Estradiol Regulates Hippocampal Dendritic Spine Density via an N-Methyl-D-Aspartate Receptor-Dependent Mechanism

Catherine S. Woolley and Bruce S. McEwen

Laboratory of Neuroendocrinology, Rockefeller University, New York, New York 10021

In the adult female rat, the densities of dendritic spines and synapses on hippocampal CA1 pyramidal cells are dependent upon the ovarian steroid estradiol; moreover, spine and synapse density fluctuate naturally as ovarian steroid levels vary across the estrous cycle. To determine whether the effects of estradiol on dendritic spine density require activation of specific neurotransmitter systems, we have treated animals concurrently with estradiol and one of four selective neurotransmitter receptor antagonists: MK 801, a noncompetitive NMDA receptor antagonist; CGP 43487, a competitive NMDA receptor antagonist; NBQX, an AMPA receptor antagonist; or scopolamine, a muscarinic receptor antagonist. Our results indicate that the effects of estradiol can be blocked by treatment with either of the NMDA receptor antagonists, but treatment with an AMPA or muscarinic receptor antagonist has no effect on spine density. Thus, we have concluded that estradiol exerts its effect on hippocampal dendritic spine density via a mechanism requiring activation specifically of NMDA receptors.

[Key words: dendritic spines, pyramidal cell, hippocampus, NMDA receptors, MK 801, Golgi impregnation]

N-methyl-D-aspartate (NMDA) receptors play a critical role in the establishment of appropriate synaptic connections in the developing nervous system (Cline et al., 1987; Kleinschmidt et al., 1987; Bear et al., 1990; Lau et al., 1992; Rabacchi et al., 1992). It has also become clear that changes in neuronal structure and synaptic connectivity occur not only during development, but in the adult brain as well (reviewed in Arnold and Breedlove, 1985; Gould et al., 1991; Horner, 1993). Determination of the factors that regulate structural changes in the adult brain is key to understanding alterations in neuronal circuitry that underlie differences in adult brain function. Currently, it is not known whether NMDA receptors are involved in structural plasticity in the adult brain. In this report, we demonstrate that estradiol-induced changes in the density of postsynaptic sites of excitatory input in the hippocampus, dendritic spines, require activation of NMDA receptors.

We first observed changes in hippocampal dendritic spine density using experimental manipulation of estradiol levels in adult female rats (Gould et al., 1990; Woolley and McEwen,

1993), and subsequently as estradiol and progesterone levels fluctuate naturally across the 5 d estrous cycle (Woolley et al., 1990; Woolley and McEwen, 1993). When circulating ovarian steroids are removed by ovariectomy, the density of dendritic spines specifically on CA1 pyramidal cells decreases; this decrease can be either prevented or reversed by treatment with estradiol. During the estrous cycle, fluctuation in dendritic spine density correlates well with hormone levels; that is, during proestrus, when ovarian steroid levels peak, spine density is highest, and 24 hr later, during estrus, steroid levels drop to their lowest values and spine density also decreases to its lowest value. We were able to confirm that estradiol-induced changes in the density of hippocampal dendritic spines reflect alteration in neuronal connectivity by showing that the density of axospinous synapses on CA1 pyramidal cell dendrites fluctuates in parallel with dendritic spine density both in the case of estradiol manipulation and during the estrous cycle (Woolley and McEwen, 1992).

The possibility that activation of specific neurotransmitter systems is involved in the effects of estradiol on hippocampal dendritic spine density was first indicated by the fact that, although spine density on CA1 pyramidal cells is sensitive to estradiol, there is evidence from *in situ* hybridization (Simerly et al., 1990; but see Pelletier et al., 1988), immunocytochemistry (Cintra et al., 1986; Don Carlos et al., 1991), and *in vivo* autoradiography (Pfaff and Keiner, 1973; Loy et al., 1988) that these neurons may not contain classical estradiol receptors. The apparent lack of receptors for estradiol in CA1 pyramidal cells suggests that the effects of this hormone on spine density might be mediated indirectly. The observation that dendritic spines are structurally dependent upon their afferents (Parnavelas et al., 1974; Benshalom and White, 1988) suggests that spine density may be regulated by an afferent population, the activity of which is sensitive to estradiol.

