA receptors, since spine-head size positively correlates with the density of AMPA-type glutamate receptors [116, 117]. These findings demonstrate the importance of the consideration of spine diameter to distinguish different types of neurotrophic effects of the steroid hormones.

The rapid effects of E2 on spinogenesis are ER-dependent, with E2-dependent spinogenesis ablated [97] and morphological changes in spine head diameter reversed to the control distribution [113] following coadministration of the ER antagonist, ICI 1822,780. E2 primarily acts through ERα to induce rapid spinogenesis, with the ERα agonist, PPT, almost as efficacious as E2 in increasing spine density [97]. However, ERβ contributes little to rapid modulation of spinogenesis by E2, since the ERβ agonist, DPN, only increased density by 10% [97]. ERα probably acts through ERK MAP kinase to modulate spinogenesis, as E2-mediated spinogenesis is completely ablated by blocking the phosphorylation of ERK MAP kinase [111]. E2 mediated spinogenesis is also inhibited by blockade of the NMDA receptors, suggesting that basal levels of calcium may be necessary for ERα induced spinogenesis [111]. NMDA receptors also play

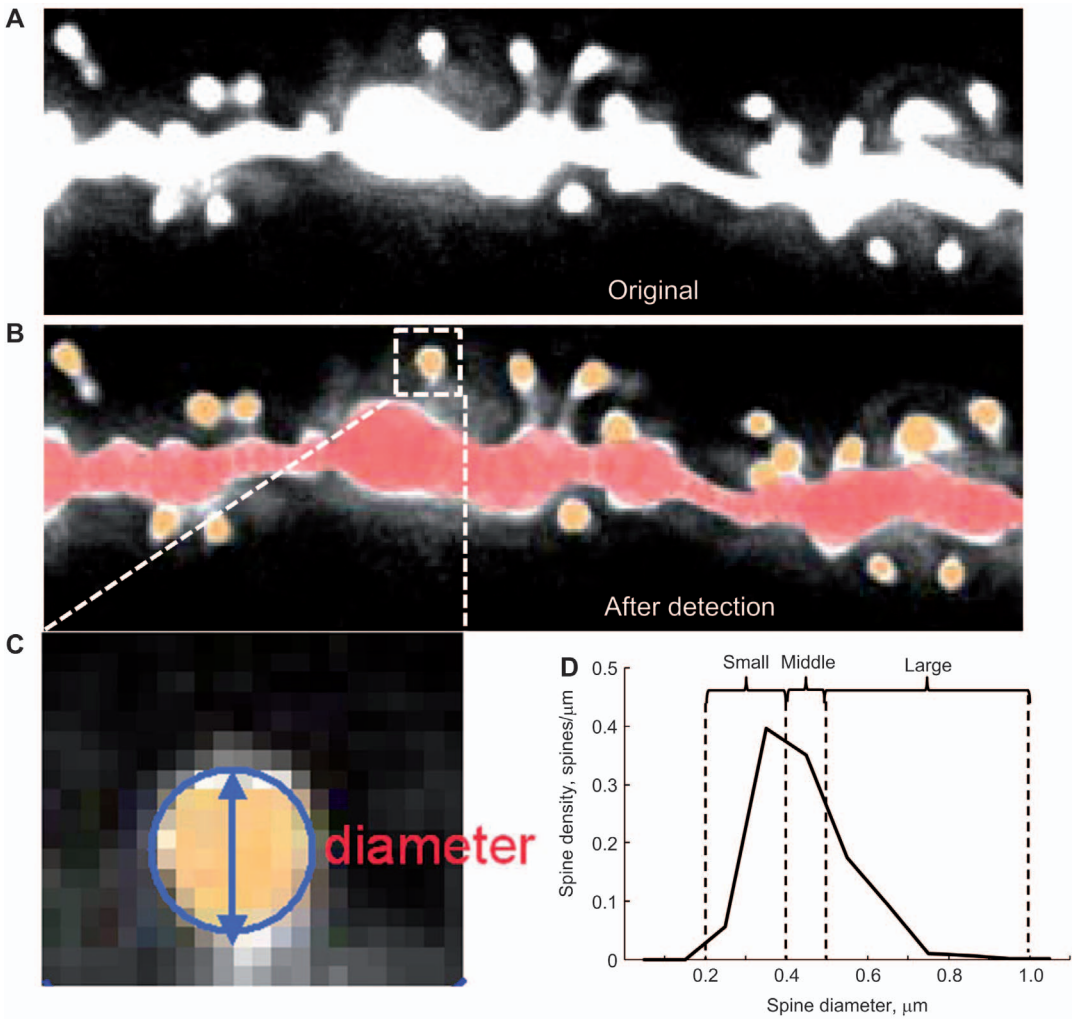

Figure 5 Automated spine density and spine head diameter analysis with Spiso-3D. (A) Original image of dendrite. (B) Traced image of dendrite (red) and spines (yellow) superimposed on original image. Traced dendrite is comprised of a connected series of circles. (C) Magnified image of identified spine head and calculated head diameter. (D) Typical spine head diameter distribution in the statum radiatum of CA1 pyramidal neurons in male rat hippocampal slices. For morphological analysis, spines are classified as small (0.2–0.4 μm), middle (0.4–0.5 μm) or large (0.5–1 μm) (modified from [113]).

