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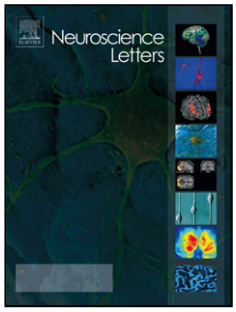

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PII: S0304-3940(21)00227-5

DOI: https://doi.org/10.1016/j.neulet.2021.135849

Reference: NSL 135849

To appear in: *Neuroscience Letters*

Received Date: 8 December 2020

Revised Date: 9 March 2021

Accepted Date: 22 March 2021

Please cite this article as: Fester L, Rune GM, Sex Neurosteroids: hormones made by the brain for the brain, *Neuroscience Letters* (2021), doi: https://doi.org/10.1016/j.neulet.2021.135849

Sex Neurosteroids: hormones made by the brain for the brain

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Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft (Ru 436/4-1).

HIGHLIGHTS

- **Estradiol and testosterone are synthesized in the hippocampus and act in a paracrine manner**
- **Neurosteroid synthesis is homeostatically regulated**
- **Stability and plasticity of hippocampal synapses depend on local neurosteroid synthesis**
- **Hippocampal neurons are responsive to estradiol in females and to androgens in males regarding synaptic plasticity**

ABSTRACT

In general, hippocampal neurons are capable of synthesizing sex steroids *de novo* from cholesterol, since the brain is equipped with all the enzymes required for the synthesis of estradiol and testosterone, the end products of sex steroidogenesis. Regarding estradiol, its synthesis in hippocampal neurons is homeostatically controlled by Ca^{2+} transients and is regulated by GnRH. Locally synthesized estradiol and testosterone maintain synaptic transmission and synaptic connectivity. Remarkably, the neurosteroid estradiol is effective in females, but not in males, and *vice versa* dihydrotestosterone (DHT) is effective in males, but not in females. Experimentally induced inhibition of estradiol synthesis in females and DHT synthesis in males resp. results in synapse loss, impaired LTP, and downregulation of synaptic proteins. GnRH-induced increase in estradiol synthesis appears to provide a link between the hypothalamus and the hippocampus, which may underlie estrous cyclicity of spine density in the female hippocampus. Hippocampal neurons are sex-dependently differentiated with respect to the responsiveness of hippocampal neurons to sex neurosteroids.

Keywords: sex steroids, hippocampus, sex-specificity, LTP, synaptic plasticity

CONTENTS

3. **Homeostasis of estradiol synthesis in hippocampal neurons**
4. **Paracrine action of hippocampus-derived estradiol in female animals**
5. **Sex-dependency in the responsiveness of hippocampal neurons to neurosteroids**
6. **Conclusion**

NEUROSTEROIDS

In the early eighties of the last century, the idea was born that the brain is a steroidogenic organ since sex steroids such as pregnenolone and dihydroepiandrosterone could be quantified in brain tissue, and its synthesis was independent from the gonads and adrenals. The pioneering work by Baulieu and colleagues demonstrating that concentrations of steroids were higher in the brain than in plasma, underscored this hypothesis, which was confirmed by numerous studies later on (for review see: [1-3]. Neurosteroids were defined as steroids that accumulate in the brain, even in the absence of steroidogenic glands. Of note in this context, initially it was believed that endogenous precursors are transported to the brain and only the late steps in sex steroidogenesis take place in the brain. [4]. The synthesis of pregnenolone and progesterone and its function in the nervous system was predominantly focussed on in these early studies, while sex steroids, such as estradiol and testosterone, were almost ignored.

It is well established that the brain, irrespective of sex, is equipped with all the enzymes required for the synthesis of estradiol and testosterone, so that a *de novo* synthesis from cholesterol is, in principle, possible [2, 5-7]. Although Naftolin had already

demonstrated the presence of aromatase, the final enzyme of estradiol synthesis in the diencephalon, as early as 1971, the expression of this enzyme in the brain, and in particular in neurones, was a matter of debate for quite a long time [8-13]. Abdelgadir et al. [14] was the first to show aromatase mRNA in rat hippocampus. Despite these early results, it took almost a further 10 years to show that this enzyme is in fact active in this part of the brain and that estradiol is synthesized and released from hippocampal neurons [15, 16]. This holds true for neurons of male and female animals [17]. Testosterone, being the substrate for aromatase, but also its stronger metabolite dihydrotestosterone, via activity of 5α-reductase which converts testosterone to dehydrotestosterone, are synthesized in hippocampal neurons irrespective of sex [18]. From the numerous studies by Kawato's laboratory, we know that the concentration of sex steroids in brain tissue is manyfold higher than in serum.

Regarding the functional roles of sex steroids in the CNS, Catherine Woolley [19-21] was the first to demonstrate that spine density, the postsynaptic partner of most excitatory synapses in the CNS, varies with the estrous cycle in the hippocampus. In females, spine density correlated positively with estradiol levels in serum. These findings pointed, for the first time, to a role of estradiol in hippocampal synaptic plasticity. Remarkably, estrous cyclicity appears to be specific to the hippocampus and is seen neither in the cortex nor in the cerebellum [22, 23]. In addition, Gould and coworkers [24] also demonstrated that removal of ovaries results in a reduction of spine numbers in the hippocampus, an effect which could be rescued by injections of estradiol. Thus, it appeared plausible that estradiol of ovarian origin regulated spine density in the female hippocampus. Similarly, following these findings in female animals, Leranth's group showed that orchiectomy results in spine synapse loss [25] in the male hippocampus, thus also pointing to a role of gonad-derived sex steroids in the regulation of synaptic plasticity in this region of the brain.