CA1 pyramidal cells receive excitatory input from glutamatergic/aspartergic and cholinergic afferents (Bayer, 1985). Our experiments were designed to test the hypothesis that the effects of estradiol on dendritic spine density require activation of either excitatory amino acid or cholinergic receptors. We have analyzed the density of dendritic spines on Golgi-impregnated hippocampal CA1 pyramidal cells in ovariectomized animals treated concurrently with estradiol and one of four specific excitatory amino acid or muscarinic receptor antagonists: MK 801, a noncompetitive NMDA receptor antagonist; CGP 43487, a competitive NMDA receptor antagonist; NBQX, an AMPA receptor antagonist; or scopolamine, a muscarinic receptor antagonist.

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Correspondence should be addressed to Catherine Woolley, Department of Neurological Surgery, RI-20, University of Washington, Seattle, WA 98195.

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The source of this difference in the effect of hormone treat-

Figure 2. Bar graphs depicting mean $\pm$ SEM dendritic spine density on the lateral branches of the CA1 pyramidal cell apical (*A*) or basal (*B*) dendritic trees in rats that were treated concurrently with estradiol and MK 801. Animals were sham ovariectomized and treated with sesame oil (SHAM+O), ovariectomized and treated with sesame oil (OVX+O), or ovariectomized and treated with estradiol (OVX+E). Half of the rats in each group received vehicle (shaded bars; $n = 5$ SHAM+O and OVX+O, $n = 6$ OVX+E), while the other half received MK 801 (solid bars; $n = 7$ SHAM+O and OVX+O, $n = 6$ OVX+E). Note that estradiol protects against an ovariectomy-induced decrease in both apical and basal dendritic spine density and that treatment with MK 801 inhibits the protective effect of estradiol. Asterisk indicates a significant difference detected using unpaired, two-tailed Student's *t* test ($p < 0.017$).

ment in MK 801-treated versus saline-treated animals was further investigated using unpaired, two-tailed Student's *t* tests. This analysis revealed that although MK 801 had no significant effect on spine density in either sham-operated or ovariectomized animals receiving sesame oil, MK 801 inhibited the ability of estradiol to protect against the ovariectomy-induced decrease in dendritic spine density (Fig. 2). That is, *t* tests indicated a significant difference between estradiol-treated animals receiving saline versus those receiving MK 801 in both apical ($p < 0.017$) and basal ($p < 0.017$) spine density (indicated by asterisks in Fig. 2*A,B*). The differences were approximately 22% for apical

and 23% for basal spine density. This analysis also indicated that MK 801 has no effect on the already low spine density in ovariectomized animals not receiving estradiol (Fig. 2). Thus, while treatment with MK 801 blocks the effect of estradiol, it results in no further decrease in spine density beyond that observed with ovariectomy. Additionally, although MK 801 treatment blocks the protective effect of estradiol on dendritic spine density, it does not appear to depress spine density in gonadally intact animals; that is, we observed no effect of MK 801 in sham-ovariectomized animals. Even in a separate experiment in which intact animals were treated with 0.5 mg/kg/d MK 801 for 7 d (2.5 times the previous dose for over twice as long), no significant decrease in spine density was observed (data not shown).

Analysis of dendritic branching demonstrated that, as previously observed (Woolley and McEwen, 1993), estradiol has no effect on either number of apical or basal dendritic branch points or total apical or basal dendritic length (Table 1). Additionally, in this study, we observed no effect of MK 801 on CA1 pyramidal cell apical or basal dendritic length, number of apical or basal dendritic branch points, or cross-sectional cell body area (Table 1).

