Gonadotropin-releasing hormone regulates spine density via its regulatory role in hippocampal estrogen synthesis

Janine Prange-Kiel,1 Hubertus Jarry,2 Michael Schoen,1 Patrick Kohlmann,1 Christina Lohse,1 Lepu Zhou,1 and Gabriele M. Rune1

1Institute of Anatomy I: Cellular Neurobiology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
2Department of Obstetrics and Gynecology, University of Göttingen, 37075 Göttingen, Germany

Spine density in the hippocampus changes during the estrus cycle and is dependent on the activity of local aromatase. In view of the abundant gonadotropin-releasing hormone receptor (GnRH-R) messenger RNA expression in the hippocampus and the direct effect of GnRH on estradiol (E2) synthesis in gonadal cells, we asked whether GnRH serves as a regulator of hippocampal E2 synthesis. In hippocampal cultures, E2 synthesis, spine synapse density, and immunoreactivity of spinophilin, a reliable spine marker, are consistently up-regulated in a dose-dependent manner at low doses of GnRH but decrease at higher doses. GnRH is ineffective in the presence of GnRH antagonists or aromatase inhibitors. Conversely, GnRH-R expression increases after inhibition of hippocampal aromatase. As we found estrus cyclicity of spine density in the hippocampus but not in the neocortex and GnRH-R expression to be fivefold higher in the hippocampus compared with the neocortex, our data strongly suggest that estrus cycle-dependent synaptogenesis in the female hippocampus results from cyclic release of GnRH.

Introduction

Woolley et al. (1990) demonstrated the correlation of changes in spine density in the CA1 region of the hippocampus with fluctuations of serum estrogen levels in intact female rats. Ovariectomy reduced spine density in the hippocampus, and subsequent estradiol (E2) substitution rescued this effect (Gould et al., 1990). These findings led to the widely accepted hypothesis that the cyclic synapse turnover in the hippocampus is regulated by gonadal estrogen (McEwen, 2002). However, estrogen is also known to be synthesized de novo in hippocampal neurons (Prange-Kiel et al., 2003; Kretz et al., 2004), and in male rats, the basal concentration of E2 produced in the hippocampus is about six times higher than the concentration in the serum (Hojo et al., 2004). Inhibition of the key enzyme of E2 synthesis, aromatase, by its inhibitor, letrozole, demonstrated the paracrine/autocrine regulation of synapse formation by E2 in the hippocampus (Kretz et al., 2004). Furthermore, the amount of E2 synthesized in the hippocampus was recently shown to be sufficient to enhance hippocampal long-term depression (Mukai et al., 2006). Paracrine regulation by E2 was also shown in neurogenesis (Fester et al., 2006) and axon outgrowth (von Schassen et al., 2006). In hippocampal cultures, treatment with E2 at physiological doses failed to induce any detectable effect, which suggests that endogenous hippocampus-derived E2, rather than gonadal E2, is essential for hippocampal synaptogenesis (Kretz et al., 2004; Fester et al., 2006; von Schassen et al., 2006). Short-term treatment of acute slices (obtained from adult male rats) with E2 at a dose of 1 nM, which roughly corresponds to physiological serum concentrations, merely induced an increase in thin but not in mature spines (Mukai et al., 2007), although it was suggested that these thin spines can be considered to be the bases for new spine synapse formation after more than 24 h.

These considerations indicate that the concept of hippocampal spine density being exclusively regulated by gonadal estrogen is questionable. Because of this, the cyclic changes in spine synapse density in the hippocampus remain to be explained.

Estrogen-regulated feedback mechanisms operating via the hypothalamo-pituitary-gonadal axis cause a gonadotrophin-releasing hormone (GnRH)—mediated cyclic release of E2 from the gonads. In this context, it is important to mention that GnRH is also capable of regulating E2 synthesis directly, for instance in
ovarian granulosa cells, where it is stimulatory at low doses and inhibitory at high doses (Parmaud et al., 1988; Janssens et al., 2000). As in the ovaries, GnRH binding sites have been demonstrated in the hippocampus of the rat by autoradiography (Badr and Pelletier, 1987; Reubi et al., 1987; Jennes et al., 1988; Leblanc et al., 1988) and GnRH receptor (GnRH-R) mRNA expression by in situ hybridization (Jennes and Woolums, 1994). These findings suggest a common regulatory mechanism of E₂ synthesis in both the ovaries and the hippocampus. In line with this, treatment of hippocampal slices with GnRH, like treatment with E₂ (Hojo et al., 2004), results in predominantly excitatory effects that are blocked by the appropriate GnRH antagonists (Wong et al., 1990; Yang et al., 1999). This strongly suggests a neuromodulatory role of GnRH in synaptic transmission.