At this point it should be mentioned that in the following years innumerable studies provided evidence that estradiol plays an important role in learning and memory, in seizure susceptibility, and it influences excitatory and inhibitory circuits in the brain [26-29]. The neuroprotective role of estradiol was also used for therapeutic approaches, in particular during menopause (for review see [30]). Most of these studies, including our own studies, were carried out in the hippocampus, which we we focus on in this review. However, to consider all these studies is beyond the scope of this review. Here we want to focus on a potential role of sex steroids, in particular estradiol, that are synthesized in the hippocampus and function locally (for review see also [31])

Nevertheless, on the one hand, the brain is obviously capable of synthesizing sex steroids, and on the other hand, gonad-derived sex steroids appear to influence synaptic plasticity in the hippocampus: in view of these discrepant results, the question arises as to what hippocampus-derived sex steroids are good for?

17β-Estradiol is synthesized *de novo* by hippocampal neurons

Cholesterol, the precursor of all steroid hormones, needs to be synthesized in the brain since it cannot pass the blood brain barrier. Regarding steroidogenesis, cholesterol is transferred into the mitochondria, as the enzyme which converts cholesterol to pregnenolone, the first step in steroidogenesis, is located at the inner mitochondrial membrane. This transfer requires the Steroid Acute Regulatory Protein (StAR). Using *in situ* hybridization and immunohistochemistry, we and others showed the expression of StAR on the mRNA level as well as on the protein level in the hippocampus [32]. Pyramidal neurons, granule cells of the dentate gyrus, and interneurons exhibited specific staining with both methods. Moreover, aromatase, the enzyme responsible for the conversion of testosterone to estradiol, and StAR showed an overlapping hippocampal expression on the mRNA and protein level [32]. The coexpression of both

proteins made a *de novo* synthesis of estradiol very likely. Findings by Kawato and coworkers [5, 6] supported the hypothesis of a *de novo* synthesis of estradiol in the hippocampus. They found that cytochrome $P_{450}17\alpha$ and P_{450} aromatase are expressed by hippocampal neurons. They further demonstrated the conversion of pregnenolone to DHEA by using radioactive metabolites and, in another experiment, the metabolism of DHEA to estradiol in hippocampal tissue [6] .

A *de novo* estradiol synthesis from cholesterol in hippocampal neurons was demonstrated for the first time by Prange-Kiel et al. [15]. They succeeded in maintaining neurons of adult rats in a serum- and steroid-free medium for a couple of days without any contamination with glia. Subsequent radioimmunoassay revealed considerable amounts of estradiol in the medium, which in this culture system could only have been of neuronal origin, thus indicating the ability of hippocampal neurons to synthesize and release estradiol. This was also shown in hippocampal slice cultures and hippocampal dissociated cultures of neonatal animals. (**Fig. 1a**).

In addition and to further substantiate the *de novo* synthesis of estradiol by hippocampal neurons, a knock-down of StAR in hippocampal neurons served as a control. For the initiation of steroidogenesis StAR is required. As expected, in the culture medium of cultures transfected with siRNA against StAR we found a significant reduction of estradiol release into the medium (**Fig.1b**; [33, 34]. These findings actually falsify the formerly held belief, that only the final steps of estrogen synthesis take place in the hippocampus. [35-37]

Homeostasis of estradiol synthesis in hippocampal neurons

A specific feature of aromatase is that in contrast to other enzymes, its activity decreases as soon as it becomes phosphorylated. This was convincingly shown by Balthazart and co-workers in numerous experiments (for review see [38, 39]). In quail preoptic-hypothalamic homogenates the enzyme is rapidly downregulated by

exposure to conditions that enhance Ca^{2+}-dependent protein phosphorylation (presence of ATP, Ca^{2+} and Mg^{2+}). Aromatase was also rapidly and reversibly downregulated in quail preoptic-hypothalamic explants exposed to high Ca^{2+} levels following a K+-induced depolarization or the stimulation of glutamate receptors. Finally, enzyme activity was significantly reduced in phosphorylating conditions due to the action of calcium-dependent kinases (PKA, PKC). The involvement of phosphorylation processes in the control of aromatase activity is also consistent with the identification of several consensus sites of phosphorylation on the deduced aromatase sequence. Comparison of the specificity of these phosphorylation sites with the effects of various specific kinase inhibitors suggests that aromatase activity is actually regulated by the phosphorylation of threonine 455 and 486. Most of these studies were performed on quail, but the authors postulated that the control of aromatase activity via phosphorylation should also be applicable to rodents and other mammals [40-43].

