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

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Spine density in the hippocampus changes during the estrus cycle and is dependent on the activity of local aromatase, the final enzyme in estrogen synthesis. 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 (E_2) 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 E_2 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 E_2 synthesis, aromatase, by its inhibitor, letrozole, demonstrated the paracrine/autocrine regulation of synapse formation by E_2 in the hippocampus (Kretz et al., 2004). Furthermore, the amount of E_2 synthesized in the hippocampus was recently shown to be sufficient to enhance hippocampal long-term depression (Mukai et al., 2006). Paracrine regulation by E_2 was also shown in neurogenesis (Fester et al., 2006) and axon outgrowth (von Schassen et al., 2006). In hippocampal cultures, treatment with E_2 at physiological doses failed to induce any detectable effect, which suggests that endogenous hippocampus-derived E_2, rather than gonadal E_2, 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 E_2 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 E_2 from the gonads. In this context, it is important to mention that GnRH is also capable of regulating E_2 synthesis directly, for instance in

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Abbreviations used in this paper: CSF, cerebrospinal fluid; E_2, estradiol; GnRH, gonadotrophin-releasing hormone; GnRH-R, GnRH receptor; IHC, immunohistochemistry; MAP-2, microtubule-associated protein 2; RIA, radioimmunoassay.

The Journal of Cell Biology, Vol. 180, No. 2, January 28, 2008 417–426
http://www.jcb.org/cgi/doi/10.1083/jcb.200707043

ovarian granulosa cells, where it is stimulatory at low doses and inhibitory at high doses (Parinaud 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 E_2 synthesis in both the ovaries and the hippocampus. In line with this, treatment of hippocampal slices with GnRH, like treatment with E_2 (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 E_2 synthesis in ovarian cells. GnRH-induced E_2 synthesis, in turn, controls synapse formation consistently. These findings suggest that cyclic GnRH release, rather than gonadal E_2, is responsible for cyclic hippocampal synapse turnover. GnRH may thereby synchronize gonadal and hippocampal E_2 synthesis, which accounts for the correlation of hippocampal synaptogenesis with the gonadal cycle.

Results

GnRH regulates hippocampal E_2 synthesis

We measured the effect of GnRH on E_2 synthesis in hippocampal slices and dissociated neurons by determination of the released E_2 in the medium using radioimmunoassay (RIA). A recent study in our laboratory demonstrated that hippocampal neurons cultivated under serum- and steroid-free conditions produce considerable amounts of E_2 and release it into the culture medium (Prange-Kiel et al., 2003). Aromatase is the final enzyme in E_2 synthesis, and treatment of these hippocampal neurons with the potent aromatase inhibitor letrozole resulted in a significant decrease in the amount of E_2 released into the medium (Prange-Kiel et al., 2003). This establishes that measurement of E_2 in the medium can be taken as a parameter for neuronal E_2 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 E_2 in a specific dose-dependent manner (Fig. 1 A). The intermediate dose of 10 nM GnRH resulted in a significant 20% increase in E_2 synthesis. However, the highest dose of 500 nM did not increase E_2 synthesis above control values, and the amount of E_2 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,