a role in the slow effects of E2 on spine density, since the slow effects of E2 on spine density are also blocked by NMDA receptor antagonists [118, 119]. However, the slow effects of E2 on spine density assessed in vivo may be the result of not only direct actions of E2 in the hippocampus, but also indirect effects of E2 on cholinergic or serotonergic neurons projecting to the hippocampus [107, 120].

The contribution of hippocampal derived E2 to the modulation of spine density is difficult to address, however, Rune and co-workers demonstrated that suppression of endogenous E2 synthesis markedly decreases the density of spines, spine-synapses, spinophilin (spine marker) and the presynaptic marker, synaptophysin, in CA1 of cultured slices [4]. Application of exogenous E2 restores synaptophysin expression following letrozole treatments. However, interestingly, E2 application had no effect on spine density in hippocampal slice cultures in the absence of letrozole. This suggests that exogenous E2 is only effective on spine density when endogenous E2 is depleted.

In contrast to the extensive investigations into the role of E2 in the modulation of spines, androgenic regulation on the formation and morphologic changes of dendritic spines is poorly understood in the hippocampus. Leranth, MacLusky and co-workers found T to be important for maintenance of normal spine density in the male rat hippocampus, observing reduced spine density in the CA1 pyramidal neurons of the hippocampus of male GDX rats, an effect that could be rescued with injection of T propionate or DHT [121]. While in males, aromatization of T to E2 contributed little to the effects of T on spine density, in females the effect of T on dendritic spines is largely mediated by local conversion to estradiol [122]. In acute hippocampal slice preparations, androgens also rapidly enhance spines in CA1 and CA3 within 1–2 h [113, 123].