Recent data from our lab confirm that this type of homeostatic control is in fact also true in rodents. (**Fig. 2a, b**). In rat dissociated hippocampal neurons, in which Ca^{2+} release from internal stores was either induced or inhibited, we were able to demonstrate a powerful Ca^{2+}-dependent control of aromatase activity [44]. Two types of receptors are located on internal Ca^{2+} stores and they control the process of Ca^{2+} release in dendrites and spines, and thus of propagating Ca^{2+} waves: the ryanodine-receptor family and the inositol-1,4,5-triphosphate-receptor (IP_3R) family [45, 46]. IP_3R-regulated channels are blocked by U73122 [47], and ryanodine receptor-regulated channels by 8Br-cADPR [48]. Thapsigargin depletes internal calcium stores, since thapsigargin inhibits sarcoplasmic or endoplasmic reticulum calcium-ATPase (SERCA), normally re-pumping calcium into the stores after Ca^{2+} release [49]. Thus, internal calcium stores become depleted and Ca^{2+} release cannot take place in the presence of thapsigargin [50].

In the presence of inhibitors of Ca^{2+}-induced Ca^{2+} (CIC) release from internal stores, the synthesis of estradiol increases, while an opposite effect is achieved if the release is stimulated. The central role of intracellular Ca^{2+} stores in the regulation of estradiol synthesis became particularly apparent in the case of ryanodine. We used ryanodine at low concentrations, which has been shown to induce calcium release, as well as at high concentrations, which have been demonstrated to block calcium release from internal stores [51]. Accordingly, we found an upregulation of estradiol synthesis at high concentrations of ryanodine and a downregulation at low concentrations of ryanodine. In order to test the regulation of aromatase activity on neuronal activity, we found a similar mechanism when we used NMDA. NMDA receptor activation, (known to induce CICR [52]), by application of NMDA for 30 min, downregulated aromatase activity and decreased the synthesis of estradiol. We speculated that continuous NMDA treatment should interfere with the reuptake of Ca^{2+} into the stores. In fact, longer-lasting NMDA treatment upregulated the protein and stimulated the synthesis of estradiol accordingly. Both the upregulation after long-term treatment and the downregulation after short-term treatment were abolished if CIC was inhibited. These findings are consistent with the findings by Balthazart et al. [43] and Hojo et al. [6]. Non-physiological conditions, such as treatment of hippocampal slice cultures with high doses of the GABAA receptor blocker bicuculline, a paradigm, which is used as an experimental approach to mimic epileptic seizures, was shown to downregulate estradiol synthesis dramatically [53].

Estradiol synthesis in hippocampal neurons also depends on substrate availability. Treatment of our cultures with testosterone or water-soluble cholesterol, both being substrates for estradiol synthesis, resulted in a highly significant increase in estradiol release in the supernatant, showing that the availability of sufficient substrate controls estradiol synthesis [54]. Application of cholesterol together with an aromatase inhibitor

resulted in a significant decrease of estradiol synthesis, as compared to cholesterol alone, and was used as a final control [55]. Together, these findings demonstrate that aromatase activity is rapidly regulated by Ca^{2+}-dependent phosphorylation, much more rapidly than by changes in concentrations of the enzyme.

Finally, the synthesis of estradiol in hippocampal neurons is homeostatically balanced by estradiol itself and thus in an autocrine manner. In hippocampal slice cultures, application of high doses of estradiol induced spine synapse density only when hippocampal estradiol synthesis was experimentally reduced [53, 55], pointing to a cross-talk of aromatase activity in the neurons and exogenously applied estradiol. At very high concentrations of estradiol no effects on synapse density were found, while at low concentrations estradiol was shown to induce spine formation ([56, 57]. Inactivation of aromatase by phosphorylation very likely accounts for this effect. (**Fig. 3**) In addition the optical density of aromatase immunoreactivity was decreased in response to estradiol [58]. As a control, we showed that the aromatase inhibitor letrozole, which is used in the therapy of breast cancer, phosphorylates aromatase (**Fig.3**), but has no effect on aromatase protein expression. Hence, our data provide evidence that aromatase activity is regulated in an autocrine manner, which is very likely mediated by the well-known Ca^{2+} influx in response to estradiol and subsequent CIC from internal stores. Estradiol induces Ca^{2+} influx into neurons via L-type channels and calcium release from internal stores [59]. This autocrine regulation, in turn, may account for the ineffectiveness of 17ß-estradiol to induce spine synapse formation in hippocampal slice cultures [60]. High doses may induce a CIC, inhibit estradiol synthesis and thus fail to induce spine formation, while the low dose of 1nM estradiol, which is commonly used to induce spine formation, fails to induce a CIC. Finally it needs to be emphasized that application of physiological concentrations of sex

steroids, as present in serum, to hippocampal neurons had no effect on spine and spine synapse density nor on the expression of synaptic proteins [60].

Paracrine action of hippocampus-derived estradiol in female animals

The first evidence that hippocampal neuron-derived estradiol acts in a paracrine manner was provided by Prange-Kiel et al. [15], who showed that in hippocampal dissociated neuronal cultures estrogen receptors (ERs) respond to varying content of estradiol in the supernatant. Low levels of estradiol in the supernatant, after treatment of the cultures with an aromatase inhibitor, induced an upregulation of ERβ and a downregulation of ERα. Vice versa, application of estradiol to the cultures resulted in upregulation of ERα and downregulation of ERβ. Very recently, this finding was confirmed by Lu et al. [61], who also reported this regulation of ER expression in a forebrain-specific conditional aromatase knock-out mouse.