The data presented in this paper confirm the hypothesis that GnRH directly regulates estrogen synthesis in the hippocampus in a similar manner to its regulation of E₂ synthesis in ovarian cells. GnRH-induced E₂ synthesis, in turn, controls synapse formation consistently. These findings suggest that cyclic GnRH release, rather than gonadal E₂, is responsible for cyclic hippocampal synapse turnover. GnRH may thereby synchronize gonadal and hippocampal E₂ synthesis, which accounts for the correlation of hippocampal synaptogenesis with the gonadal cycle.

**Results**

**GnRH regulates hippocampal E₂ synthesis**

We measured the effect of GnRH on E₂ synthesis in hippocampal slices and dissociated neurons by determination of the released E₂ in the medium using radioimmunoassay (RIA). A recent study in our laboratory demonstrated that hippocampal neurons cultured under serum- and steroid-free conditions produce considerable amounts of E₂ and release it into the culture medium (Prange-Kiel et al., 2003). Aromatase is the final enzyme in E₂ synthesis, and treatment of these hippocampal neurons with the potent aromatase inhibitor letrozole resulted in a significant increase in the amount of E₂ released into the medium (Prange-Kiel et al., 2003). This establishes that measurement of E₂ in the medium can be taken as a parameter for neuronal E₂ synthesis.

Hippocampal slice cultures from rats at postnatal day 5 were precultured for 14 d, after which they were treated with GnRH doses ranging from 1 to 500 nM for 8 d. This type of organotypic neonatal hippocampal cultures has been demonstrated to develop connectivity after 3 wk, which is characteristic for the adult hippocampus in vivo (Frotscher et al., 1995).

Treatment with GnRH affected the release of E₂ in a specific dose-dependent manner (Fig. 1 A). The intermediate dose of 10 nM GnRH resulted in a significant 20% increase in E₂ synthesis. However, the highest dose of 500 nM did not increase E₂ synthesis above control values, and the amount of E₂ released into the medium was therefore significantly lower than after the treatment with 10 nM GnRH. Toxic effects of GnRH at higher doses were ruled out because the morphological integrity of the hippocampus was unaffected, as judged by morphological inspection of semithin sections (unpublished data). Moreover,

![Figure 1](image)

**Figure 1.** Hippocampal E₂ synthesis is regulated by GnRH. [A] E₂ synthesis of hippocampal slices obtained from young rats (postnatal day 5, 14 d of preculture) was measured by RIA after treatment with GnRH for 8 d. E₂ synthesis was significantly increased after the treatment with 10 nM GnRH compared with the control. No such increase was seen with the highest dose of 500 nM GnRH (mean ± SEM; n = 10). [B] Similar results were obtained when dispersion cultures of hippocampal neurons were treated for 8 d with the same doses of GnRH. Intermediate doses of 10 and 100 nM GnRH significantly increased E₂ synthesis, whereas after treatment with the highest dose of 500 nM, E₂ synthesis did not differ from the control (mean ± SEM; n = 5). [C] The increase of E₂ synthesis induced by 10 nM GnRH was blocked by simultaneous treatment of the dispersion cultures with 100 nM of the GnRH antagonist antide. 100 nM antide alone did not affect E₂ synthesis compared with the control. Treatment of the cultures with 100 nM of the aromatase inhibitor letrozole also inhibited GnRH-induced E₂ synthesis. Treatment of hippocampal neurons with 100 nM letrozole alone resulted in a significant down-regulation of E₂ synthesis, as demonstrated in earlier studies (Prange-Kiel et al., 2003; Kretz et al., 2004; mean ± SEM; ★, P < 0.05 compared with control; n = 5).
simultaneously applied to the cultures (Fig. 1 C). The application of antide alone did not affect the baseline $E_2$ release. Collectively, these findings demonstrate the specificity of GnRH effects on estrogen synthesis.

