Sexual dimorphism in estrogen-induced synaptogenesis in the adult hippocampus

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ABSTRACT It has long been known that estradiol influences synaptic plasticity in the female hippocampus. The density of dendritic spines varies during the estrous cycle and correlates positively with varying levels of estradiol in serum. In accordance, ovariectomy results in a loss of spines that can be rescued by estradiol treatment in animals, suggesting that estradiol originating from the ovaries induces spine formation in the hippocampus. More recent studies point to a role of hippocampus-derived estradiol in synaptogenesis in the female hippocampus, rather than of estradiol of ovarian origin. In our studies, we have shown that inhibition of hippocampal estrogen synthesis results in spine synapse loss in female animals and, more importantly, also in ovariectomized animals. Surprisingly, inhibition of local estradiol synthesis had no effect on synapse formation in males, in spite of a similar capacity to synthesize estradiol in male and female hippocampal neurons. In females, neuro-sexual steroid production is promoted by hypothalamic, cyclic GnRH release and likely underlies the estrus cyclicity of spine synapse density in the hippocampus. As a result, peripheral serum concentrations of estradiol determine the amount of estradiol synthesis in the hippocampus. This paradigm may also be true in males. In support of this hypothesis, we found that the content of estradiol in hippocampal tissue is higher in female compared to that in male animals, with low levels of estradiol in serum and tonic and acyclical GnRH release. In summary, our data point to important sex-specific differences in sexual steroid-induced synaptogenesis.

KEY WORDS: *aromatase, estrogen, synaptogenesis, hippocampus, sexual dimorphism*

Introduction

In the early nineties, it became evident that hormones, namely estrogens, influence synaptic plasticity. At that time, McEwen and co-workers discovered that ovariectomy results in loss of postsynaptic dendritic spines on hippocampal neurons of the CA1 region (Gould *et al.*, 1990). As systemic injections of estradiol into these animals restored dendritic spine reduction, it appeared plausible to believe that estradiol originating from the ovaries influences spine density in the hippocampus. The subsequent finding, showing that spine density varies with the estrous cycle, was consistent with this hypothesis, i.e. that ovary-derived estradiol in serum is capable of controlling spine density in the hippocampus (Woolley *et al.*, 1990). When estradiol concentrations are highest in serum at proestrus, shortly before ovulation, spine density has reached its maximum and the density of spines decreases thereafter, along with decreasing levels of estradiol in serum. Altogether, these data underline the high plasticity of synapses, or at least of postsynaptic spines, in the hippocampus and show estrogen-induced synaptogenesis

to be a prominent example of adult synaptogenesis.

Despite the great number of studies that followed the initial studies on estrogen-induced synaptogenesis and related phenomena (for review see Spencer *et al.*, 2008), many questions, in particular many basic endocrinological questions, remained unanswered or were ignored. Two points may be particularly emphasized: serum concentrations in female rats are around 0.1 nM and are even lower in males. An increase in spine density due to estradiol, however, is generally accepted to take place in response to 1nM estradiol *in vitro* in males as well as in females (Kramar *et al.*, 2009). To detect estrogenic effects in females, ovariectomized animals were commonly used for systemic treatment, which does not allow for the discrimination of a direct estrogenic effect from an indirect effect, since the removal of ovaries includes many more factors than merely estradiol. Another important issue is the relevance of systemic estrogenic effects in males, which were frequently

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Abbreviations used in this paper: EGFP, enhanced green fluorescent protein.

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taken for the experiments to avoid cyclicity. Not only were the doses applied tremendously higher than the physiological serum concentrations, but also the question as to how the rather high doses of estradiol could interfere with other metabolic processes, such as the synthesis of other sexual steroids in the animals, was never considered.