Since the first evidence of a paracrine regulation of estradiol in the hippocampus [15], subsequent studies confirmed the hypothesis of estradiol made in the brain for the brain. The aromatase inhibitor letrozole, knock-down of StAR, various hippocampal *in vitro* models, thus in the absence of any other sources of estradiol, and the classical aromatase knock-out mouse and ovariectomized animals were used to confirm that locally synthesized estradiol is essential for hippocampal synaptic plasticity.

The inhibiting effect of letrozole on estrogen synthesis turned out to be dose-dependent, as demonstrated by using hippocampal slice cultures. The effects were reversible within hours, and any cytotoxic effects could be ruled out [62]. Moreover, immunoreactivity of StAR [32, 62] and aromatase expression varied in hippocampal tissue sections, which suggests that local synthesis of estradiol differs in various regions of the hippocampus. According to our immunohistochemical findings the synthesis should be highest in the CA3 region. Since estradiol upregulates ERα in

hippocampal neurons [15, 63], stronger nuclear expression of ERα in CA3 pyramidal neurons than in CA1 pyramidal neurons is consistent with this hypothesis [15, 63].

In sum, under *in vitro* conditions, and thereby strongly underscoring the paracrine mode of estradiol action, letrozole downregulated the number of dendritic spines, spine synapse density (**Fig. 4**) [60, 64], synaptic proteins, [55, 62], inhibited axon out-growth , neurogenesis [33], and impaired long-term potentiation (LTP, **Fig. 5**) [64, 65] in organotypic hippocampal slice cultures and hippocampal dissociated neuronal cultures of exclusively female animals (for review see [66]).

Consistent results were found in cultures after knock down of StAR [33]. This downregulation could be restored by adding estradiol to the medium, which proves that downregulation of synaptic proteins following letrozole treatment is actually due to the lack of estrogen, but not to potentially increased levels of the precursors of estrogen synthesis in response to the inhibition of either aromatase and StAR [53]. Similar rescue experiments were performed regarding synapse density, axon outgrowth, and LTP. Most importantly, our *in vitro* findings were confirmed using aromatase-deficient mice and ovariectomized animals [23, 67]. Eventually, our results regarding estradiol-induced synaptic transmission and synaptic maintenance in females are nicely confirmed by a recent study by Lu et al. [61] in a forebrain-specific aromatase deficient mouse.

A further study by Prange-Kiel and coworkers [62] addressed the paracrine mode of action of estradiol using a specific methodological approach in hippocampal slice cultures. As already mentioned in this culture model, aromatase and StAR expression was stronger in the CA3 region than in the CA1 region and the dentate gyrus, pointing to varying estradiol levels in hippocampal regions. Accordingly, Prange-Kiel et al. found a region-specific downregulation of synaptic proteins after application of letrozole into

the culture medium of the slices. In response to letrozole, expression of spinophilin, a specific postsynaptic protein, downregulation was strongest in the CA1 region, where aromatase and StAR expression were lowest, as compared to CA3, where enzyme expression was considerably higher. Upon the strict dose-dendency of aromatase inhibitors [60], letrozole, at a defined dose, was consistently more effective in CA1 than in CA3. Similarly, synaptophysin, a specific presynaptic protein, was most strongly downregulated in stratum lucidum of CA3, thus in mossy fibres, which originate from granule cells with low aromatase expression. It was downregulated to a lesser extent in stratum radiatum of CA1, because the axons of CA3 neurons, which strongly express aromatase, terminate at the apical dendrites of CA1 pyramidal cells. Both the region-specific expression of steroidogenic enzymes and region-specific downregulation of synaptic proteins indicate that estradiol, synthesized and released in defined regions within the female hippocampus, also exerts its effects in exactly these areas. Likewise, estradiol, synthesized in the CA3 region, also acts on CA3 cells, but not on cells of the dentate gyrus and cells of the CA1 region. These findings underscore hippocampus-derived estradiol as a potent paracrine neuromodulator in female animals.

Hypothalamo-Hypophysial axis

In view of a strong paracrine mode of estradiol action, the question as to why spine and spine synapse density varies with the estrous cycle in the female hippocampus remains an open question [19, 68]. The idea came up that GnRH could provide a link since GnRH is a master regulator of estrous cyclicity. Its pulsative release from the hypothalamus controls the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary, which, in turn, regulate steroid hormone synthesis in the gonads. In ovarian granulosa cells, however, GnRH has also been