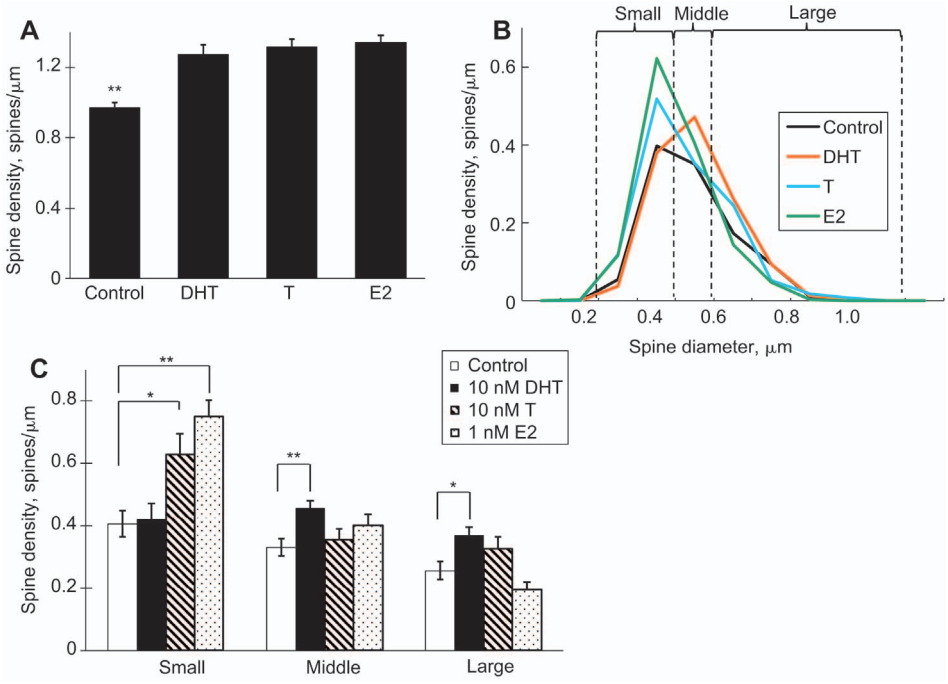

Figure 6 E2 and androgens differentially regulate spine morphology. Spine density and head diameter were assessed using Spiso-3D in hippocampal slices that had been treated for 2 h with either vehicle, DHT, T or E2. (A) DHT, T and E2 promoted a rapid increase in total spine density. (B) DHT, T and E2 altered the hippocampal spine head diameter distribution; for morphological analysis and comparison of steroid hormone treatments, spine heads were classified as either small (0.2–0.4 μm), middle (0.4–0.5 μm) or large (0.5–1 μm). (C) Density of small-, middle- and large-spine head subtypes. E2 promoted an increase in small-headed spines, T increased both small and large head spines, while DHT increased middle and large headed spines. Results are reported as mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs. control (modified from [113]).

Hippocampal E2 in aging and disease

While an age-related decline in circulating levels of E2 at menopause has been well characterized, the relationship between neurosteroidogenesis, age and disease is not well understood [124]. Few studies have addressed the effect of aging on brain hormone levels and investigations have been limited by low neurosteroid assay sensitivity and difficulty in obtaining human tissue, resulting in small sample sizes and limited capacity to analyze the differences in neurosteroid levels between gender [125–129] or brain regions [130]. Nonetheless, neurosteroids have been identified as potential biomarkers of cognitive aging [124, 131]. Female brain levels of E2 have been found to qualitatively mirror circulating E2 levels, with significant declines observed in the brains of postmenopausal women compared to premenopausal women [127, 130]. Age-related declines in brain T levels in men have been described, with brain T levels depleted to negligible levels by 80 years of age [130, 132]. However, whether hippocampal neurosteroidogenesis becomes impaired during aging as it does in gonad, is yet to be determined. Interestingly, despite much higher serum T and E2 levels in aged men than women [133–135], brain concentrations of T and E2 are similar between aged men and post-menopausal women [130]. Depleted brain E2 levels may be a risk factor for neurodegenerative disease. Multiple studies have described a relationship between low circulating levels of E2 in women and an increased risk of Alzheimer's disease [136, 137]. Likewise, depleted E2 is observed in female AD brains compared to age-matched cognitive controls [130, 132, 138, 139].

In addition to declining neurosteroid levels, hippocampal responsiveness to E2 may also diminish, due to age-related changes in ER expression and subcellular localization. The first evidence that ER expression and distribution may change with age, was that E2 binding decreased in nuclear extracts from the whole brain of middle-aged rats [140, 141]. Immunohistochemical studies have shown that ERα and ERβ levels are decreased in the hippocampus of aged female rats [80, 142]. Expression of ERα and ERβ is reduced at the pre- and post-synaptic densities [80], with spines containing ERα reduced by 50% in aged rat hippocampus [143]. Further, while hippocampal expression of ERα and ERβ increased following E2 treatment in young adult rats, ERα expression is not altered by E2 treatment in aged rats [80]. In contrast to the rat, an age-related increase in ERα immunoreactivity has been observed in the human hippocampus [144]. Age-related changes in ER expression may alter E2 mediated synaptic plasticity. For example, although long-term E2 administration increases spine density in the dentate gyrus of young-OVX rats, the opposite is true in aged-OVX rats, where short-term, but not long-term E2 administration increased spine density [145]. E2 administration has also been found to differentially alter the synaptic distribution of NMDA receptors in the hippocampus of young and aged-

OVX rats [146]. These age-related changes in E2-mediated synaptic plasticity may also alter the functional outcomes of E2 on learning and memory. Some behavioral effects of E2 are aged-dependent, for example, improved T-maze performance observed following E2 treatment in young adult, but not reproductively senescent, rats challenged with the muscarinic receptor antagonist scopolamine [147]. Further, OVX impaired performance in the Morris water maze in young-adult but not middle-aged rats and E2 supplementation had diminished benefits in middle aged rats [148].