Hippocampal neurons synthesize estradiol independent of gender

Naftolin and co-workers (Naftolin *et al.*, 1971) were the first to describe aromatase expression in the diencephalon. Aromatase is the final enzyme of estradiol synthesis and catalyzes the conversion of testosterone to estradiol. It has meanwhile been established that the brain is equipped with all the enzymes of estrogen synthesis, including aromatase (Shibuya *et al.*, 2003; Do Rego *et al.*, 2009; Pelletier, 2010). The actual synthesis and secretion of estradiol from hippocampal neurons, however, was not shown until many years later (Prange-Kiel *et al.*, 2003). We showed that adult cultured hippocampal neurons are capable of synthesizing estradiol *de novo*, which was confirmed by Hojo and co-workers (Hojo *et al.*, 2004). Prange-Kiel and co-workers cultured isolated adult neurons under serum- and steroid-free conditions, which allowed them to postulate that the neurons synthesize estradiol *de novo* from cholesterol. To further substantiate the *de novo* synthesis of estradiol in hippocampal neurons, we performed a knockdown of steroidogenic acute regulatory protein (StAR). StAR transports cholesterol through the mitochondrial membranes, since the first enzyme of estrogen synthesis, the side chain cleavage enzyme (SCC P_{450}), is located at the inner mitochondrial membrane, where steroidogenesis commences (Jefcoate, 2002; Rone *et al.*, 2009). An earlier study by our group had demonstrated the expression of StAR together with aromatase in the hippocampus (Wehrenberg *et al.*, 2001). After transfection of hippocampal neurons with siRNA against StAR, estradiol synthesis in the neurons was clearly reduced, further indicating that estradiol is synthesized *de novo* from cholesterol in hippocampal neurons (Fester *et al.*, 2006; Fester *et al.*, 2009a).
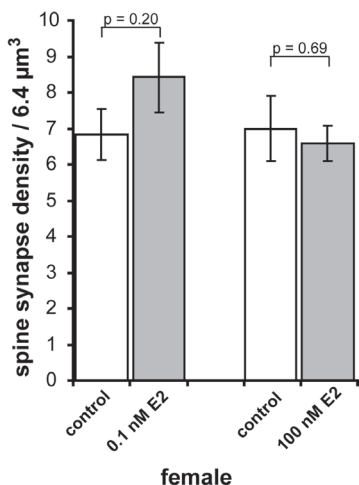

Fig. 1. Spine synapse density in female hippocampal slice cultures after estradiol treatment at a dose of 0.1 nM (physiological female serum concentration) and 100 nM estradiol for 7 days. *No significant differences were detected between treated and non-treated cultures. (mean±SEM, n=5; *Student t-test, p≤0.05).*
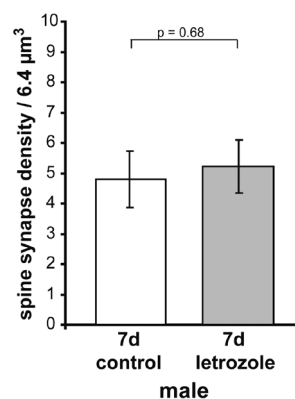

Fig. 2. Spine synapse density in male hippocampal slice cultures in response to inhibition of aromatase at a dose of 100 nM letrozole for 7 days. *No significant differences were detected between treated and non-treated cultures. (mean±SEM, n=5; *Student t-test, p≤0.05).*

With respect to our basic question regarding the role of local estradiol synthesis in estrogen-induced synaptic plasticity, we manipulated the amount of neuronal estradiol secretion by upregulation and downregulation of aromatase as the last step of estrogen synthesis. For the upregulation we added substrates of estrogen synthesis, such as cholesterol and testosterone, to the cultures and for the downregulation we used aromatase inhibitors, which are commercially available and commonly used in the therapy of postmenopausal women who suffer from breast cancer.

Cholesterol, as well as testosterone, the direct substrate of aromatase, stimulate estradiol synthesis dramatically, cholesterol to a higher degree than testosterone (Fester *et al.*, 2009a). The aromatase inhibitor letrozole, which we used for our experiments, is a non-steroidogenic, reversible inhibitor that reduces the synthesis and release of estradiol from newborn hippocampal slice cultures and dissociated hippocampal neurons dose-dependently, by a maximum of 40% (for review see Fester *et al.*, 2011). Potential toxic effects could be ruled out up to a dose of 100 nM and the reversibility of the effects was seen within 32 hours (Fester *et al.*, 2006; Prange-Kiel *et al.*, 2006).

