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Estrogen and the Aging Brain: an elixir for the weary cortical network?

Dani Dumitriu¹, Peter Rapp¹, Bruce McEwen², and John Morrison¹

¹Department of Neuroscience, Mount Sinai School of Medicine, One Gustave L Levy Place, New York, NY 10029, USA

²Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

Abstract

The surprising discovery in 1990 that estrogen modulates hippocampal structural plasticity launched a whole new field of scientific inquiry. Over the past two decades, estrogen-induced spinogenesis has been described in several brain areas involved in cognition, in a number of species, in both sexes, and on multiple time-scales. Exploration into the interaction between estrogen and aging has illuminated some of the hormone's neuroprotective effects, most notably on age-related cognitive decline in non-human primates. While there is still much to be learned about the mechanisms by which estrogen exerts its actions, key components of the signal transduction pathways are beginning to be elucidated and non-genomic actions via membrane bound estrogen receptors are of particular interest. The goal of this line of investigation is the eventual development of new therapeutics that can prevent or reverse age-related cognitive impairment.

Keywords

estrogen; aging; hippocampus; prefrontal cortex; rat; monkey; dendritic spines; cognition

Key experiments on female rats in the McEwen laboratory in the early 1990s showed that the dendritic spine density on the neurons in the CA1 region of the hippocampus varies over the course of the ovarian cycle¹, and surgical ovariectomy (OVX) leads to a 30% loss in spine density that can be rescued by estrogen replacement². The estrogen-induced spinogenesis is mirrored by an equal increase in synapses³, pointing to potential integration of the new spines into the hippocampal network. The effects of fluctuating hormone levels, OVX and estrogen replacement on spine density have now been extended to the somatosensory and motor cortex of rats⁴.

Classically, it has been thought that the induction of spine formation happened over one to two days. Recently however, induction of both spinogenesis⁵ and synaptogenesis⁶ have been demonstrated to also occur within half an hour of a subcutaneous injection of estrogen. Since cFos activation following estrogen administration is biphasic, with one peak at one hour and a second peak at 24 hours⁷, and since both the spinogenesis and the synaptogenesis are reduced at two and four hours post injection respectively, it is possible that estrogen induces two distinct phenomena over the two different time courses. In a powerful parallel to the structural plasticity body of work, estrogen has been shown to enhance hippocampal-dependent memory both over the course of days⁸ and rapidly, over the course of minutes⁹.

The existence of non-genomic mechanisms of actions for estrogen were postulated early on, following the initial discovery of estrogen's effect on hippocampal electrophysiology¹⁰ and the first observation of rapid spinogenesis in cultured hippocampal cells¹¹. Both of these phenomena occurred on the order of seconds to minutes and thus precluded the possibility of being mediated via gene transcription and translation. In fact, the first evidence of estrogen's ability to bind plasma membranes came as early as 1983¹², but it would take almost two more decades before the receptors would begin to be described.

It is now clear that ER α and ER β as well as additional potential ERs are present in multiple neuronal compartments in hippocampus, including synapses^{13, 14}. Through these receptors estrogen activates multiple signaling pathways and cellular processes via both genomic and non-genomic actions¹⁵. The McEwen lab has been characterizing multiple pathways linked to the synaptic effects of estrogen in hippocampus, particularly those linked to PI3K (fig 2)¹⁶ (See Spencer et al.¹⁶ for review). For example, they have shown that estrogen rapidly activates dendritic and synaptic Akt that leads to the stimulation of local translation of PSD95, a key scaffolding protein required for synaptic expansion¹⁷ (Fig 2). We have also been particularly interested in the serine/threonine kinase LIM Kinase (LIMK), which plays a key role in actin polymerization through phosphorylation of cofilin¹⁸. LIMK is present in CA1 synapses¹⁹, and critically important to the dynamics of actin polymerization required for spine formation¹⁶ (fig 2). Further characterization of these rapidly activated signaling cascades will be required to reveal the mechanisms underlying the non-genomic effects of estrogen on synapse formation and synaptic plasticity linked to learning and memory.