In a further control experiment, we tested the specificity of GnRH on aromatase-dependent $E_2$ synthesis. If GnRH indeed stimulates estrogen synthesis, then the GnRH-induced increase in $E_2$ release should be abolished by coapplication of the aromatase inhibitor letrozole. Letrozole, at a dose of 100 nM, has previously been demonstrated to suppress $E_2$ synthesis in hippocampal cultures without any undesired side effects (Prange-Kiel et al., 2003; Kretz et al., 2004). In line with our hypothesis, GnRH-induced $E_2$ synthesis in hippocampal dispersion culture was clearly inhibited by simultaneous treatment with letrozole (Fig. 1 C).

**GnRH influences spinophilin expression via its impact on $E_2$ synthesis**

Spinophilin is an actin-associated scaffold protein that is enriched in dendritic spines (Allen et al., 1997), where it is involved in regulating the morphology, function, and formation of the spines (Feng et al., 2000; Muly et al., 2004; Sarrouilhe et al., 2006). Spinophilin has been demonstrated to be a reliable spine marker (Tang et al., 2004) and previous experiments have demonstrated that spinophilin expression is sensitive to changes in hippocampal estrogen synthesis (Kretz et al., 2004; Prange-Kiel et al., 2006). We speculated that GnRH influences synaptogenesis via its regulatory role on hippocampal $E_2$ synthesis and, therefore, studied the effects of GnRH on spinophilin expression.

After preculture hippocampal slices were treated with 1–500 nM GnRH for 8 d, the effects were evaluated by immunohistochemistry (IHC) and confocal fluorescence microscopy of cryostat sections of the cultured slices (Fig. 2 A), followed by image analysis. For the quantitation of the spinophilin protein expression, we determined an index for the spinophilin immunostaining that integrates staining intensity and the number of stained pixels in a defined area. Most importantly, treatment with 10 nM GnRH resulted in a significant 70% increase of the staining index (Fig. 2 B), whereas treatment with 500 nM GnRH did not result in any change in spinophilin expression in comparison to the control. The staining index was significantly lower after treatment with 500 nM GnRH and was not significantly altered compared with the control (mean $\pm$ SEM; $n = 10$).

![Figure 2. GnRH regulates spinophilin expression in hippocampal slice cultures. (A) Cy3-coupled anti-spinophilin was used to detect spinophilin in the CA1 region of hippocampal slice cultures after treatment with GnRH. The staining intensity varied depending on the treatment. The nuclei were counterstained with DAPI [blue]. Bar, 20 μm. (B) Image analysis of IHC for the postsynaptic protein spinophilin in hippocampal slice cultures after 8 d of GnRH treatment demonstrated that an intermediate dose of 10 nM GnRH resulted in a significant increase of the staining index compared with the control. The staining index was significantly lower after treatment with 500 nM GnRH and was not significantly altered compared with the control (mean $\pm$ SEM; $n = 10$).](image-url)
by letrozole resulted in a significant reduction of spinophilin expression. GnRH treatment in combination with letrozole did not result in an increase in spinophilin expression. In fact, this combined treatment had the same effect as treatment with letrozole alone. These findings demonstrate that the stimulatory action of GnRH on spinophilin expression is mediated by its influence on estrogen synthesis.