In vitro, the capacity of estradiol synthesis was independent of gender. When we compared the amount of estradiol synthesis in dissociated cultures that originated from male animals with cultures that originated from female animals, no difference was found in the amount of estradiol in the supernatant, as evidenced by radioimmunoassay (Fester *et al.*, 2012).

Local estradiol synthesis controls spine synapse density in the hippocampus of female but not of male animals

In our first experiments on the role of local estradiol synthesis on synaptogenesis, we used hippocampal cultures to exclude any potential estrogenic influence from peripheral sources. In hippocampal slice cultures, the density of spine synapses was stereologically determined in electron micrographs. For determination of spine density we used EGFP-transfected, serum- and steroid-free hippocampal dissociated neurons.

In previous experiments, we had not found estradiol treatment to have any effect on spine synapse density in hippocampal slice cultures; neither with physiological serum concentrations of estradiol nor with high doses of 100 nM estradiol for 7 days, was an

increase in spine synapse density detected (Fig. 1). This holds true for cultures originating from female, as well as for those originating from male animals (Kretz *et al.* 2004).

In contrast, the inhibition of aromatase by letrozole and the consequent lowering in estradiol synthesis in cultures that were prepared from female animals, resulted in a significant and clear reduction in the number of spine synapses in the CA1 hippocampal region (Kretz *et al.*, 2004). This effect was restored by simultaneous application of estradiol and letrozole, the aromatase inhibitor we used in our experiments (Zhou *et al.*, 2007). No effect was seen after letrozole treatment in cultures that originated from male animals (Fig. 2). Spine synapse loss in "female" hippocampal cultures is likely to result from the dramatic impairment of long term potentiation in response to letrozole treatment, since LTP impairment precedes spine synapse loss and LTP was shown to induce spine formation (Yuste and Bonhoeffer, 2001, 2004). No such effect on LTP in response to inhibition of aromatase was seen in males (Vierk *et al.*, 2012)

In order to be sure that the effects we found after inhibition of aromatase were not due to elevated levels of precursors of estrogen synthesis, we had previously tested in mixed hippocampal slice cultures of male and female animals the effects of cholesterol and testosterone. Spine synapse density and estradiol levels, as described above, were both significantly increased (Fester *et al.*, 2009a). However, the effects after treatment with cholesterol and testosterone respectively were abolished in the presence of letrozole, pointing to the specificity of estradiol synthesis and showing that cholesterol and testosterone are ineffective unless they are metabolized to estradiol.

Two types of spines are present in the hippocampus and in the neocortex: thin spines, which are considered to be more dynamic and transient in character, and mushroom spines, which are believed to be mature, stable spines that can persist for months (Holtmaat *et al.*, 2005; Zuo *et al.*, 2005). Mushroom spines have larger postsynaptic densities (Harris *et al.*, 1992) that anchor more AMPA receptors and make these synapses functionally stronger (Matsuzaki *et al.*, 2001; Ganeshina *et al.*, 2004; Ashby *et al.*, 2006; Majewska *et al.*, 2006). Compared to thin spines, mushroom spines often contain polyribosomes for local protein synthesis (Haber and