CGP 43487 treatment

Analysis of CA1 pyramidal cell dendritic segments from rats treated with the competitive NMDA receptor antagonist CGP 43487 confirmed that the effect of estradiol on dendritic spine density requires NMDA receptor activation. Quantitative analysis of CA1 pyramidal cell dendritic segments from animals treated concurrently with estradiol and CGP 43487 showed that, in a pattern very similar to that observed with MK 801, CGP 43487 blocks the protective effect of estradiol without decreasing spine density any further in ovariectomized animals (Fig. 4). Two-way ANOVA indicated a significant main effect of estradiol on apical ($F[1,20] = 59.283$, $p < 0.01$) and basal ($F[1,20] = 6.716$, $p < 0.05$) spine density as well as a significant main effect of CGP 43487 on apical ($F[1,20] = 56.513$, $p < 0.01$) and basal ($F[1,20] = 5.477$, $p < 0.05$). In addition, a significant interaction between estradiol and CGP 43487 was detected for both apical ($F[1,20] = 26.535$, $p < 0.01$) and basal ($F[1,20] = 5.331$, $p < 0.05$) spine density. Unpaired, two-tailed Student's *t* tests were then used to compare spine density in estradiol-treated animals that received either CGP 43487 or saline. This analysis indicated a significant decrease of approximately 32% in apical spine density ($p < 0.01$, indicated by asterisk in Fig. 4*A*) and 20% in basal spine density ($p < 0.05$, indicated by an asterisk in Fig. 4*B*). CGP 43487 has no effect on spine density in ovariectomized rats treated with sesame oil alone.

NBQX or scopolamine treatment

In contrast to the experiments involving NMDA receptor blockade, the third and fourth experiments in which animals were treated concurrently with estradiol and either the AMPA receptor antagonist NBQX (Fig. 5) or the muscarinic receptor antagonist scopolamine (Fig. 6) indicated no effect of these drugs on dendritic spine density; that is, these drugs failed to inhibit the protective effect of estradiol. Analysis of apical and basal dendritic segments from animals treated with these blockers demonstrated that, as previously observed, estradiol protects against an ovariectomy-induced decrease in spine density. However, estradiol had the same protective effect in animals treated

Figure 3. Photomicrographs (*A–C*) or camera lucida drawings (*D–F*) of representative apical dendritic segments from ovariectomized rats treated with sesame oil and vehicle (*A, D*), ovariectomized rats treated with estradiol and vehicle (*B, E*), or ovariectomized rats treated with estradiol and MK 801 (*C, F*). Some dendritic spines are indicated by arrows; the same dendritic spines are indicated in the drawing and the photomicrograph of each particular dendritic segment. Note that while estradiol treatment increases dendritic spine density in an ovariectomized rat, MK 801 treatment inhibits this effect. Scale bar, 10 μ m (for *A–F*).

with NBQX or scopolamine as in those treated with vehicle alone.

It is important to note that the doses of NBQX and scopolamine used in these experiments have previously been shown to be centrally effective: NBQX to protect CA1 pyramidal cells from ischemic damage (Buchan et al., 1991) and scopolamine to disrupt septohippocampal activity and classical conditioning (Salvatierra and Berry, 1989) as well as cause deficits in a spatial learning task (Spangler et al., 1990). Thus, given our findings, it appears that the functional interaction of estradiol and neurotransmitter receptor blockade on the density of dendritic spines is specific to the NMDA receptor.

Discussion

The results of these experiments demonstrate that the protective effect of estradiol on hippocampal dendritic spine density occurs

via a mechanism requiring NMDA receptor activation. Inhibition of estradiol's effects on spine density is observed using either MK 801, a noncompetitive NMDA receptor antagonist, or CGP 43487, a competitive antagonist of the NMDA receptor. Treatment with either of these compounds does not further decrease the already low spine density in ovariectomized animals, indicating that low spine density in estradiol-treated animals given NMDA receptor antagonists most likely reflects an actual inhibition of estradiol's protective effect on spine density rather than merely additive, opposite effects of NMDA receptor antagonists and estradiol. In contrast to the results using NMDA receptor antagonists, treatment with AMPA or muscarinic receptor antagonists has no effect on spine density, indicating that these receptor types are probably not involved in the effects of estradiol on spine density.