Regulation of GnRH-R by hippocampus-derived E2

Earlier studies have demonstrated that E2 prevents GnRH-R mRNA expression in ovarian cells (Nathwani et al., 2000), and this raises the question of whether the expression of GnRH-R in the hippocampus is influenced by locally derived or exogenously applied E2. To examine this, hippocampal neurons were treated for 8 d with either 100 nM letrozole, to inhibit hippocampal E2 synthesis, or with 100 nM E2. For the analysis of GnRH-R expression in neurons, GnRH-R staining was exclusively analyzed in microtubule-associated protein 2 (MAP-2)–positive cells. Double labeling of both antigens resulted in an evenly distributed MAP-2 staining and a more punctuate staining for GnRH-R. GnRH-R as a membrane-bound receptor has also been shown to be internalized upon stimulation by its ligand (Hazum et al., 1980). Accordingly, GnRH-R immunoreactivity was primarily detected at the periphery of the hippocampal neurons but signals were also localized in the cytoplasm (Fig. 5 A). Confocal imaging (Fig. 5, B–D) and subsequent image analysis (Fig. 5 E) showed that treatment with letrozole resulted in a significantly higher staining index (170% of control) for GnRH-R in treated cells compared with untreated controls. However, treatment with E2 did not change GnRH-R expression in hippocampal neurons, which suggests that there is a ceiling of E2-mediated GnRH expression.

Spine synapse density varies in the hippocampus, but not in the neocortex, during the estrus cycle

Although the cycling of hippocampal spine synapse density during the estrus cycle has been extensively studied, it is as yet unknown whether this phenomenon is restricted to the hippocampus or whether it also occurs in the more highly developed neocortex. To this end, regularly cycling female rats were staged and perfused either in proestrus, when they have high E2 serum levels, or at estrus, when E2 serum levels are low. To eliminate the peripheral source of E2, another group of females was ovariectomized and perfused 14 d later. Stereological calculation of spine synapse density confirmed the findings of Woolley et al. (1990). In regularly cycling female rats, the density of spine synapses in CA1 was significantly higher during proestrus, the phase of high estrogen levels in serum, than in the stage of estrus, when peripheral estrogen levels are relatively low (Fig. 6, A and C). Ovariectomy resulted in a decrease in spine synapse density as compared with rats at proestrus. The decrease of 20% was of the same magnitude as found in previous studies (Woolley et al., 1990). In these animals, however, no such effect was observed in the neocortex. As previously shown (Deller et al., 2003), the number of spine synapses in the external pyramidal layer (layer III) of the neocortex was about one third lower than in the hippocampus. Here, however,
GnRH-R mRNA expression was strongest in the hippocampus, where it was almost three times higher than in the hypothalamus and more than five times higher than in the neocortex. The high concentration of GnRH-R mRNA in the hippocampus points to the specific responsiveness to GnRH in this brain area.

To validate the culture systems used for the expression of GnRH-R mRNA, we also performed real-time RT-PCR on hippocampal and neocortical tissue obtained from day-5 neonatal rats with similar results (unpublished data).

**GnRH-R mRNA expression is unusually high in the hippocampus**

Finally, we posed the question of whether GnRH responsiveness differs in the hippocampus and the neocortex of adult female rats. Real-time RT-PCR was used to compare the amount of GnRH-R mRNA in both regions and for control purposes in the hypothalamus, which has the highest density of GnRH neurons in the central nervous system (Spergel et al., 1999; Fig. 7).

**Discussion**

Our results demonstrate that GnRH binding to its receptor regulates hippocampal E2 synthesis, which, in turn, influences synaptogenesis. This may explain the estrus cycle–regulated cycling of spine density that is seen specifically in the hippocampus.
ovarian steroidogenic enzymes are also expressed in the hippocampus (Compagnone and Mellon, 2000). Our recent studies have shown that these hippocampal enzymes are functional (Prange-Kiel et al., 2003; Kretz et al., 2004). E₂ synthesis in neurons depends on aromatase, and the activity of this enzyme in neurons is regulated by neuronal activity (Zhou et al., 2007) and Ca²⁺-dependent phosphorylation (Balthazart et al., 2003).

GnRH is the first peptide described to regulate hippocampal E₂ synthesis. The dose dependency of this GnRH effect is most striking, as doses of 10 and 100 nM in organotypic and dissociated cultures, respectively, had the maximal effect on E₂ synthesis. A further increase in the GnRH concentration did not result in an additional increase in E₂ synthesis but, rather, in its inhibition. Notably, an inverted U-shaped dose–response curve for E₂ synthesis has also been described in cultured granulosa cells that were treated with a GnRH agonist (Parinaud et al., 1988). This type of dose–response curve is typical of G protein–coupled receptors such as the GnRH-R and is caused by receptor desensitization brought about by receptor internalization (McArdle et al., 2002).