shown to exert a direct stimulatory action on estradiol synthesis [69]. This prompted us to test whether GnRH controls estradiol synthesis also directly in the hippocampus. GnRH receptors are abundantly expressed in the hippocampus, the expression was considerably higher in the hippocampus than in the hypothalamus and in the neocortex [68]. Very similar to the effect of GnRH on granulosa cells, treatment of rat hippocampal slice cultures with GnRH upregulated the synthesis of estradiol in a specific dose-dependent manner by up to 20% [68], resulting in an inverted U-shaped dose-dependency curve. The dose-dependency was similar when we measured estradiol release in dispersion cultures. The stimulatory effect of GnRH was abolished in the presence of antide, an GnRH receptor antagonist, in the culture medium. We also tested the specificity of GnRH on aromatase-dependent estradiol synthesis by application of the aromatase inhibitor letrozole together with GnRH. GnRH-induced increase of estradiol release was clearly inhibited by simultaneous treatment with letrozole, indicating that the enhanced estradiol synthesis in reponse to GnRH is mediated by aromatase. To our knowledge, GnRH is the first peptide described to regulate estradiol synthesis in the hippocampus. The inverted U-shaped dose-response curve of estradiol synthesis is a typical feature of G-protein-coupled receptors such as GnRH-R. Receptor desensitization, mainly due to receptor internalization, accounts for this phenomenon [70].

As expected, we found that GnRH induced spine and spine synapse formation in hippocampal slice cultures [68] and in rats, when GnRH was continuously pumped into the hippocampus or into the ventricle [71]. The contralateral side of the brain served as a control. Obviously, cyclic release of GnRH from the hypothalamus induces varying levels of estradiol, which in turn result in varying estrous cycle-dependent densities of spines in the hippocampus. This hypothesis was nicely confirmed by Kato et al. [72], who showed that in fact, estradiol levels vary with the estrous cycle in the

hippocampus. Furthermore, our data were confirmed by a study of Nelson et al. [73] on the behavioral background. They used very similar methodological approaches and identified GnRH in rats as a regulator in hippocampus-related behavioral tests. Similarly, Gonzales-Torres and coworkers [74] demonstrated that chronic administration of GnRH improves learning in gonadectomized rats, which was paralleled by enhanced expression of spinophilin. Nevertheless, it still remains elusive as to how GnRH reaches the hippocampus. Two possibilities should be tested: either GnRH reaches the hippocampus via the liquor ventriculi or by synaptic release from GnRH neurons in the hippocampus. The presence of GnRH neurons has been demonstrated by Merchenthaler et al. [75] and more recently by Ferris et al. [76].

Sex-dependency in the responsiveness of hippocampal neurons to estradiol

Finally and most importantly, we found a clear sex-dependency in estradiol-induced synaptic plasticity (for review see: [66]). The results listed above refer exclusively to the female hippocampus, either *in vivo* or in hippocampal tissue which originates from female animals. Inhibition of estradiol synthesis has no effect on synaptic connectivity and almost no effect on LTP in the male hippocampus [64]. Numerous studies provide increasing evidence supporting the idea that every cell in the brain of males may differ from those in females due to differences in sex chromosome complement, as well as in response to hormonal effects. In a recent study [77] we addressed the question as to whether actions of neurosteroids, thus steroids, which are synthesized and function within the brain, contribute to sex-specific hippocampal synaptic plasticity. We found that testosterone and its metabolite dihydrotestosterone are the players in sex steroid-induced synaptic plasticity specifically in males. This also holds true for the density of

mushroom spines and of spine synapses. Similar to our findings in females, we obtained similar sex-dependent results using primary hippocampal cultures of male animals. Since these cultures originated from perinatal animals, our findings argue for sex-dependent differentiation of hippocampal neurons regarding their responsiveness to sex neurosteroids up to birth, which persist during adulthood. Hence, our *in vitro* findings may point to a developmental effect, either directly induced by sex chromosomes or indirectly, by fetal testosterone secretion during the perinatal critical period, when developmental sexual priming takes place.

Conclusion

In the female, but not in the male hippocampus, locally synthesized estradiol maintains hippocampal connectivity. Inhibition of estradiol synthesis results in a decrease in spine density, spine synapse density, in the expression of synaptic proteins in mice and in rat hippocampal slice cultures, and impaired long-term potentiation in acute and cultivated hippocampal slices. All effects could be rescued by estradiol. Gonadal estradiol very likely exerts an effect via regulation of GnRH release from the hypothalamus, which in turn regulates hippocampal estradiol synthesis, but estradiol of gonadal origin and present in serum is unlikely to have any direct effect in the hippocampus. Comparing the male and female hippocampus, it becomes evident that female neuons specifically respond to estradiol, while male neurons respond to androgens regarding synaptic plasticity in the hippocampus.