Interestingly, we saw no significant effect of MK 801 on spine

Table 1. Effect of concurrent treatment with estradiol and MK 801 on morphologic parameters of hippocampal CA1 pyramidal cells

Morphologic parameter	OVX + O	OVX + E	OVX + O/MK	OVX + E/MK
Apical dendritic length (μ m)	1692.6 $\pm$ 66.5	1738.6 $\pm$ 84.2	1790.4 $\pm$ 82.8	1841.2 $\pm$ 115.3
Basal dendritic length (μ m)	982.6 $\pm$ 59.3	993.2 $\pm$ 78.5	1005.0 $\pm$ 75.5	915.8 $\pm$ 73.8
Number of apical dendritic branch points	23.9 $\pm$ 0.6	22.4 $\pm$ 1.7	24.4 $\pm$ 1.4	22.9 $\pm$ 0.6
Number of basal dendritic branch points	11.4 $\pm$ 0.8	10.5 $\pm$ 1.1	11.5 $\pm$ 0.7	11.4 $\pm$ 1.0
Cross-sectional cell body area (μ m ²)	236.1 $\pm$ 38.9	228.8 $\pm$ 17.2	254.7 $\pm$ 31.6	239.0 $\pm$ 21.2

Values represent mean $\pm$ SEM of morphologic parameters for CA1 pyramidal cells from ovariectomized rats treated with either sesame oil vehicle (OVX + O, n = 5; OVX + O/MK, n = 7) or estradiol benzoate (OVX + E, n = 6; OVX + E/MK, n = 6). No differences were observed in any parameter.

Figure 4. Bar graphs depicting mean $\pm$ SEM dendritic spine density on the lateral branches of the CA1 pyramidal cell apical (A) or basal (B) dendritic trees in rats that were treated concurrently with estradiol and CGP 43487. Animals were ovariectomized and treated with sesame oil (OVX+O) or ovariectomized and treated with estradiol (OVX+E). Half of the rats in each group received vehicle (shaded bars; $n = 6$), while the other half received CGP 43487 (solid bars; $n = 6$). Note that estradiol protects against an ovariectomy-induced decrease in both apical and basal dendritic spine density and that treatment with CGP 43487 inhibits the protective effect of estradiol. Asterisk indicates a significant difference detected using unpaired, two-tailed Student's t tests ($p < 0.01$, apical; $p < 0.05$, basal).

Figure 5. Bar graphs depicting mean $\pm$ SEM dendritic spine density on the lateral branches of the CA1 pyramidal cell apical (A) or basal (B) dendritic trees in rats that were treated concurrently with estradiol and NBQX. Animals were ovariectomized and treated with sesame oil (OVX+O) or ovariectomized and treated with estradiol (OVX+E). Half of the rats in each group received vehicle (shaded bars; $n = 6$ OVX+O, $n = 5$ OVX+E), while the other half received NBQX (solid bars; $n = 5$ OVX+O, $n = 4$ OVX+E). Two-way ANOVA indicated a significant effect of estradiol treatment but no effect of NBQX on spine density in ovariectomized rats treated with either sesame oil or with estradiol.

density in gonadally intact animals. This observation and the finding that NMDA receptor blockade did not decrease spine density in ovariectomized animals are consistent with the possibility that the NMDA receptor dependence of dendritic spines is limited to a time during which estradiol is acting. Our laboratory (Woolley and McEwen, 1993) has shown that during the estrous cycle, estradiol acts transiently to increase dendritic spine density. The estradiol-induced increase in spine density is then followed by a biphasic effect of progesterone, initially to increase spine density further, and then to cause a decrease. The lack of an MK 801 effect in the sham-operated group may be related to the fact these animals were in various stages of the estrous cycle and therefore differed in levels of circulating es-

tradiol and progesterone. If the progesterone-mediated effects on spine density are not NMDA receptor dependent, then MK 801 would not be expected to have decreased spine density uniformly in this group, since in some of these animals spine density was probably under the control of progesterone. In the future, the regulation of hippocampal dendritic spine density will undoubtedly be shown to involve factors in addition to NMDA receptor activation.

The differences in the density of dendritic spines reported here are likely to reflect differences in the overall number of spines per neuron. While differences in the number of spines per unit length of dendrite could theoretically result either from changes in spine number or from expansion and shrinkage of the dendritic tree, our data indicate that the latter is not the

case. Our analysis indicated that, in animals in which spine density was affected by estradiol, there were no concomitant changes in dendritic branching or dendritic length. Thus, in the absence of any overall changes in the dendritic tree, differences in spine density must result from alterations in spine number.