The importance of hippocampus-derived E₂ for synaptic plasticity has been unequivocally demonstrated using the aromatase inhibitor letrozole. Inhibition of hippocampal E₂ synthesis resulted in a significant decrease in spines and spine synapses in the CA1 region (Kretz et al., 2004). This effect was rescued by supplementing the medium with high pharmacological doses of E₂ but not with amounts corresponding to serum E₂ concentrations. Concomitantly, Hojo et al. (2004) have shown that the basal concentrations of E₂ in hippocampi of male rats are six times higher than the concentrations in serum. This suggests that the serum E₂ concentration available in vivo may be too low to effectively modulate spine density.

A recent study, however, demonstrated that the short-term treatment (2 h) of acute hippocampal slices obtained from adult male rats with 1 nM E₂ resulted in an increase in the number of thin spines (Mukai et al., 2007). Although these newly generated spines did not form new synapses within 2 h, as judged from electrophysiological measurements (Mukai et al., 2007), they may, nevertheless, acquire synapses within a short period of time, as shown by Pozzo-Miller et al. (1999). These experiments, using

GnRH regulates E₂ synthesis and, as a consequence, spine density in the hippocampus

GnRH is the key regulator of reproduction, as its pulsatile release from the hypothalamus controls the secretion of follicle-stimulating and luteinizing hormones in the pituitary, which, in turn, regulate steroid hormone synthesis in the gonads. In recent years, GnRH has also been shown to directly influence E₂ synthesis in the ovary (Parinaud et al., 1988; Janssens et al., 2000). However, the gonads are not the only site of E₂ synthesis. The hippocampus has been shown to be a prominent extragonadal site of E₂ synthesis, and all of the ovarian steroidogenic enzymes are also expressed in the hippocampus (Compagnone and Mellon, 2000). Our recent studies have shown that these hippocampal enzymes are functional (Prange-Kiel et al., 2003; Kretz et al., 2004). E₂ synthesis in neurons depends on aromatase, and the activity of this enzyme in neurons is regulated by neuronal activity (Zhou et al., 2007) and Ca²⁺-dependent phosphorylation (Balthazart et al., 2003).

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short-term E2 treatment of acute slices from adult rats, may correspond more closely to the physiological situation in cycling animals. However, our experimental design, which includes long-term cultivation, requires hippocampal slices and dispersion cultures of prenatal day-5 animals. As a consequence, developmental effects should be considered in the interpretation of our data.

Here, treatment with GnRH influenced spinophilin protein expression as well as spine synapse density in hippocampal slices in the same dose-dependent manner as seen in E2 synthesis. Intermediate doses of GnRH stimulated spinophilin expression, whereas high doses had no effect. Moreover, when GnRH-induced hippocampal E2 synthesis was blocked by the aromatase inhibitor, the spinophilin-stimulating effect of GnRH was abolished. This finding shows that the GnRH effect on spine formation is mediated by its influence on E2 synthesis.

The effects of GnRH on spine synapse density and spinophilin were highly correlated, which confirms that spinophilin is a reliable spine marker. Slight differences were observed only in regard to the dose dependency of the phenomena. Even the lowest dose induced an increase in spine synapse number and the highest dose still resulted in an elevated spine synapse density compared with the control. Differences in the sensitivity of spine synapse formation and spinophilin expression to E2 might explain these differences. Other GnRH effects on these parameters that are not mediated by E2 cannot be completely ruled out.