Murai, 2006; Witcher *et al.*, 2007). Accordingly, mushroom spines are proposed to be memory spines (Tada and Sheng, 2006; Bourne and Harris, 2007; Kasai *et al.*, 2010). By using EGFP-transfected dissociated hippocampal neurons, we counted the number of spines and determined the phenotype of spines (thin versus mushroom spine) on "male" as well as on "female" neurons. In the controls, we found that male and female neurons differ in the composition of the spine phenotype. "Female" neurons exhibited more mushroom spines than "male" neurons, while it was vice versa with respect to thin spines: the proportion of thin spines was larger in neurons from males than in neurons from females (Vierk *et al.,* 2012). Treatment of these cultures with letrozole resulted in considerable loss of spines on female neurons but not on male neurons. Total spine number was reduced by less than 9% in male neurons, compared to roughly 40% in female neurons (Fig. 3). Most importantly, spine loss on female neurons in response to letrozole consisted predominantly of mature, mushroom spines and to a lesser degree of thin spines. In contrast, mature mushroom spines were unaffected after aromatase inhibition on male neurons and spine loss, if any, included only thin spines (Vierk *et al.,* 2012). Aside from the clear-cut differences between genders, the results show that in females, aromatase inhibition preferentially effects mushroom spines, which are considered to represent memory spines. The reduction in mature spines in females and in female hippocampal slice cultures after letrozole treatment is in agreement with the findings of Matsuzaki *et al.* (Matsuzaki *et al.,* 2004) showing that LTP, which is strongly impaired after letrozole treatment and precedes spine synapse loss, causes spines to develop a round configuration. As a conclusion, spine synapse loss in females appears to preferentially affect synapses with large spines, rather than thin spines. In contrast, the number of spine synapses and the number of mature spines were unchanged in males and were very much consistent, with no change in LTP. Consistent with the *in vitro* data on spine density on male single neurons and spine synapse density in male hippocampal slice cultures in response to letrozole, systemic treatment of male adult animals with the aromatase inhibitor had no effect, even after 4 weeks of treatment with letrozole (Zhou *et al.*, 2010; Fester *et al.*, 2012).

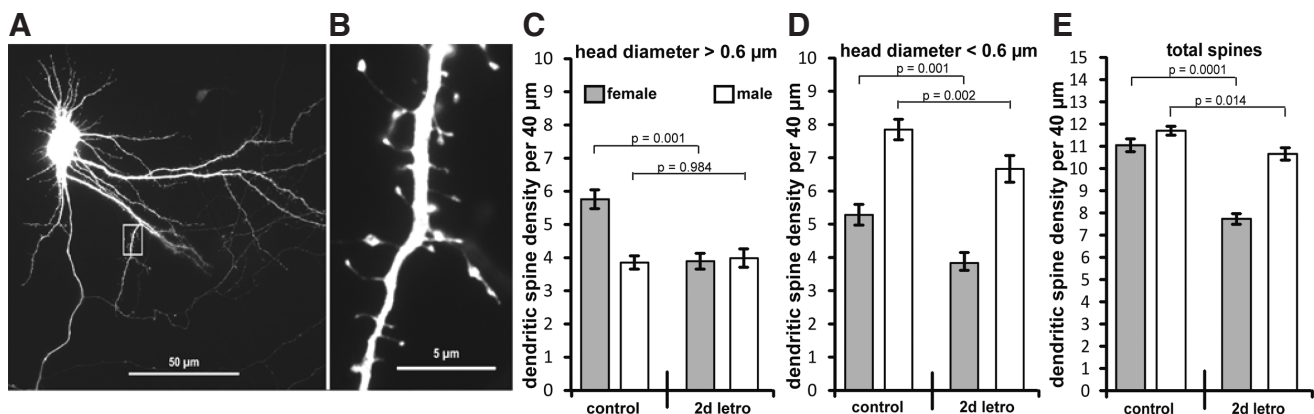Finally, when we treated adult female animals daily with letro-

Fig. 3. Spine density along dendrites of EGFP transfected neurons. (A) *Spine densities on dendrites of single dissociated neurons transfected with EGFP.* **(B)** *Enlargement of the framed dendrite section in A.* **(C, D, E)** *Determination of spine types (mature spines with a head diameter of more than 0.6 μm and thin spines with a head diameter less than 0.6 μm) revealed more mature spines on female neurons than on male neurons. After 2 days of treatment of the cultures with letrozole (letro), loss of mature and thin spines was observed on female dendrites, while on male neurons solely a reduction in thin spines was seen. (means±SEM; n=3 independent cultures; a total of 30 neurons in each group were used).*

zole for 7 days and 4 weeks we found, as expected, a reduction in spine synapse number, not only in intact females but also in ovariectomized animals (Zhou *et al.,* 2010). These data nicely confirm our *in vitro* results and most importantly, strongly suggest and support our hypothesis that estradiol of ovarian origin does not play the primary role. Altogether, our findings show that local estradiol controls spine synapse density in the hippocampus of females, rather than estradiol from peripheral sources.

How can the estrus cyclicity of synaptogenesis in females be explained?