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Legends to the figures

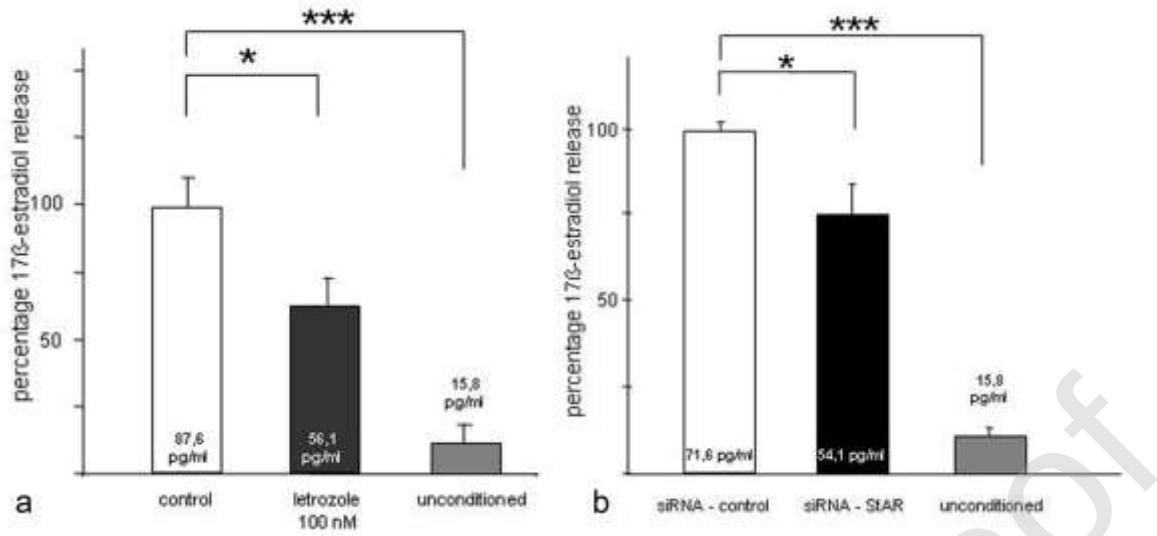

Fig. 1 Estradiol synthesis after knock-down of StAR and after pharmacological inhibition of aromatase using letrozole.

(a) Estradiol synthesis was significantly reduced by 40% at a dose of 100 nM letrozole in dispersion cultures. (b) The release of estradiol into the medium was significantly decreased after StAR knock-down. Unconditioned medium: freshly prepared medium that was not used for culturing; mean ± SEM; n=3 cultures per group;*p<0.05, ***p<0.01

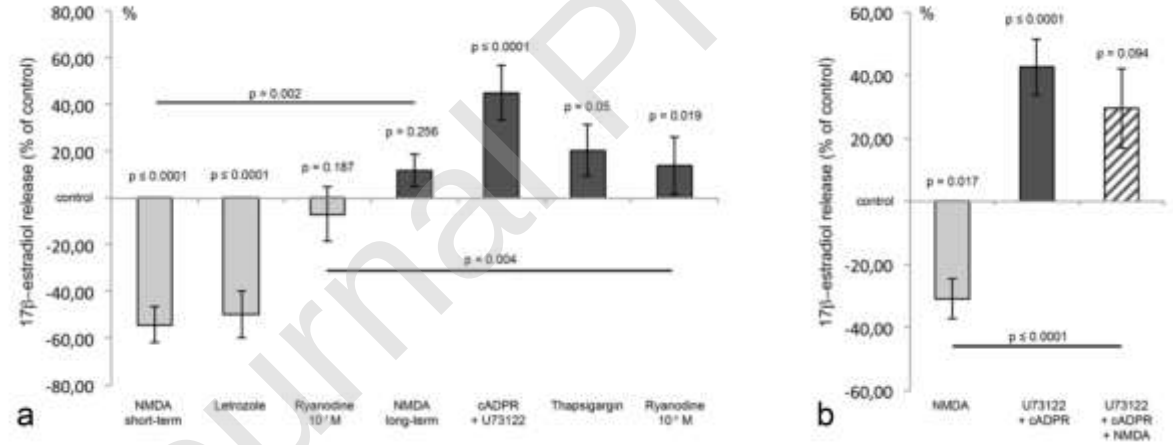

Fig. 2 Estradiol synthesis in response to Ca^{2+} transients in neurons.

(a) Measurements of estradiol by radioimmunoassay in the supernatant of dispersed cultures after various treatments. Short-term treatment with NMDA and with ryanodine at low concentrations, inducing calcium release from internal stores, and with letrozole, phosphorylating aromatase, decreases the release of estradiol into the medium, while U73122/cADPR and ryanodine at high concentrations increase estradiol release by inhibiting calcium release from internal stores. Similarly, thapsigargin, long-term treatment with NMDA (48 h), depleting calcium stores, also increased estradiol secretion into the culture medium. (b) Rescue experiment. Down-regulation of estradiol synthesis in response to short-term treatment with NMDA is rescued in the presence of the Ca^{2+} channel blockers U73122/cADPR.