Hippocampus-derived E2 regulates GnRH-R expression

In a gonadotrope-derived cell line, treatment with E2 results in a decrease of GnRH-R expression (McArdle et al., 1992). As treatment with letrozole up-regulated GnRH-R expression in our study, we conclude that hippocampus-derived E2 limits GnRH-R in hippocampal neurons. Surprisingly, treatment with additional E2 does not result in a further down-regulation of GnRH-R. A similar effect was observed in a study of a human neuronal cell line. GnRH-R promoter activity was not inhibited by treatment with GnRH agonists but was enhanced by GnRH antagonist treatment. Yeung et al. (2005) interpreted that this resulted from an autoregulation of the promoter by endogenously produced GnRH. By analogy, hippocampus-derived estrogen may keep GnRH-R expression down to a constitutive minimum that is not influenced by E2. Our findings are further supported by the observations of Jennes et al. (1995, 1996), which demonstrate changes in GnRH-R mRNA levels in the rat hippocampus during the estrus cycle and after gonadectomy. In summary, our data show a fine-tuned loop of GnRH action on E2 synthesis via its receptor regulation. The regulation of GnRH-R by E2 may indicate a negative-feedback mechanism that prevents excessive E2 production and, thus, balances the system.

The regulatory role of GnRH on hippocampal estrogen synthesis accounts for estrus cycling of spine density in the hippocampus

Gould et al. (1990) demonstrated that systemic treatment of ovariectomized female rats with E2 results in an increase in spines in the CA1 region of the hippocampus. Concomitantly, Woolley et al. (1990) showed a correlation of changes in E2 serum levels during the phases of the rat estrus cycle with changes in spine density. Since then, the replication of experiments by Gould et al. (1990) in various species has led to the conclusion that fluctuation in spine synapse density in the hippocampus is regulated by gonadal E2 (McEwen, 2002). However, recent findings from our laboratory emphasized the importance of hippocampus-derived E2 and questioned the effects of gonadal E2 on hippocampal synaptogenesis (Kretz et al., 2004; Rune et al., 2006). Our present findings may help to explain the phenomenon of varying spine density during the estrus cycle that is, nevertheless, dependent on hippocampal aromatase activity. Cycling of spine density may be a distinctive feature of the hippocampus because it was not found in other regions of the neocortex. In addition, the expression of GnRH-R mRNA in the hippocampus is five times higher than in these parts of the neocortex, which suggests that the neocortex is much less responsive to GnRH. Indeed, only 7% of the cortical neurons have been demonstrated to be GnRH-R immunopositive (Quintanar et al., 2007). A lack in responsiveness to circulating E2 of the rat neocortex seems to be unlikely, as the regions under investigation (parts of the motor cortex and the primary sensory cortex) have been demonstrated to be immunopositive for estrogen receptor beta (Shughrue and Merchenthaler, 2001).

Based on our finding that GnRH regulates E2 synthesis in cultured hippocampal neurons, it is tempting to speculate that hypothalamic GnRH also regulates hippocampal estrogen synthesis in vivo. Hypothalamic neurons release GnRH into the hypophysial portal blood stream, whereas the amplitude and frequency of GnRH pulses regulate the cyclic follicle-stimulating hormone/luteinizing hormone release from the pituitary. GnRH pulses have also been detected in the cerebrospinal fluid (CSF; Skinner and Caraty, 2002) and they are coincident with peripheral luteinizing hormone pulses. The median eminence, the organum vasculosum of the lamina terminalis, and retrograde blood flow have all been suggested as possible sources of GnRH in the CSF (Lehman et al., 1986; Skinner and Caraty, 2002). Intracerebroventricular injection of GnRH induced changes in the sexual behavior of sheep (Caraty et al., 2002) and rodents (Pfaaff et al., 1994), suggesting that GnRH in the CSF influences adjacent brain regions.

GnRH might also reach the hippocampus by neurons projecting from other brain regions because GnRH fibers have been demonstrated in the hippocampus (Jennes and Stumpf, 1980; Witkin et al., 1982). However, as tracer studies to investigate this have so far yielded inconsistent results (Senut et al., 1989; Dudley et al., 1992), the origin of these fibers remains to be resolved.