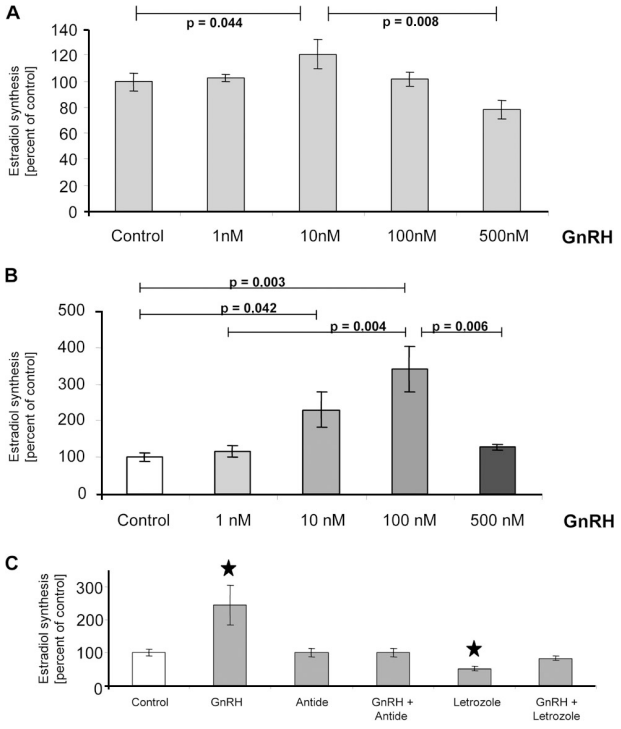

Figure 1. **Hippocampal E_2 synthesis is regulated by GnRH.** (A) E_2 synthesis of hippocampal slices obtained from young rats (postnatal day 5, 14 d of preculture) was measured by RIA after treatment with GnRH for 8 d. E_2 synthesis was significantly increased after the treatment with 10 nM GnRH compared 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 E_2 synthesis, whereas after treatment with the highest dose of 500 nM, E_2 synthesis did not differ from the control (mean ± SEM; *n* = 5). (C) The increase of E_2 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 E_2 synthesis compared with the control. Treatment of the cultures with 100 nM of the aromatase inhibitor letrozole also inhibited GnRH-induced E_2 synthesis. Treatment of hippocampal neurons with 100 nM letrozole alone resulted in a significant down-regulation of E_2 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).

TUNEL and lactate dehydrogenase assays did not show any signs of apoptosis or necrosis (unpublished data).

Because cultivation of hippocampal slice cultures requires serum containing undefined components that might interfere with the RIA, we confirmed these results using hippocampal dispersion cultures. These cultures were maintained under serum- and steroid-free conditions for a total of 12 d (4 d of preculture and 8 d of treatment). A similar inverted U-shaped dose–response curve was observed (Fig. 1 B), although the effects in these dispersion cultures were more clear-cut than in the slice cultures. Intermediate doses of 10 and 100 nM GnRH resulted in a significant increase in E_2 synthesis compared with controls (~130 and 240% above control level, respectively). At the highest dose of 500 nM GnRH, E_2 synthesis was inhibited as compared with the intermediate doses and did not differ from the untreated control.

The stimulatory effect of GnRH at the intermediate dose of 10 nM was abolished when the GnRH antagonist antide was

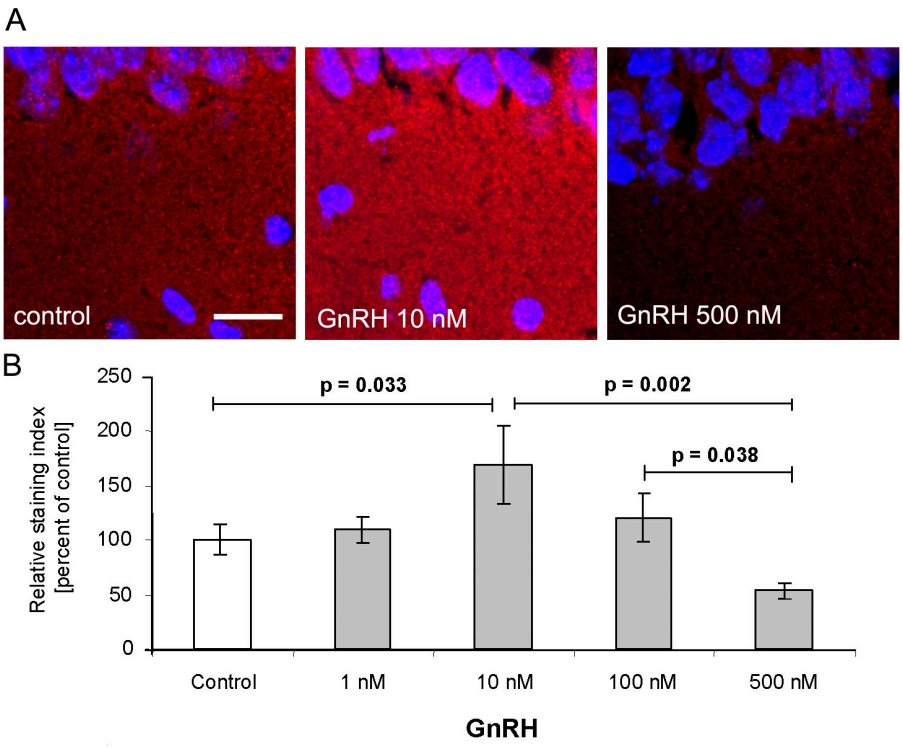

Figure 2. **GnRH regulates spinophilin expression in hippocampal slice cultures.** (A) Cy3-coupled anti-spinophilin was used to detect spinophilin in the CA1 region of hippocampal slice cultures after treatment with GnRH. The staining intensity varied depending on the treatment. The nuclei were counterstained 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 ± SEM; n = 10).