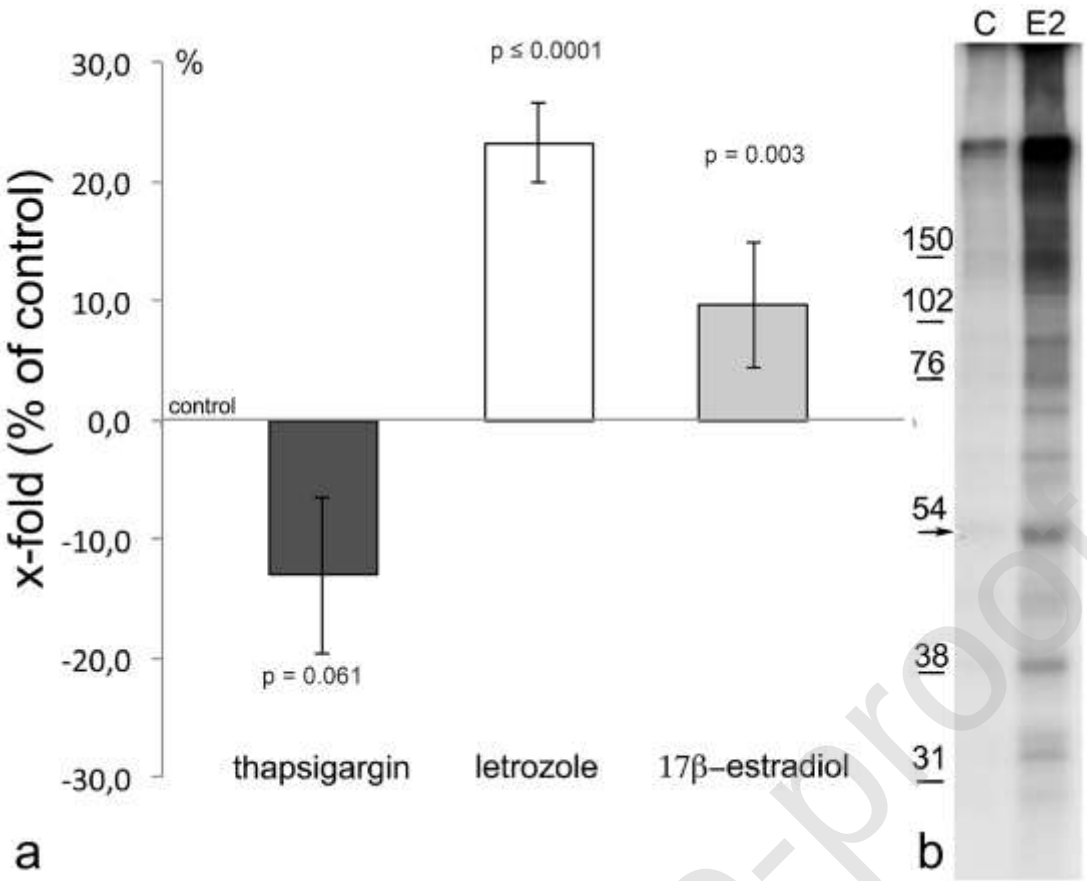

Fig. 3 Estradiol phosphorylates aromatase.

(a) Primary embryonic neurons in culture after DIV7 were short-term stimulated with thapsigargin 10^5 M, letrozole 10^7 M or 17b-estradiol 10^7 M and used for immunoprecipitation. Aromatase was precipitated with a polyclonal antibody to aromatase and subjected to SDS-PAGE. The subsequent Western blot was analyzed with either a monoclonal antibody to aromatase (Acris, SM2222P) or a mixture of a monoclonal antibody to phosphoserine/threonine (PM3801, ECM Biosciences) and a monoclonal antibody to phosphotyrosine (Millipore, 05-321). The level of phosphorylation of immunoprecipitated aromatase after treatment of neurons with thapsigargin is decreased as compared to control levels. In contrast, both letrozole and 17b-estradiol treatment resulted in significantly increased levels of phosphorylation. (Mean% SEM%. p 0.05, n = 3 for thapsigargin, n = 19 for 17b- estradiol, n = 5 for letrozole).
(b) Aromatase is phosphorylated in female neurons after 17b-estradiol treatment. Female primary hippocampal neurons in culture at DIV10 were used for immunoprecipitation and treated either under control conditions or with 17b-estradiol (10^7 M). Aromatase was labeled with ^{32}P-orthophosphate and immunoprecipitated with an antibody directed to aromatase (Acris, SM2222P) and subjected to SDS-PAGE. The autoradiogram of ^{32}P-labeled aromatase is shown above. The band at 54kDa indicates phosphorylated aromatase. Aromatase is more strongly phosphorylated after application of 17b-estradiol in female neurons. (n=3).