While it is not known which cell type(s) NMDA receptor antagonists act upon directly to inhibit the effect of estradiol, it seems likely that the effects of MK 801 and CGP 43487 are mediated, at least in part, at the level of the CA1 pyramidal cells themselves. Compared to other regions of the brain, as well as within the hippocampus, the CA1 region contains a particularly high density of NMDA receptors (Monaghan et al., 1988). Furthermore, it is known that CA1 hippocampal NMDA receptors are sensitive to estradiol; estradiol has been shown to upregulate agonist binding to NMDA receptors specifically within the CA1 region and with a time course very similar to estradiol's effect on dendritic spines (Weiland, 1992a; C. S. Woolley, N. G. Weiland, and B. S. McEwen, unpublished observations). Interestingly, the effect of estradiol on glutamate receptor binding appears to be specific for NMDA receptors as AMPA receptor binding in the CA1 region of the hippocampus is unaffected by estradiol treatment (Woolley, Weiland, and McEwen, unpublished observations).

There are at least two possible locations for a direct action of estradiol to regulate the density of dendritic spines on CA1 pyramidal cells. Loy et al. (1988) have demonstrated a small number of relatively large cell bodies within or near the CA1 pyramidal cell layer that concentrate ^3H -estradiol and thus are likely to be estrogen sensitive. The morphological characteristics of these cells are consistent with those of locally projecting inhibitory neurons (LaCaille et al., 1989). Given the fact that the effect of estradiol is NMDA receptor dependent and that activation of NMDA receptors requires sufficient depolarization to remove an Mg^{2+} block of the channel (Collingridge and Singer, 1990), it is possible that changes in spine density involve estradiol action upon local inhibitory neurons to disinhibit CA1 pyramidal cells. Such disinhibition could theoretically depolarize CA1 pyramidal cells sufficiently to unmask an NMDA receptor-dependent effect. Two lines of evidence favor direct action of estradiol on inhibition in the CA1 region. First, two types of studies have demonstrated that GABAergic function in this region is sensitive to estradiol: both GABA_A receptor binding (Schumacher et al., 1989) and levels of mRNA for glutamic acid decarboxylase, the rate-limiting enzyme in GABA synthesis (Weiland, 1992b), are regulated by estradiol. Second, the effects of estradiol treatment on the electrophysiology of CA1 pyramidal cells are consistent with disinhibition (see below).

Wong and Moss (1992) have recently shown that, in a subpopulation of CA1 pyramidal cells, estradiol treatment results in prolongation of the EPSP and increased probability of repetitive firing in response to Schaffer collateral stimulation. At least two, not mutually exclusive, explanations exist for these findings. Estradiol's effects could result from a potentiation of the slower NMDA component of the CA1 pyramidal cell EPSP or from a reduction of the disynaptic IPSP that results from activity of inhibitory basket cells. The authors (Wong and Moss, 1992) favored the former explanation, given the fact that neither the duration nor amplitude of the IPSP recorded in CA1 pyramidal cells was altered by estradiol treatment. However, as mentioned above, it is possible that an estradiol-induced disinhibition of CA1 pyramidal cells could depolarize them suf-

Figure 6. Bar graphs depicting mean $\pm$ SEM dendritic spine density on the lateral branches of the CA1 pyramidal cell apical (*A*) or basal (*B*) dendritic trees in rats that were treated concurrently with estradiol and scopolamine. Animals were ovariectomized and treated with either sesame oil (*OVX+O*) or estradiol (*OVX+E*). Half of the rats in each group received vehicle (shaded bars; $n = 4$ *OVX+O*, $n = 5$ *OVX+E*) and the other half received scopolamine (solid bars; $n = 4$ *OVX+O*, $n = 5$ *OVX+E*). Two-way ANOVA indicated a significant effect of estradiol treatment but no effect of scopolamine on spine density in ovariectomized rats treated with either sesame oil or with estradiol.

ficiently to remove the Mg^{2+} block of the NMDA receptor channel and increase its frequency of opening, thereby potentiating the NMDA component of the pyramidal cell EPSP. Future experiments will be required to test this possibility.