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. Thus, after treatment with GnRH, the dose dependency of spinophilin expression mirrors that of E_2 synthesis.

To verify the effects of GnRH on spinophilin expression, spine synapse density was determined by stereological calculation in organotypic slice cultures treated with GnRH at doses from 1 to 500 nM (Fig. 3, A–F). Under all conditions, neuronal ultrastructure and the typical cellular arrangement of the pyramidal layer were well preserved in the cultures and did not differ from the in vivo situation. Using electron microscopy, we found no qualitative differences between treatments. Morphologically intact spine synapses were located in untreated slices and in slices treated with GnRH. The quantitation of spinophilin expression and of spine synapses in CA1 both showed a dose-dependent response to GnRH (Fig. 3 G). Low doses of GnRH (1 and 10 nM) resulted in a significant increase in spine synapses of ~42 and 36%, respectively, whereas the treatment with 100 nM GnRH increased spine synapse density by 91%. However, the highest dosage used (500 nM) resulted in an increase of only 42%. This result correlates well with our findings on spinophilin immunoreactivity.

To test whether the stimulatory effect of GnRH on spinophilin expression is mediated by its stimulatory effect on E_2 synthesis, hippocampal neurons were treated with GnRH,

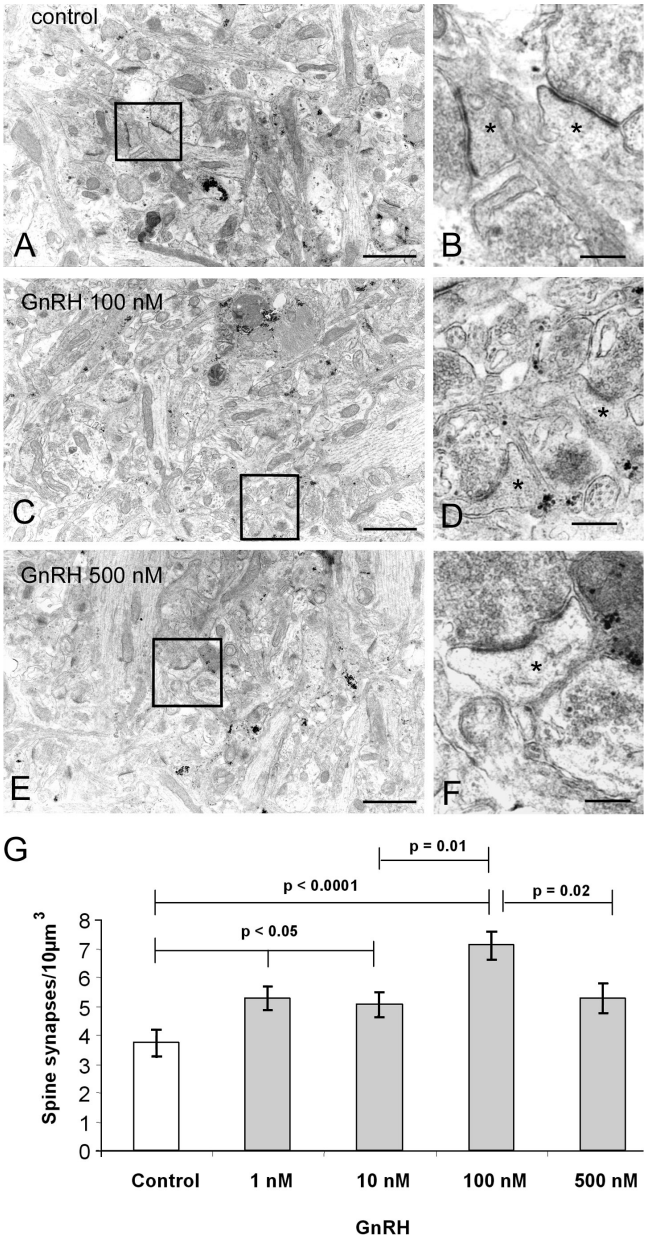

Figure 3. **Spine synapse density in the CA1 region of hippocampal slices increases in a dose-dependent fashion after treatment with GnRH.** (A–E) Electron micrographs of the stratum radiatum of the CA1 region after treatment with GnRH. (A, C, and E) No differences between treatments with increasing GnRH doses were observed in the neuropil. Bars, 1.5 µm. (B, D, and F) High magnification of the framed areas in A, C, and E. In all groups, morphologically intact synapses were found (*). Bars, 0.2 µm. (G) The maximal increase in spine synapse number was observed after treatment with 100 nM GnRH (mean ± SEM; $n = 5$).