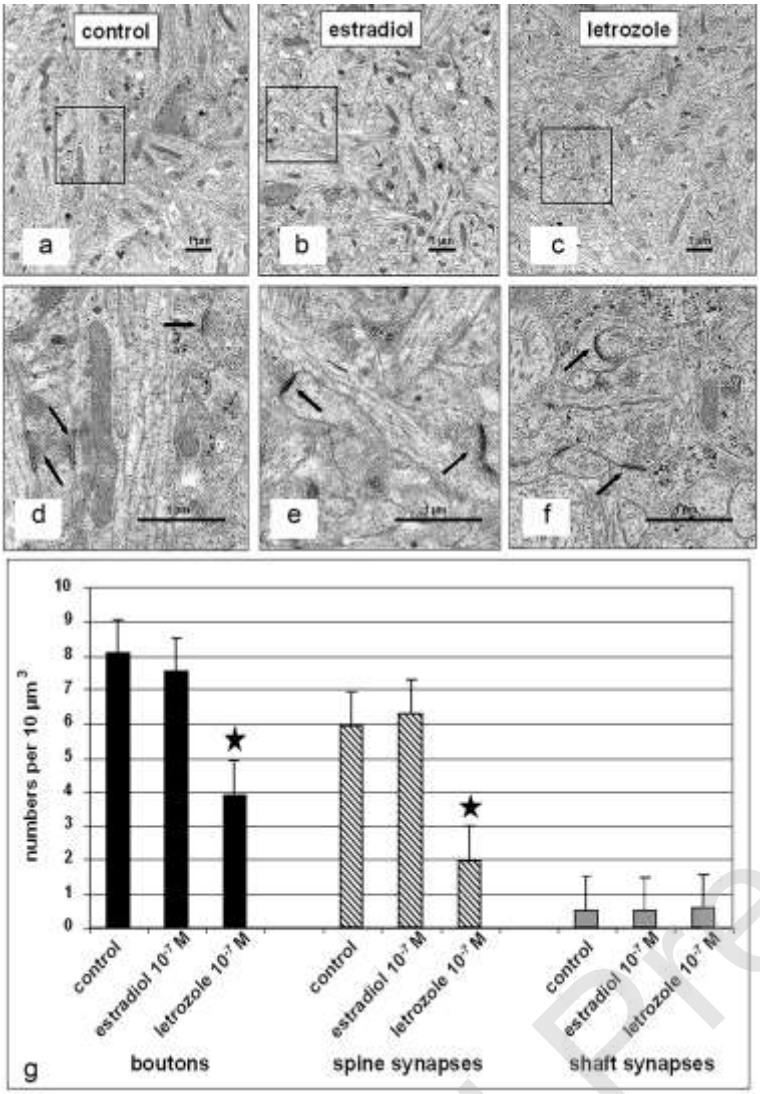

Fig. 4 Letrozole reduces spine synapse number in hippocampal slice cultures of female animals.

Electron micrographs of the stratum radiatum of the CA1 region after treatment with letrozole or estradiol. (a–c) No differences between different treatments were observed in the neuropil. (d–f) Framed areas at higher magnification. In all groups, morphologically intact synapses were found (arrows).(g) Quantitative evaluation of synapses in the stratum radiatum of the CA1 region in slice cultures. No differences in the number of shaft synapses were found between treatments. A significant decrease in spine synapses and in bouton number was seen after treatment of the slices with letrozole, whereas spine synapse and bouton numbers did not increase in response to estradiol (n=5; mean±SD;$p<0.05$ to control)

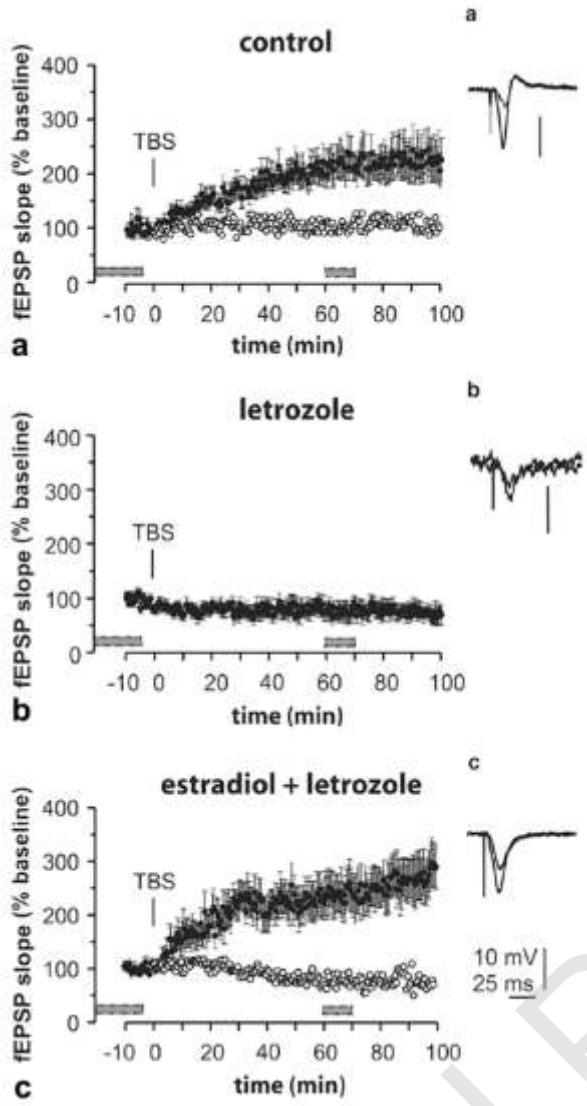

Fig. 5 Letrozole impairs LTP in hippocampal slice cultures of female animals.

LTP in hippocampal slice cultures of female animals. Theta-burst stimulation was performed after 20 min of stable baseline recording (10 min are shown) and 60 –70 min after theta-burst stimulation (as indicated by gray bars); changes in mean fEPSP slopes were determined. (a) In untreated slices, the average time course of the fEPSP slopes had significantly increased after 60 min up to 215 ± 34%. (b) Theta-burst stimulation did not induce LTP in slice cultures treated with letrozole for 7 d. (c) LTP is rescued by estradiol slice cultures treated with letrozole. After 60 min, the time course of the fEPSP slope had significantly increased to 239 ± 35% (mean ± SEM; *n* = 6 slice cultures of 3 animals per group; a, time course of LTP; b, superimposed recordings of fEPSPs 10 min before and 60 min after theta-burst stimulation).