Estrogen and the aging hippocampus: lessons learned from the rat model

Convincing evidence that estrogen modulates synaptic plasticity in young female rats naturally led us to the clinically relevant question of estrogen's role in age-related cognitive decline. Our initial task was simply to examine if the synaptic remodeling observed in response to estrogen in young animals would also occur in the hippocampus of aged OVX rats. For this purpose, we used electron microscopy (EM) to quantify the axospinous synapse density in young (3-4 month) and old (23-24 month) OVX rats in the presence or absence of estrogen. While we were able to replicate previous findings² of a 30% increase in synapse density in the young group, estrogen treatment failed to increase synapse density in CA1 of aged OVX rats (figure 3a)²⁰. Interestingly, when we quantified synaptic levels of the obligate NMDA receptor subunit NR1 in the same animals, we found that estrogen increased the synaptic pool of NR1 (defined as being located within 30 nm of the postsynaptic membrane) in the aged but not the young cohort²⁰. Consistently, we later found a trend ($p=0.06$) of higher representation of the NMDA receptor subunit NR2A in the same synaptic region and a significant increase in the representation of the NR2B subunit in the lateral portion of the postsynaptic density of aged animals, again with no estrogen-induced change in the young group²¹. These important findings indicate that the aged synapse is not entirely insensitive to the circulating estrogen. Though estrogen-induced hippocampal structural plasticity is restricted to young animals, the estrogen-induced molecular plasticity with respect to NMDA receptor composition might allow the aged network to partly compensate for the loss of youthful connectivity.

An interesting parallel could be drawn between the age-dependent estrogen-induced molecular adaptations in NMDA receptor profile described above and electrophysiological findings by Thompson and colleagues on the interactions between aging, stress and estrogen in acute slices from male rats. For example, long term depression (LTD) is enhanced by age and this age-induced enhancement is blocked by estrogen. Furthermore, estrogen is protective against changes in both long-term potentiation (LTP) and LTD following stress in aged animals. Specifically, estrogen ameliorates the stress-induced decrease in LTP and blocks the stress-induced enhancement in LTD²². Both LTP and LTD are NMDA receptor-dependent forms of

plasticity. Estrogen exerts its effect within seconds to minutes, indicating that genomic mechanisms can also be excluded here. One potential non-genomic mechanism underlying these functions is the phosphorylation of NR2 subunit via the src/MAPK pathway²³. It is interesting to note that this phosphorylation of NR2 is diminished in aging²⁴, which could explain why estrogen confers stronger protection in LTD than LTP changes in slices from aged stressed animals²². In summary, this is another model in which, by altering NMDA receptor function, estrogen is able to maintain select network properties in the aged hippocampus.

We next investigated some of the possible mechanisms underlying the loss of structural plasticity in the aged hippocampus by quantitative ultrastructural analysis on tissue from the same animals in which we conducted our initial study. We first decided to look at the distribution of ER α since it had recently been found to localize in dendritic spines in CA1¹³ and therefore was ideally positioned to induce local, non-genomic structural changes. We found that only about half as many synapses contain ER α in aged versus young animals (fig 3b,c), irrespective of estrogen treatment. In addition, though subtle, an estrogen-induced decrease in synaptic ER α levels was observed in both axon terminals and dendritic spines selectively in the young cohort, once again pointing to a loss of responsivity by the neurons in the aged CA1 subfield²⁵.

As discussed above, we have been particularly interested in phosphorylated LIMK (pLIMK) located within the CA1 synapses activated by estrogen, due to its critically important role in regulation of actin dynamics linked to spine formation. As in the case of ER α , we found selective responsivity of synaptic pLIMK to estrogen in the young cohort, where we observed a 30% increase in the number of synapses containing pLIMK in the presence of estrogen¹⁹ (fig 3d,e). In contrast, the aged animals had decreased synaptic pLIMK levels in CA1 and this decrease was not reversed by estrogen treatment. Taken together, the following model emerges:

1. *Young animals*: estrogen replacement induces an increase in pLIMK via ER α , which then leads to phosphorylation of cofilin. Phosphorylation of cofilin inhibits its binding to actin, promoting actin polymerization required for the formation of new spines.
2. *Aged animals*: estrogen is unable to increase levels of pLIMK, possibly due to the low levels of synaptic ER α . This results in decreased deactivation of cofilin and ultimately a disruption of actin polymerization required for formation of new spines.