with the aromatase inhibitor letrozole, and with a combination of both substances. As previously described (Kretz et al., 2004), immunostaining for spinophilin in dispersion cultures resulted in a punctate staining of the cytoplasm and the dendrites (Fig. 4 A). The stained area and its staining intensity depended on the treatment. Image analysis (Fig. 4 B) demonstrated that treatment with 10 nM GnRH resulted in an increase in spinophilin expression, which is in agreement with the results from the hippocampal slice cultures. As previously shown (Kretz et al., 2004; Prange-Kiel et al., 2006), inhibition of endogenous E_2 synthesis 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 E_2

Earlier studies have demonstrated that E_2 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 E_2. To examine this, hippocampal neurons were treated for 8 d with either 100 nM letrozole, to inhibit hippocampal E_2 synthesis, or with 100 nM E_2. 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 E_2 did not change GnRH-R expression in hippocampal neurons, which suggests that there is a ceiling of E_2-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 E_2 serum levels, or at estrus, when E_2 serum levels are low. To eliminate the peripheral source of E_2, 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,

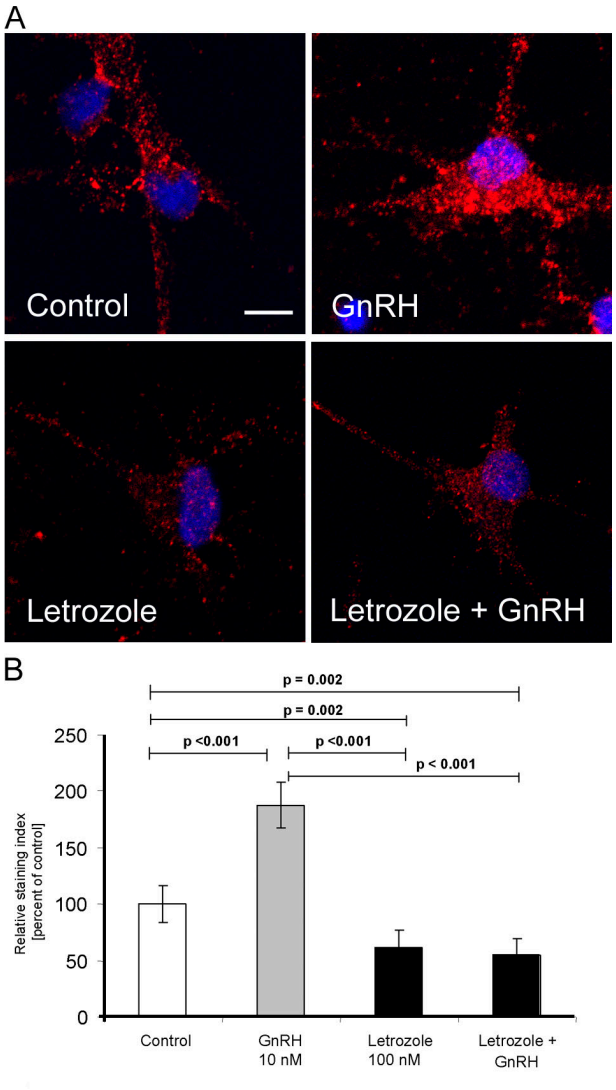

Figure 4. **Effects of GnRH on spinophilin expression are mediated by estrogen synthesis in hippocampal dispersion cultures.** (A) Punctate spinophilin immunoreactivity was detected on single pyramidal neurons (Cy3, red). The stained area and its staining intensity varied with the different treatments. The nuclei were counterstained with DAPI (blue). Bar, 10 μm. (B) The GnRH-induced increase in spinophilin expression was blocked when E_2 synthesis was inhibited by 100 nM letrozole. In contrast to the treatment with GnRH alone, the combination of 10 nM GnRH and 100 nM letrozole did not result in an increase of the spinophilin staining (mean ± SEM; $n = 5$).

