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Estradiol and the Relationship between Dendritic Spines, NR2B Containing NMDA Receptors, and the Magnitude of Long-Term Potentiation at Hippocampal CA3-CA1 Synapses

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Summary

When circulating estrogen levels decline as a natural consequence of menopause and aging in women, there is an increased incidence of deficits in working memory. In many cases, these deficits are rescued by estrogen replacement therapy. These clinical data therefore highlight the importance of defining the biological pathways linking estrogen to the cellular substrates of learning and memory. It has been known for nearly two decades that estrogen enhances dendritic spine density on apical dendrites of CA1 pyramidal cells in hippocampus, a brain region required for learning. Interestingly, at synapses between CA3-CA1 pyramidal cells, estrogen has also been shown to enhance synaptic NMDA receptor current and the magnitude of long term potentiation, a cellular correlate of learning and memory. Given that synapse density, NMDAR function, and long term potentiation at CA3-CA1 synapses in hippocampus are associated with normal learning, it is likely that modulation of these parameters by estrogen facilitates the improvement in learning observed in rats, primates and humans following estrogen replacement. To facilitate the design of clinical strategies to potentially prevent or reverse the age-related decline in learning and memory during menopause, the relationship between the estrogen-induced morphological and functional changes in hippocampus must be defined and the role these changes play in facilitating learning must be elucidated. The aim of this report is to provide a summary of the proposed mechanisms by which this hormone increases synaptic function and in doing so, it briefly addresses potential mechanisms contributing to the estrogen-induced increase in synaptic morphology and plasticity, as well as important future directions.

Keywords

long term potentiation; LTP; NR2B; NMDA receptor; NMDAR; AMPA receptor; AMPAR; spine density; glutamate synapses; estradiol; estrogen; ER α ; ER β

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Introduction

Clinical studies demonstrate that in women cognitive performance declines as a result of endogenous loss of estrogen (17β -estradiol; E2) during menopause, which is reversed following hormone replacement therapy (Sherwin, 1988). Consistent with a role for E2 in improving learning in women, in mammalian models, including rats and primates, E2 replacement in ovariectomized (OVX) female animals enhances hippocampal dependent spatial memory (Daniel et al., 1997; Gibbs, 1999a; Maren, 2001; Rapp et al., 2003; Simpkins, 1994). However, recent reports (Women's Health Initiative, WHI) challenge the beneficial effects of E2 replacement in menopause (Shumaker et al., 2004; Shumaker et al., 2003). The discrepancy in the value of E2 replacement therapy in restoring normal learning and memory may be reconciled when considering the WHI used conjugated equine estrogens (premarin) (Shumaker et al., 2004; Shumaker et al., 2003), a hormone combination which might not be able to stimulate the cellular mechanisms required for enhancing cognition (Bimonte and Denenberg, 1999; Gibbs, 1999a; Rapp et al., 2003; Simpkins, 1994). Furthermore, the WHI enrolled subjects that had been hormone deprived for 10-20 years prior to the initiation of hormone replacement (Shumaker et al., 2004; Shumaker et al., 2003). Menopause is a natural consequence of aging in women. Therefore, it is critical that the cellular effectors which mediate the E2 enhancement in memory be discovered so that new interventions can be developed that are needed to sustain mental and cognitive health throughout the lifespan.

Proestrous levels of E2 are correlated with increased apical dendritic spine density on CA1 pyramidal cells, which is believed to contribute to the memory enhancing effects of E2 (Bi et al., 2001; Cordoba Montoya and Carrer, 1997; Cyr et al., 2000; Daniel and Dohanich, 2001; Gould et al., 1990; Hao et al., 2003; Maren, 2001; McEwen, 1994; Smith and McMahon, 2005, 2006; Woolley et al., 1997). In 1990, Gould et al published the first report demonstrating an increase in spine density on CA1 pyramidal cells in adult OVX rats receiving exogenous E2 (Gould et al., 1990). This study was quickly followed by a second manuscript from this group, Woolley et al., 1990 showing that during proestrus in adult cycling female rats, CA1 pyramidal cells experience a 20-30% increase in apical dendritic spine density (Woolley et al., 1990a). This effect of E2 on CA1 pyramidal cell spine density was later confirmed by studies from several other laboratories in both rats and monkeys (Adams et al., 2001; Hao et al., 2003; Leraneth et al., 2000; Leraneth et al., 2002).