Interestingly, the above-described collection of age-related losses of responsivity to estrogen with respect to structural plasticity, molecular rearrangement and biochemical function correlate with an age-related loss of estrogen-induced memory enhancement. In young, but not aged OVX rats, estrogen rescues the learning deficits associated with experimentally induced cholinergic impairment. When given the drug scopolamine, rats perform worse on a T maze active avoidance task. Estrogen remains protective against scopolamine during middle age, as defined by irregular cyclicity, but loses its effect at more advanced stages in reproductive aging²⁶.

Estrogen and the aging prefrontal cortex: lessons learned from the non-human primate model

Menopause is an important physiological and psychological milestone in the lives of half of our population. While some clinical studies have shown that hormone treatment (HT) following menopause can protect against cognitive decline, other studies have shown an increased risk for neurologic complications with HT (see Sherwin and Henry²⁷ for review). The inconsistencies within the clinical studies likely result from multiple differences in design, primary among them, differences in the timing of treatment relative to the menopausal transition in women²⁷. A further impediment to the emergence of a consensus on HT for women

has been the disconnect between basic science and the medical reality of menopause²⁸. Until recently, the investigation into estrogen's role on brain function has largely been restricted to young OVX rats. Extending the model into the aged rat was an important first step toward increasing the clinical relevance of the animal work. The translational power of the animal studies has now been enhanced further by the design of experiments on non-human primates (NHPs) modeled after our findings in the rat described above²⁸.

The NHP model is ideal for preclinical studies on age-related cognitive decline and HT for two main reasons. First, unlike the rat which experiences a chronic high estrogen state at reproductive senescence, the NHP menopause is very similar to humans, characterized by loss of circulating estrogen. Second, NHPs are an excellent model to investigate age-dependent cognitive impairment linked to the dorsolateral prefrontal cortex (dlPFC), which is comparable to that of humans²⁹. In order to look at the interaction between aging and estrogen, both young (~10 years) and aged (~22 years) female rhesus monkeys had surgical OVX and were given an injection of either 100 µg estradiol cypionate (E) or Vehicle (V) every 21 days. Note that this is a cyclical treatment, designed to mimic the physiological peak in estrogen at ovulation and is therefore very different from the HT that women are currently prescribed by their physicians. At various stages pre- and post-operatively, the monkeys are cognitively assessed on multiple tasks including delayed response (DR), a task which is dependent on dlPFC and a delayed non-matching to-sample (DNMS) task that is more closely tied to the medial temporal lobe system, which includes the hippocampus. In this review, we will focus our discussion on our dlPFC findings, a brain area that is particularly vulnerable to cognitive aging in both NHPs²⁹ and humans³⁰.

Our first important finding is that estrogen restores performance on the DR task in the aged cohort³¹, but has no effect in the young monkeys, where resilience in cognitive function is observed even in the absence of estrogen (fig 4a)³². Thus, the only group that experiences cognitive decline is the group that models peri-menopausal women, and we provide evidence that cyclic treatment with estrogen initiated shortly after OVX restores cognitive function to levels seen in young monkeys. In order to examine the underlying neural basis for this observation, we turned our attention to the layer III neurons in area 46 of the dlPFC, some of the key neurons that execute the brain computations underlying a monkey's performance in the DR task³³. Detailed quantitative morphometric analyses were performed by microinjection of individual cells with the fluorescent dye Lucifer Yellow followed by 3-dimensional tracing and high resolution confocal imaging of dendritic spines at systematic distances from the soma (fig 4c)³². Interestingly, although we found that estrogen induces spinogenesis in both young and aged monkeys (fig 4b), there were two key age-dependent differences that can account for the selective decrease in performance in DR in the aged vehicle group. First, the decrease in spine density in the young OVX+V group compared to OVX+E occurred against a background of an adaptive increase in dendritic length in the young V-treated animals. This implies that, in spite of the higher spine density in the E-treated group, overall there is no difference in the number of spines per neuron in the young cohort. Second, the estrogen-induced spinogenesis in the aged cohort occurred against the background of an age-related "second hit" to the spine density of the aged V-treated group. Thus, the vulnerability to cognitive decline in the aged untreated group can be explained in terms of a "double hit" to the connectivity in the dlPFC of these animals: an age-induced loss of spines coupled with an estrogen deficiency-induced loss of spines (fig 5).