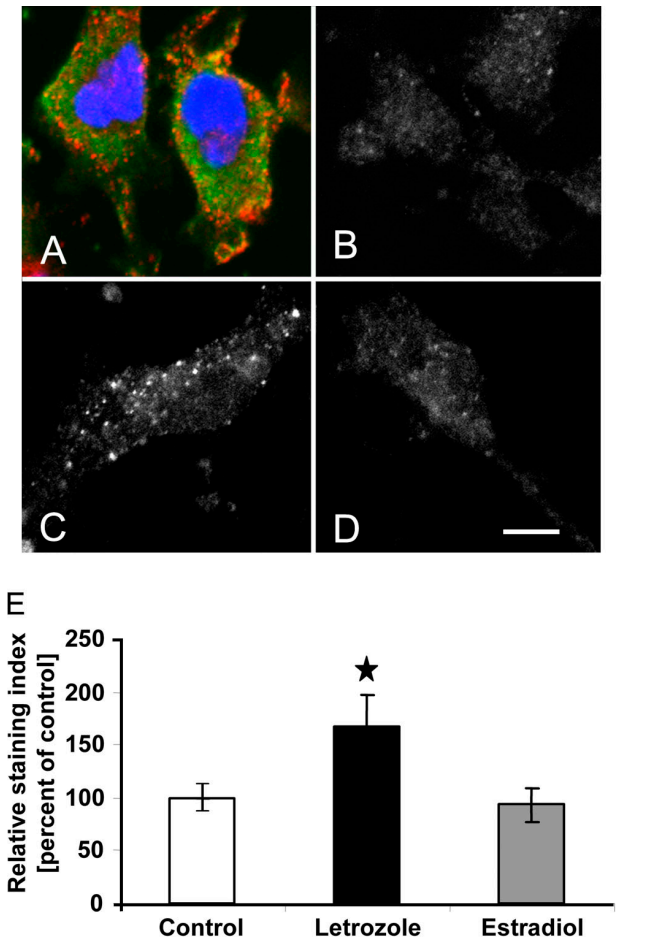

Figure 5. **Hippocampus-derived E_2 inhibits GnRH-R expression.** (A) Hippocampal neurons in dispersion cultures were identified by the expression of MAP-2 (FITC, green). Coincubation with an antibody against GnRH-R (Cy3, red) resulted in punctuate staining primarily located at the periphery of the cells. The nuclei were counterstained with DAPI (blue). (B–D) Confocal images of single hippocampal neurons that were either untreated (B) or treated with letrozole (C) or E_2 (D). These images were subsequently used for image analysis. Bar, 10 μM. (E) In dispersion cultures of hippocampal neurons, image analysis of GnRH-R staining demonstrated that treatment with 100 nM letrozole, which inhibits E_2 synthesis, resulted in a significant up-regulation of the GnRH-R staining index. Treatment with 100 nM E_2 did not affect GnRH staining in these cultures (mean ± SEM; ★, $P < 0.05$; $n = 12$).

calculation of spine synapse density failed to show any correlation to the ovarian cycle or to the ovarian status of the animals (Fig. 6 E). Thus, cycling of spine synapse density is not a general phenomenon seen throughout the entire neocortex.

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). 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).

Discussion

Our results demonstrate that GnRH binding to its receptor regulates hippocampal E_2 synthesis, which, in turn, influences synaptogenesis. This may explain the estrus cycle–regulated cycling of spine density that is seen specifically in the hippocampus.