In spite of spine synapse loss in ovariectomized mice in response to letrozole and the ineffectiveness of treatment of our hippocampal slice cultures with estradiol, which both argue for a primary role of local estradiol synthesis in estrogen-induced synaptic plasticity, the estrus cyclicity of spine density in the female hippocampus appeared to be discrepant with our hypothesis and needed to be addressed.

Serum levels of estradiol, which preferentially correspond to the synthesis of estradiol in ovarian granulosa cells, control the hypothalamo-pituitary-gonadal axis via a feed-back mechanism. Classically, estradiol serum levels control the release of GnRH from the hypothalamus, which in turn stimulates the pituitary to secrete FSH/LH, which enhances the synthesis of estradiol in ovarian granulosa cells. It was, however, also shown that GnRH is able to directly stimulate granulosa cells with regard to their ability to synthesize estradiol. Much to our surprise, GnRH receptors are abundant in the hippocampus and mRNA is at its highest levels in this brain region, compared to the cortex and the hypothalamus (Prange-Kiel *et al.*, 2008). Based upon the idea that GnRH could also stimulate estradiol synthesis in the hippocampus, similar to its effect on granulosa cells, we treated our hippocampal cultures with GnRH. GnRH stimulated both estradiol synthesis in hippocampal neurons and spine synapse density in an identical dose-dependent manner. The effect on synapse density was abolished when we applied GnRH together with letrozole, the aromatase inhibitor, to the cultures, showing that GnRH regulates spine synapse density
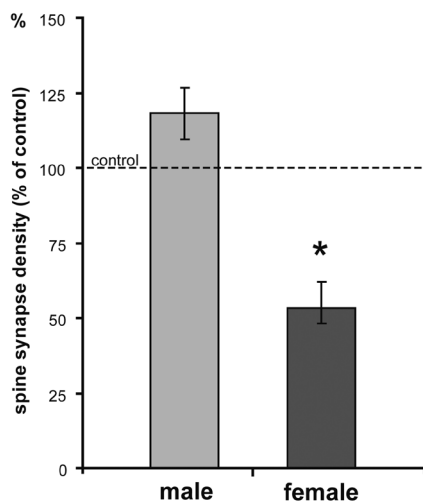
via regulation of the estradiol synthesis in hippocampal neurons. These *in vitro* findings strongly suggest that the release of GnRH upon varying estradiol levels during the estrus cycle synchronizes the ovarian and the "hippocampal" cycle. Increasing levels of estradiol in serum during the follicular phase give rise to an increasing release of GnRH from the hypothalamus, which not only stimulates estradiol synthesis in the ovaries via LH, but also directly stimulates estradiol synthesis in the hippocampus. Finally, injections of GnRH into the hippocampus of female animals also had promoting effects on synaptogenesis (unpublished data). Although we do not know yet how GnRH from the hypothalamus reaches the hippocampus, as direct neuronal connections do not exist, GnRH is found in cerebrospinal fluid (Skinner and Caraty, 2002) and thus GnRH could have access to the hippocampus via the median eminence, organum vasculosum of the lamina terminalis, and retrograde blood flow (Lehman *et al.*, 1986). All have been suggested as a potential source of GnRH in the cerebrospinal fluid. In addition, sexual behaviour was shown to be influenced by injection of GnRH into the ventricles (Pfaff *et al.*, 1994). Our findings were confirmed by Meethal *et al.* (2009), showing that peripheral sex steroids modulate GnRH-receptor signalling and gonadotropin-induced StAR-mediated uptake of mitochondrial cholesterol for neurosteroid synthesis, such as estrogen. These data support the dependency of neurosteroid synthesis on the hypthalamo-hypophyseal-gonadal axis.

This dependency could also help to understand why the content of estradiol in hippocampal tissue as measured by mass spectrometry differs between males and proestrus females (Fester *et al.*, 2012), although the capacity of estradiol synthesis in dissociated single hippocampal neurons does not differ between males and females. Robust functional sex differences have been identified in hypothalamic circuitry regulating reproductive function. Increasing levels of estradiol synthesized by granulosa cells in ovaries and released into the blood stream trigger the release of GnRH from neurons scattered throughout the hypothalamus, which in turn induce the mid-cycle surge in LH, which is essential for ovulation. In contrast, in male rats the pattern of LH release upon GnRH stimulation is tonic and acyclic leading to a steady state of testosterone, which always exerts a negative feed back on GnRH release (for review see Gillies and McArthur, 2010). The lack of cyclic variations of GnRH/LH in males, together with the findings by Meethal *et al.* (2009) showing that periperhal concentrations of estradiol influence StAR, could account for the difference in the content of estradiol in hippocampal tissue between males and females.