Expert opinion

E2 is recognized as an important modulator of hipppocampal synaptic plasticity. Much of our understanding of the role of E2 in the modulation of hippocampal synaptic plasticity comes from the manipulation of gonadal and exogenous sources of E2. However, in addition to peripherally derived E2, the hippocampus endogenously synthesizes E2 from cholesterol which can elicit rapid synaptocrine, autocrine and paracrine effects in the hippocampal microenvironment. Hippocampal fluctuations in E2 concentrations rapidly potentiate synaptic signalling at existing synapses and induce an increase in spine density, most likely mediated by ERα located at the synapse. E2 action through both synaptic and nuclear ERα also sets genomic effects in motion, increasing functional synaptic contacts and facilitating LTP. Brain-derived sex hormones may play a crucial role in the rapid and continuous modulation of synaptic plasticity and cognitive functions.

Outlook

The potential use of E2-based hormone therapy (HT) to protect against age-related cognitive decline and/or neurodegenerative diseases, including Alzheimer's disease, has been extensively studied in animal models with promising results [149–151]. However, translation of these therapies to the clinical setting has thus far proven unsuccessful [152, 153]. The clinical failure of hormone therapy has been partly the result of deleterious effects of HT including increased risk of breast cancer, cardiovascular disease and stroke [154]. This has led to investigation of selective estrogen receptor modulators (SERMs) as the next generation of HT [155]. SERMs elicit tissue specific agonist and antagonistic effects. For example, the SERM raloxifene is currently used in the treatment of osteoporosis, acting as a partial E2 agonist to prevent bone loss, while functioning as an antiestrogen in breast and endometrial tissue [156–158]. Understanding of the role of ER in E2 mediated synaptic plasticity is crucial to successful develop pharmacological interventions which could protect against cognitive aging.

One of the key questions remaining to be addressed is the role of neurosteroidogenesis in aging, neurotrauma and disease. As our understanding of factors regulating brain E2 synthesis increase, drug targets which promote brain neurosteroidogenesis could offer a therapeutic alternative to conventional hormone therapy, not only to protect against age-related cognitive decline, but also as a potential therapeutic intervention in neurodegenerative diseases such as Alzheimer's disease.

Highlights

- CA1–3 pyramidal neurons and granule cells neurons of the dentate gyrus are the predominant steroidogenic cells in the hippocampus, expressing the complete complement of necessary steroidogenic machinery for de novo E2 synthesis.
- Although steroidogenic enzymes are expressed at very low levels in the hippocampus, compared to classic endocrine organs, small quantities (but high concentrations) of hippocampally synthesized E2 act locally through synaptocrine, autocrine and paracrine actions to modulate synaptic plasticity.
- Hippocampal E2 synthesis is stimulated by synaptic activity through NMDA.
- E2 modulates synaptic plasticity through classic ERα and ERβ receptors located at the synapse where they rapidly (<2 h) modulate synpatic plasticity and at the nucleus where they elicit slow-genomic effects on synaptic plasticity.
- E2 rapidly induces spinogenesis through synaptic ERα which activates the ERK MAP kinase signalling pathway.
- Synaptic ERα may also contribute to the slow genomic effects of E2 on spinogenesis through ERK/MAP kinase phosphorylation of CREB.
- Endogenously synthesized hippocampal E2 plays an important role in the maintenance of hippocampal spine density, since inhibition of E2 synthesis has been shown to markedly decrease CA1 spine density.
- Using Spiso-3D software to classify spine morphology by head diameter, E2 promoted the formation of small headed spines, while androgens increased large and middle headed spines.
- Age-related changes in hippocampal E2 synthesis and ER expression and distribution may alter effects of E2 on synaptic plasticity.

Acknowledgements

AMB is funded by a Japan Society for the Promotion of Science Postdoctoral Fellowship.

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