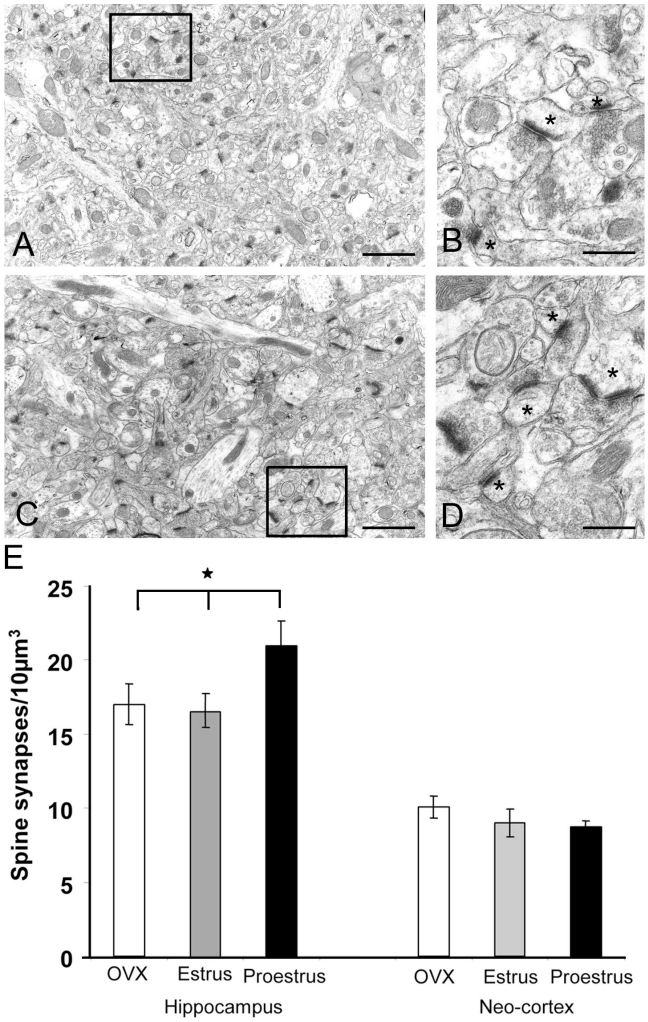

Figure 6. **Spine synapse density varies during the estrus cycle in the hippocampus but not in the neocortex.** (A and C) Electron micrographs of the stratum radiatum of the CA1 region of the hippocampus (A) and layer III of the neocortex (C) of a rat during estrus. Bars, 1 µm. (B and D) High magnification of the framed area in A and C revealed morphologically intact synapses (*). Bars, 0.25 µm. (E) Unbiased electron microscopic stereological calculations showed that spine synapse density in the stratum radiatum of the CA1 region in female rats at the stage of proestrus was 20% higher (mean ± SEM; ★, $P < 0.05$; $n = 3$) than in females at the stage of estrus or after ovariectomy. However, in the external pyramidal layer of the neocortex of these animals no such difference was detected. In this region, spine synapse density remained stable during the estrus cycle and ovariectomy did not change the number of spine synapses. As previously shown, the mean spine synapse density in the hippocampus was ~40% higher than in the neocortex (Deller et al., 2003).

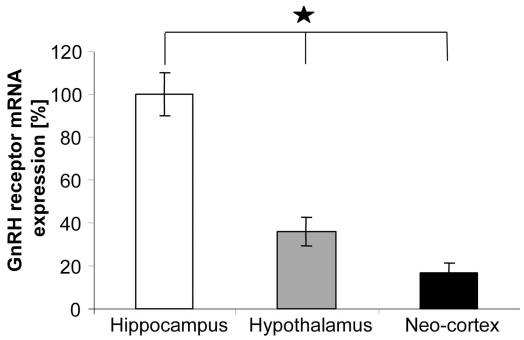

Figure 7. **GnRH-R mRNA expression is strongest in the hippocampus.** Real-time RT-PCR demonstrated that the amount of GnRH-R mRNA in the hippocampus was significantly higher than in the hypothalamus and in the neocortex of adult female rats (mean ± SEM; ★, $P < 0.0001$; $n = 10$). The difference in GnRH-R mRNA expression between the hypothalamus and neocortex was slightly higher than the level of significance ($P = 0.071$).

GnRH regulates E_2 synthesis and, as a consequence, spine density in the hippocampus

GnRH is the key regulator of reproduction, as its pulsative 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 E_2 synthesis in the ovary (Parinaud et al., 1988; Janssens et al., 2000). However, the gonads are not the only site of E_2 synthesis. The hippocampus has been shown to be a prominent extragonadal site of E_2 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). E_2 synthesis in neurons depends on aromatase, and the activity of this enzyme in neurons is regulated by neuronal activity (Zhou et al., 2007) and Ca^{2+}-dependent phosphorylation (Balthazart et al., 2003). GnRH is the first peptide described to regulate hippocampal E_2 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 E_2 synthesis. A further increase in the GnRH concentration did not result in an additional increase in E_2 synthesis but, rather, in its inhibition. Notably, an inverted U-shaped dose–response curve for E_2 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 E_2 for synaptic plasticity has been unequivocally demonstrated using the aromatase inhibitor letrozole. Inhibition of hippocampal E_2 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 E_2 but not with amounts corresponding to serum E_2 concentrations. Concomitantly, Hojo et al. (2004) have shown that the basal concentrations of E_2 in hippocampi of male rats are six times higher than the concentrations in serum. This suggests that the serum E_2 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 E_2 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

short-term E_2 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 E_2 synthesis. Intermediate doses of GnRH stimulated spinophilin expression, whereas high doses had no effect. Moreover, when GnRH-induced hippocampal E_2 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 E_2 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 E_2 might explain these differences. Other GnRH effects on these parameters that are not mediated by E_2 cannot be completely ruled out.