Our data strongly suggest that cycling of spine density in the hippocampus results from cyclic regulation of hippocampal E2 synthesis in response to the pulsatile release of GnRH from the hypothalamus. Thus, GnRH synchronizes both gonadal and hippocampal E2 synthesis and, as a consequence, E2 serum levels and hippocampal spine density change in parallel. Although the source of GnRH in the hippocampus remains to be clarified, earlier data on E2-induced increase in spine density (Gould et al., 1990; McEwen, 2002) in ovariectomized animals now need to
be reinterpreted. Although the regulation of GnRH release is far from being understood, there is strong evidence that ovario-
extomy of rats results in a significant increase in pro-GnRH mRNA and GnRH mRNA expression in the hypothalamus (Toranzo et al., 1989; Pelletier et al., 2001). Enhanced GnRH mRNA expression has also been observed in the medial basal hypothalamus of postmenopausal women (Rance and Uswandi, 1996), and in pubertal nonhuman primates, ovarioectomy resulted in augmented GnRH release (Chongthammakun et al., 1993). However, as we show here, high GnRH inhibits hippocampal E₂ synthesis and reduces spine density. This provides an explana-
tion for the reduced spine density seen after ovarioectomy. Systemic treatment of ovarioctomized animals with E₂, in turn, may normalize hypothalamic GnRH release and so result in an increase in spine density.

In vivo experiments, including the application of GnRH into the hippocampus and the ventricle system of adult rats, will be required to further substantiate the hypothesis that GnRH synchronizes hippocampal and ovarian E₂ synthesis under in vivo conditions in cycling animals.

In summary, the interplay of GnRH on E₂ synthesis and, thus, on synaptogenesis offers a novel explanation for the regulation of hippocampal steroidogenesis and, together with previous work (Hojo et al., 2004; Kretz et al., 2004; Prange-Kiel et al., 2006), supports the role of hippocampus-derived E₂ in synaptogenesis.

For almost two centuries, circulating estrogens were con-
"Materials and methods"

**Animals**

Wistar rats [Charles River Laboratories] were maintained under controlled conditions and water and food were available ad libitum. All experiments were performed in accordance with the institutional guidelines for animal welfare and approved by the Behörde für Wissenschaft und Gesundheit (Ludwig-Maximilians-Universität München). A group of 10-week-old females was deeply anesthetized (3.3 ml/kg of a ketamine-xylazine mixture, i.p.) and ovarioctomized. 14 d after sur-
gery the animals were perfused. Another group underwent determination of the stage of the cycle. Vaginal smears were analyzed every morning over a period of at least four cycles. Animals at a defined stage of the es-
trus cycle (proestrus or estrus) were perfused in the morning to assure maxi-
mal E₂ serum levels in animals in the stage of proestrus. The results of the staging were confirmed by determination of serum E₂ levels of the animals by a commercial E₂ RIA (Beckman Coulter).

**Dispersion cultures**

Cell culture preparations from day-5 postnatal rats were performed as described by Brewer (1997), with slight modifications (Prange-Kiel et al., 2003). Cells were seeded on 20-μg/ml poly-D-lysine-coated (Sigma-
aldrich) coverslips in 8-mm-diameter 24-well culture dishes (Thermo Fisher Scientific) at a density of 5 × 10⁵ cells/well. The cells were incub-
bated in estrogen-free culture medium [Neurobasal A [without phenol red]; Invitrogen], 1% B27, 500 nM t-glutamine (Invitrogen), 1% anti-
biotics [Invitrogen], and 50 ng/ml basic FGF [Invitrogen]. The medium was changed every second day. This protocol results in a culture consisting of 80% neuronal cell, 12% astroglia, and negligibly few oligodendrocytes and microglial cells (von Schassen et al., 2006).

**Organotypic cultures**

400-μm slices of hippocampus and entorhinal cortex from day-5 newborn rats were prepared and cultivated according to the method introduced by Stoppini et al. (1991) and as described elsewhere (Kretz et al., 2004).

In brief, selected sections were placed on moistened translucent membranes (0.4-μm culture plate insert, 30-mm diameter; Millipore), which were in-
serted in 6-well plates (35 mm in diameter) filled with 0.8 ml of medium (50% MEM, 25% Hanks’ balanced salt solution, and 25% heat-inactivated horse serum) with a final concentration of 2 mM glutamine and 0.044% NaHCO₃. The pH was adjusted to 7.3. Before the experiments, the slices were preincubated for 14 d at 37°C in a humidified CO₂-enriched atmo-
sphere and the culture medium was changed three times a week.