Pre- and postsynaptic marker proteins are regulated by local estradiol synthesis, independent of the regulation of synapse maintenance

Parallel to our studies on estrogen-controlled spine and spine synapse density in the hippocampus we studied pre- and postsynaptic marker proteins, which are frequently taken to be indicative of synapse formation. We used synaptophysin as a presynaptic marker and spinophilin as a postsynaptic marker. Synaptophysin is a constituent of the transmitter vesicle membrane and spinophilin is an actin-associated protein which is enriched in spines (Feng *et al.*, 2000). Both proteins turned out to be highly responsive to estradiol (Murphy and Segal, 1996; Brake *et al.*, 2001; Rune *et al.*, 2002; Hao *et al.*, 2003; Kretz *et al.*, 2004; Fester *et al.*, 2009b). In contrast to our findings on synapse formation, application of estradiol to the

Fig. 4. Changes in spine synapse density in male and female hippocampi after systemic treatment with letrozole for 4 weeks. *Letrozole significantly reduced synapse density in females but not in males (mean±SEM, n=5; *p≤0.05).*

cultures upregulated the protein and, consistent with our findings on synapse formation, they were downregulated in response to letrozole, thus after inhibition of aromatase in the hippocampal cultures. As far as the upregulation after estradiol is concerned, it has to be mentioned that the upregulation was seen at 100 nM of 17ß-estradiol but not at physiological serum concentrations of estradiol, neither in cultures originating from males nor in cultures originating from females (Kretz *et al.*, 2004). After knockdown of StAR, thus after inhibition of estrogen synthesis from its beginning, the proteins were also downregulated. This downregulation was rescued when we applied estradiol to the cultures that were transfected with siRNA against aromatase. It is noteworthy in this context that cholesterol and testosterone failed to rescue the downregulation of the proteins after knockdown of StAR, showing the specificity of estrogenic effects on the expression of synaptic proteins (Fester *et al.*, 2009b).

By using spinophilin and synaptophysin expression and their responsiveness to estradiol we were best able to demonstrate the auto/paracrine character of estrogen-regulated synaptogenesis, since steroidogenic proteins, such as StAR and aromatase, are expressed in a region-specific manner and the proteins were downregulated in a similar region-specific manner (Prange-Kiel *et al.*, 2006). Both the region-specific expression of steroidogenic enzymes and the identical region-specific downregulation of synaptic proteins in response to a defined dose of letrozole strongly suggest that estradiol, synthesized and released in defined regions within the hippocampus, also exerts its influence in exactly these areas.

In intact female animals, synaptophysin and spinophilin were also downregulated after daily injections of letrozole for 7 days and 4 weeks. It is notable that the proteins were dramatically upregulated in ovariectomized animals after 4 weeks of letrozole treatment, in spite of persistent spine synapse loss. This effect was not seen after 7 days of treatment. Obviously, protein expression only transiently correlates with spine and spine synapse formation. This finding led us to speculate that long-lasting estradiol depletion may induce a counter regulation in order to strengthen synaptic functionality.

In a more general sense, it appears questionable from these findings to take the expression of synaptic proteins to be indicative of synapse formation, since in the case of estrogen-induced structural synaptic plasticity, synaptic proteins are regulated independently from spinogenesis and synaptogenesis respectively.

Conclusion

Our findings over the last years strongly suggest that hippocampus-derived estradiol regulates synaptogenesis in the hippocampus rather than estradiol originating from peripheral sources such as the ovaries. Regulation of synapse density by local estradiol synthesis, is however, only valid in female, but not in male animals. This clear-cut sex-specific difference points to the necessity for further studies on the potential influence of sexual steroids on synaptogenesis in male animals.

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