Because spine size is highly correlated with both synapse size as well as glutamate receptor number³⁴, we were very interested in examining dlPFC spine morphology in our model. We found that estrogen shifts the distribution of spine head diameter toward smaller size in both the young and aged animals (fig 4d). However, most remarkably, we found that aging dramatically reduced the representation of spines with small heads (fig 4d) and long necks³².

This age-related selective loss of small spines (and the partial recovery with estrogen) fits in nicely with a developing framework in neurobiology of the essential role that small spines play in learning and memory. Both Kasai and Harris have recently suggested that thin spines are “learning” while big, mushroom-type spines represent “memory” traces^{34, 35}. Spontaneous spinogenesis visualized by two-photon time-lapse microscopy has shown that new spines are small, highly plastic, responsive to external manipulations, and form synapses within days of appearing³⁶. In addition, small spines can enlarge and stabilize in response to LTP³⁷, which has been suggested as a potential mechanism for learning³⁵. Therefore, the “double hit” to the aged V-treated group in our model (fig 5) might likely be most detrimental for the animal’s cognitive performance precisely because small, plastic spines are missing in the dIPFC. When estrogen replacement is provided to the aged animals, their DR performance matches the performance of young animals (fig 4a) in spite of an overall smaller spine density than the young estrogen-treated animals (fig 4b). This could indicate that a modest increase in small spines goes a long way in providing neurobiological resilience.

Future directions: the bench science with the potential to alter clinical practice

We have developed an NHP model for the age-related cognitive decline experienced by postmenopausal women and have shown that long-term cyclic treatment with estrogen is protective against both cognitive impairment as well as some of the structural changes that occur with aging and low hormone status. This is an important first step in the long-term goal of identifying the best HT in the clinic. Currently we are investigating the difference between continuous and cyclic estrogen treatment, as well as the effects of estrogen with progesterone.

Following the identification of the best HT, we plan to test the idea that there is a “window of opportunity” within which treatment must be started in order to retain beneficial effects. This is a question of enormous clinical relevance in light of the recent findings from the Women’s Health Initiative (WHI) that HT does not confer any protection against age-related cognitive decline in women when the onset of therapy lags by many years the transition into menopause. The idea of a “window of opportunity” has already been suggested from work in the rat model, where it has been shown that HT initiated immediately after surgery but not at 10 month post-OVX is effective at preventing cognitive decline³⁸. A reduction in estrogen-induced spinogenesis in CA1 following long-term estrogen deprivation has also been shown³⁹.

Another important clinical question is the role of locally synthesized estrogen. The identification of the presence of the enzymatic machinery capable of synthesizing estrogen from cholesterol in the brain⁴⁰ has brought a spotlight onto the interaction between the two sources of estrogen in the female. It is possible for example that the observed resilience in our young untreated monkeys is a result of local compensatory production of estrogen within the cortical network. In addition, an investigation into the role of local estrogen has the potential to extend our findings to males, as mouse male brains have been found to contain estrogen levels similar to females, presumably from local synthesis⁴¹. Although estrogen is not thought to affect spine density in the hippocampus of males^{42,43}, it does have a spinogenic effect in male rat prefrontal cortex⁴⁴.

Finally, we plan to continue to probe for molecular targets in the rat model for subsequent evaluation of their relevance in our NHP model. Given the large number of signaling cascades activated by estrogen in neurons, therapeutic strategies based on naturally occurring hormones will continue to be a major focus. However, we will also seek to identify new pharmaceutical targets within the signal transduction pathways initiated by estrogen. Such refined analysis of the molecular mechanisms and signaling pathways activated by estrogen in brain regions linked

to cognition will likely lead to new treatments that could also be used to protect against age-related cognitive impairment in males.

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Figure 1.