**Culture treatment**

After 4 (dispersion cultures) and 14 d (organotypic cultures) in vitro, the incubation media were supplemented with 1, 10, 100, and 500 nM GnRH (luteinizing hormone-releasing hormone; Sigma-Aldrich) and/or 100 nM of the GnRH antagonist antide (Sigma-Aldrich) for another 8 d. For some experiments, cultures were treated with 100 nM of the aromatase inhibitor letrozole (Novartis). Media and supplements were changed every second day and the used media were collected for the RIA.

**E₂ RIA**

The medium of treated and nontreated cultures was collected every sec-
ond day and the medium of each well was pooled over the duration of the experiment. The processing of the medium and the E₂ measurement was performed as previously described (von Schassen et al., 2006). In brief, 3.5 ml of culture supernatant was loaded on a Sep-Pak cartridge (Milli-
pore), which had been preconditioned with 5 ml methanol and equili-
"Image analysis"
This threshold was applied to every image under analysis. The imaging software considered only pixels with a gray value higher than the threshold for analysis.

To assay GnRH or spinnophilin staining in the dispersion cultures, pictures of single neurons, identified by MAP-2 staining, were taken with the LSM and analyzed by Openlab. In each cell, four areas of fixed size were selected, and a relative staining index was determined for each cell by multiplying the intensity of staining (value on a grayscale) by the stained area (number of pixels). In each experiment, 12 cells of each treatment were analyzed and a mean was calculated for every group. In organotypic slice cultures, the spinnophilin expression was analyzed in the stratum radiatum. For this purpose, five sections were used per treatment and six pictures were taken from each section. An area of defined size was analyzed in every image. The relative staining index was determined by multiplying the intensity of staining by the stained area and a mean for each group was calculated.

Calculation of spine synapse density

Adult female rats in proestrus or estrus or 14 d after ovariectomy were perfused with 3% glutaraldehyde in PBS. The brains were removed and postfixed overnight. Subsequently, the hippocampi were dissected out and treated according to our standard protocol for electron microscopy (Kretz et al., 2004). Likewise, a part of the neocortex was dissected out and prepared for electron microscopy. To obtain matchable regions, the brain was dissected coronally at the level of the optic chiasm (approximately breoganum 50 mm) and a 3-mm-thick slice was cut from the rostral part of the brain. The dorsolateral part of the cortex (~5 mm in width) containing parts of the motor cortex and primary sensory cortex was separated from the remaining tissue and used for further analysis. Hippocampal slice cultures were fixed with 2.5% glutaraldehyde in phosphate buffer overnight and were treated according to the same standard protocol. An unbiased stereological method was used to evaluate the spine synapse density in tissues and slice cultures, as previously described (Prange-Kiel et al., 2004). In brief, pairs of consecutive serial ultrathin sections were cut and collected on Formvar-coated single grids. The sections contained either the upper and middle third of the CA1 stratum radiatum of the hippocampus or the outer pyramidal layer (III) of the neocortex. Electron micrographs were taken at a magnification of x6,600, with the observer blinded to the experimental groups. To obtain a comparable measure of synaptic numbers unbiased for possible changes in synaptic size, the dissector technique was used (Sterio, 1984). The density of spine synapses of pyramidal cell dendrites was calculated with the help of a reference grid superimposed on the electron microscope prints. Only those spine synapses were counted that were present on the reference section but not on the lookup section. The dissector volume was calculated by multiplying the intensity of staining by the stained area and a mean for each group was calculated.

Apoptosis and necrosis

A kit obtained from Boehringer Ingelheim was used for TUNEL, which was performed according to the instructions of the suppliers. Cytochemical lactate dehydrogenase was determined in the medium of slice cultures by using a calorimetric kit (Roche). For evaluation of both tests, five cultures (n = 5) in each group were used.

Statistical analysis

In all experiments, the means ± SEM were calculated. For large n values (n = 10 or more) with normally distributed data, statistical analysis was performed by analysis of variance followed by a post-hoc (LSD) test. For smaller n values, the bootstrap method was used, as it allows for the analysis of small datasets with unclear distributional assumptions (Henderson, 2005). P < 0.05 was considered to be significant.

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