An alternative possibility for the location of direct action of estradiol lies in the observation that neurons in the entorhinal cortex also concentrate ^3H -estradiol (Pfaff and Keiner, 1973; Loy et al., 1988). Loy et al. (1988) reported that many of these neurons are found in layer II, the layer that provides input to dentate gyrus granule cells via the perforant path. This projection is the first step in the hippocampal "trisynaptic" circuit (Bayer, 1985) that drives hippocampal activity. It is possible that estradiol acts directly on entorhinal neurons to regulate overall activity of the trisynaptic circuit. If a certain level of activation of CA1 pyramidal cells is required to maintain dendritic spines, then NMDA receptor antagonists could act at any

step within the trisynaptic circuit to block the action of estradiol, thereby affecting spine density. It is important to note, however, that differences in activity alone cannot account for all changes in spine density. Since NMDA receptor antagonists failed to decrease spine density in gonadally intact animals, it is clear that the complete mechanism producing a fluctuation in spine density will involve additional factors, perhaps related to the action of progesterone (as discussed above).

It is tempting to speculate that antagonists of NMDA receptors block the effect of estradiol by inhibiting activation of NMDA receptors at estradiol-sensitive synapses themselves; that is, the synapses that would be lost with ovariectomy. The NMDA receptor channel is permeable to Ca^{2+} (MacDermott et al., 1986), and hippocampal dendritic spines have been shown to regulate Ca^{2+} independently from their parent dendrite (Guthrie et al., 1991). Because dendritic spines contain several components of a potentially Ca^{2+} -sensitive spine cytoskeleton, for example, actin (Fifkova and Delay, 1982; Matus et al., 1982), myosin (Morales and Fifkova, 1989a), and possibly MAP2 (Morales and Fifkova, 1989b), a localized increase in Ca^{2+} concentration resulting from NMDA receptor activation is likely to be an important factor in the extension and retraction of dendritic spines. Interestingly, Halpain and Greengard (1990) have shown that in a hippocampal slice preparation, NMDA receptor activation results in dephosphorylation of MAP2, a process shown to affect its interaction with tubulin (Murthy and Flavin, 1983) and actin (Seldon and Pollard, 1983).

It is possible that NMDA receptor activation is a generalizable requirement in synaptogenesis and the establishment of neuronal connections. Given our finding that hippocampal dendritic spine (and therefore synapse) formation is dependent upon activation of NMDA receptors, together with the findings of others that NMDA receptors are involved in the formation of neuronal connections in several different systems (Cline et al., 1987; Kleinschmidt et al., 1987; Bear et al., 1990; Lau et al., 1992; Rabacchi et al., 1992), it could be that various different factors that regulate these processes (e.g., hormones, neurotransmitters, neurotrophins) have in common that they exert their effects, at least in part, through activation of NMDA receptors. Because activation of NMDA receptors requires binding of several ligands as well as sufficient postsynaptic depolarization, NMDA receptor activation has been proposed to signal concurrent pre- and postsynaptic activity (Collingridge and Singer, 1990). NMDA receptors may thus serve to stabilize synaptic connections in a use-dependent manner.

The hippocampus is a site of epileptiform activity in both rats and humans. In both species there is evidence linking estradiol to seizure activity. In the rat hippocampus, estradiol has been shown to decrease the threshold for localized seizure induction (Terasawa and Timiras, 1968) as well as facilitate the induction of kindled seizures (Buterbaugh and Hudson, 1991). In humans, in some cases, epileptic activity can be related to events associated with large changes in ovarian hormone secretion such as puberty, pregnancy, or menopause (Rosiewicz et al., 1986); in the case of catamenial epilepsy, seizure frequency is linked to the menstrual cycle such that when estradiol levels are elevated, seizure frequency is increased (Crawford, 1991). If changes in hippocampal connectivity are causally related to alterations in hippocampal excitability, then our present observation that treatment with NMDA receptor antagonists can inhibit changes in neuronal connectivity may provide a means to control alterations in hippocampal physiology.

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