Spine density fluctuations in the CA1 region of the hippocampus from naturally cycling female rats. **A** Manual quantification of spine density from micrographs of Golgi-impregnated CA1 pyramidal cells. Values represent mean+SEM; asterisks denotes significant difference from proestrus ($p < 0.01$). **B** and **C** are representative Camera Lucida drawings of apical dendrites from proestrus and estrus females respectively. Scale bar 10 μ m. Adapted from Woolley 1990.

Figure2.

Non-genomic effects of estrogen. Estrogen initiates a complex set of signal transduction pathways in the hippocampal neuron via several membrane bound receptors. Above are two examples of estrogen-initiated signal transduction leading to spinogenesis and changes in synapse size. Rapid activation of Akt (protein kinase B) via PI3K is thought to be mediated by ER α . Subsequently, activated Akt initiates translation of PSD-95 by removing the repression of the initiation factor 4E- binding protein 1 (4E-BP1). Estradiol-mediated phosphorylation of cofilin has been shown to occur via activation of LIMK. Cofilin is an actin depolymerization factor and it is inactivated by phosphorylation. Therefore, in the presence of estrogen, cofilin repression of actin polymerization is removed, resulting in an increase in filopodial density. The signal transduction pathways illustrated here are an oversimplification of a large body of work done in an *in vitro* cell line.

Figure 3.

Interaction between estrogen and aging in stratum radiatum of the CA1 subfield of the hippocampus from ovariectomized rats. **A** Synapse density in young and aged animals treated with either estrogen or vehicle. For unbiased quantitative ultrastructural analysis, we took 15 pairs of adjacent electron micrographs from each animal and counted synapses using the dissector method. A significant increase was observed in the young estrogen treated as compared to vehicle treated animals (30%, $p < 0.005$). No synapse density changes occurred in the aged cohort. *Adapted from Adams 2001.* **B** Percentage of ER α -immunoreactively-labeled synapses. Our data illustrate that ER α is significantly reduced with age ($p < 0.0001$) but does not respond to estrogen treatment. **C** Sample electron micrographs showing the distribution of

ER α in the synapse. Gold particles were observed to be localized both pre- and postsynaptically. Scale bar, 100 nm. *B & C adapted from Adams 2002.* **D** Percentage of synapses immunoreactive to pLIMK. The graph illustrates that the percentage of pLIMK containing synapses increases with estrogen treatment in the young cohort (30%, $p=0.02$), and decreases with age in the presence of estrogen (43%, $p=0.04$). **E** Representative electron micrographs of pLIMK immunoreactivity. Gold particles are observed both pre- and post-synaptically and are often affiliated with the PSD (arrow). Scale bar, 100 nm. *D & E adapted from Yildirim 2008.* YV=young vehicle treated, YE=young estrogen treated, AV=aged vehicle treated, AE=aged estrogen treated, ax=axon, sp=spine, psd=post-synaptic density, t=terminal.

Figure 4.

Interaction between estrogen and aging on cognitive performance and dendritic spines from layer III neurons in area 46 of the monkey dlPFC. **A** Performance on a delayed response (DR) task. While young animals perform well on the task irrespective of their estrogen status, aged vehicle treated animals perform significantly worse than all other groups. **B** Apical spine density of layer III pyramidal neurons from the dlPFC. Dendritic segments were imaged at high resolution using confocal microscopy, followed by manual, blind counts of all dendritic protrusions. Significant treatment effects were observed in both young and aged animals. In addition, there was an age-related decrease in spine density. **C** Representative Lucifer Yellow loaded pyramidal neuron. In order to ensure systematic sampling for spine density analysis, neurons were first traced in 3D and concentric circles were drawn at multiples of 60 μm from

the cell body. Dendritic analysis was subsequently restricted to segments intersecting the circles. **D** Cumulative frequency analysis of spine head diameter. A significant increase in spine head is observed with aging, while estrogen shifts the distribution in favor of spines with smaller heads. *Adapted from Hao 2007.*

Figure 5.

“Double-hit” to the dIPFC connectivity of aged vehicle treated NHPs. Our observations of estrogen-induced spinogenesis and age-related spine loss have led us to the hypothesis that the cognitive impairment observed in the aged vehicle-treated monkeys could be explained by a double-hit to their neural connectivity in the dIPFC. The diagram illustrates that this group lacks the threshold density of small spines seemingly necessary for cognitive function.