Hippocampus-derived E_2 regulates GnRH-R expression

In a gonadotrope-derived cell line, treatment with E_2 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 E_2 limits GnRH-R in hippocampal neurons. Surprisingly, treatment with additional E_2 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 E_2. 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 E_2 synthesis via its receptor regulation. The regulation of GnRH-R by E_2 may indicate a negative-feedback mechanism that prevents excessive E_2 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 E_2 results in an increase in spines in the CA1 region of the hippocampus. Concomitantly, Woolley et al. (1990) showed a correlation of changes in E_2 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 E_2 (McEwen, 2002). However, recent findings from our laboratory emphasized the importance of hippocampus-derived E_2 and questioned the effects of gonadal E_2 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 E_2 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 β (Shughrue and Merchenthaler, 2001).

Based on our finding that GnRH regulates E_2 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 (Pfaff 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 E_2 synthesis in response to the pulsative release of GnRH from the hypothalamus. Thus, GnRH synchronizes both gonadal and hippocampal E_2 synthesis and, as a consequence, E_2 serum levels and hippocampal spine density change in parallel. Although the source of GnRH in the hippocampus remains to be clarified, earlier data on E_2-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 ovariectomy 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, ovariectomy resulted in augmented GnRH release (Chongthammakun et al., 1993). However, as we show here, high GnRH inhibits hippocampal E_2 synthesis and reduces spine density. This provides an explanation for the reduced spine density seen after ovariectomy. Systemic treatment of ovariectomized animals with E_2, 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 E_2 synthesis under in vivo conditions in cycling animals.

In summary, the interplay of GnRH on E_2 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 E_2 in synaptogenesis.

For almost two centuries, circulating estrogens were considered to be the exclusive source of estrogenic action in the hippocampus and many in vivo studies promoted this idea. It is now clear that this picture is inadequate. Further studies will show to what extent gonadal and hippocampus-derived steroids are involved in the regulation of neuronal plasticity.

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.

A group of 10-wk-old females was deeply anesthetized (3.3 ml/kg of a ketamine–xylazine mixture, i.p.) and ovariectomized. 14 d after surgery 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 estrus cycle (proestrus or estrus) were perfused in the morning to assure maximal E_2 serum levels in animals in the stage of proestrus. The results of the staging were confirmed by determination of serum E_2 levels of the animals by a commercial E_2 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/cm^2 poly-D-lysine–coated (Sigma-Aldrich) coverslips in 8-mm-diameter 24-well culture dishes (Thermo Fisher Scientific) at a density of 5×10^5 cells/well. The cells were incubated in estrogen-free culture medium (Neurobasal A [without phenol red]; Invitrogen), 1% B27, 500 mM L-glutamine (Invitrogen), 1% antibiotics (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 inserted 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% $NaHCO_3$. The pH was adjusted to 7.3. Before the experiments, the slices were precultured for 14 d at 37°C in a humidified CO_2-enriched atmosphere 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 with100 nM of the aromatase inhibitor letrozole (Novartis). Media and supplements were changed every second day and the used media were collected for the RIA.

E_2 RIA

The medium of treated and nontreated cultures was collected every second day and the medium of each well was pooled over the duration of the experiment. The processing of the medium and the E_2 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 (Millipore), which had been preconditioned with 5 ml methanol and equilibrated with 5 ml water. After a wash with ammonium acetate buffer (0.1 M, pH 4, 5 ml) to remove hydrophilic compounds, the retained analyte was eluted with 2 ml methanol. The eluate was dried in vacuo and redissolved in 250 μl of RIA buffer. 25 μl of the sample was analyzed in the E_2 RIA in duplicate. The assay has a high analytical sensitivity and little cross-reactivity with other steroids (von Schassen et al., 2006). Values measured in the unconditioned medium (pure medium, which had not been used for culture) were subtracted as background. For each treatment and each dose, 10 cultures were measured. To calculate the percentage values, the mean of the E_2 concentrations determined in the medium collected from the control slice cultures (which was in the range of 200 pg/ml) was set at 100%, and the values determined in the treatment groups were related to it.

IHC

The dispersion cultures were fixed in 4% PFA for 10 min and stored in PBS at 4°C until further use. Organotypic slices were fixed in 4% PFA overnight and incubated in 25% sucrose (in PBS) for another 6 h. The slices were then transferred to methylbutane and quick-frozen in liquid nitrogen. 12-μm-thick cryostat sections were cut, put on microscope slides, air dried, and fixed in cold acetone for 15 min.

IHC was performed as described previously (Rune et al., 2002). The sections or cultures were incubated overnight at 4°C with primary antibodies against GnRH-R (1:100; Santa Cruz Biotechnoloy, Inc.), MAP-2 (1:200; Millipore), or spinophilin (1:750; BIOMOL International, L.P.). When double labelling was required, the corresponding antibodies were applied simultaneously. Staining was visualized by the use of the appropriate fluorescence-labeled secondary antibodies (Cy3- and FITC-labeled anti–mouse or anti–rabbit antibodies, 1:350; Millipore). Nuclei were counterstained with DAPI (1:10,000 in PBS; Sigma-Aldrich).

Image analysis

To avoid bias, all analyses were performed with coded slices and the investigator was unaware of the protocol of the sample under study.

For the observation and documentation of IHC, a laser-scanning microscope (LSM; Carl Zeiss, Inc.) was used. For image acquisition, a 63×/1.4 NA oil objective lens (Plan-Apochromat; Carl Zeiss, Inc.) was used, and the region of interest was further magnified by using the LSM-Meta software (Carl Zeiss, Inc.) zoom function. Two- and fourfold zoom were used for the slice sections and neurons from dispersion cultures, respectively. Once conditions for data collection were optimized, the chosen parameters were kept constant for the documentation of the entire experiment.

For the subsequent analysis of the digitized pictures, the cell-imaging software Openlab 2.3.1. (Improvision) was used. In a first step, the specific staining for each experiment was defined and discriminated from the background. For that purpose, a threshold was determined using control sections/cell cultures immunostained without the primary antibody. A gray value that was slightly higher than the background staining of the control sections was chosen as the appropriate threshold.

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-R or spinophilin 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, 15 cells of each treatment were analyzed and a mean was calculated for every group. In organotypic slice cultures, the spinophilin 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 bregma 1.60 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 ultra-thin 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 made at a magnification of 6,600, 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 miscroscope 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 unit area of the reference by the distance (0.09 µm) between the reference and the lookup section. At least 20 neuropil fields per tissue sample and animal were analyzed.

Real-time RT-PCR

10 5-d neonatal rats and 10 adult 10-wk female rats were anesthetized and decapitated. The brains were removed and tissue samples of the same size were taken from the hypothalamus, hippocampus, and neocortex. The neocortex was prepared in the same way as described for electron microscopy. The fresh weights of the samples were determined and they were immediately quick frozen in liquid nitrogen and stored at −80°C. The total RNA was isolated with the RNeasy Total RNA kit (QIAGEN), including the removal of DNA with DNase, according to the manufacturers instructions. RT reaction and real-time PCR were performed as previously described (Roth et al., 2001), using an ABI Prism 7700 sequence detection system (TaqMan; Applied Biosystems). For analysis, a standard curve was generated by plotting known cDNA concentrations versus the corresponding threshold cycle (Ct) value obtained in the real-time PCR reaction. To determine the relative expression levels of the tissue samples, the respective Ct values were interpolated from the standard curve.

Apoptosis and necrosis

A kit obtained from Boehringer Ingelheim was used for TUNEL, which was performed according to the instructions of the suppliers. Cytoplasmic 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.

The authors wish to thank Brigitte Asmus, Helga Herbort, Bettina Kruck, Maria Metten, Claudia Neitzel, and Christiane Schroeder for their excellent technical assistance, Robert Jack (University of Greifswald, Greifswald, Germany) for reviewing the paper, and Elisabeth Grundy for proofreading the manuscript.

Submitted: 5 July 2007
Accepted: 18 December 2007

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