In addition to increasing spine density, elevated circulating E2 levels *in vivo* increases NMDA receptor (NMDAR) expression and transmission and enhances the magnitude of long term potentiation (LTP) (Bi et al., 2001; Cordoba Montoya and Carrer, 1997; Cyr et al., 2000; Daniel and Dohanich, 2001; Gould et al., 1990; Hao et al., 2003; Maren, 2001; McEwen, 1994; Smith and McMahon, 2005, 2006; Woolley et al., 1997). When considering that LTP at CA3-CA1 synapses is a cellular correlate of learning and memory (Malenka and Bear, 2004; Whitlock et al., 2006), it is important to determine the E2 stimulated mechanisms responsible for enhancing synaptic function. Furthermore, given that enhanced dendritic spine density and increased NMDAR expression correlate with an increase in learning (Leuner and Shors, 2004; Tang et al., 1999), it is possible the heightened LTP magnitude is dependent upon the increase in spine density and NMDAR function. It is known that the E2-induced increase in spine density requires activation of NMDARs, is decreased by coadministration of E2 with progesterone, and is prevented by the estrogen receptor (ER) modulator tamoxifen, suggesting a role for classical estrogen receptors (Murphy and Segal, 1996).

The effects of E2 in hippocampus are complex and identifying which estrogen receptor (ER) is responsible for mediating these effects is still up for debate. ER α the predominant estrogen receptor in hippocampus (Mitra et al., 2003), resides in the cytosol as a classical steroid hormone receptor, and is localized in CA1 pyramidal cell dendritic spines where it activates

intracellular signaling pathways important for stimulating the increase in dendritic spine growth and expression of NMDARs (Bi et al., 2001; Gibbs, 1999b; Solum and Handa, 2002). ER α is also localized to presynaptic terminals of CA3 afferents (Adams et al., 2002; Hideo Mukai, 2007), is colocalized with glutamate decarboxylase (GAD) in GABAergic interneurons (Hart et al., 2001), and is localized at cholinergic terminals where it is clustered with small synaptic vesicles (Towart et al., 2003). ER β on the other hand is expressed in much lower density than ER α (Mitra et al., 2003) and is exclusively found in postsynaptic spines (Jelks et al., 2007) and in astrocytes (Azcoitia et al., 1999). Interestingly, as animals age there is decreased expression of both ER α and ER β (Adams et al., 2002; Mehra et al., 2005), correlated with decreased circulating E2, suggesting a role of endogenous hormone in preserving estrogen receptor density. Importantly, exogenous E2 treatment and locally synthesized E2 up regulates ER α nuclear staining in hippocampal cultures (Prange-Kiel et al., 2003; Rune et al., 2002) and down regulates ER β expression (Prange-Kiel et al., 2003).

Growing evidence supports that E2-induced changes in synaptic morphology and function are mediated through ER α . Treatment of embryonic hippocampal cell cultures with E2 increases dendritic spine density which is blocked by treatment with tamoxifen, a classical estrogen receptor antagonist (Murphy and Segal, 1996). Tamoxifen *in vivo* also blocks the E2-induced increase in spines, NMDAR transmission and LTP magnitude implicating a role for a classical estrogen receptor, likely ER α as previously proposed (Rudick et al., 2003) (Smith and McMahon, 2005). Further, acutely treating hippocampal slices with E2 or the ER α selective agonist PPT for 2 hours increases dendritic spines and increases LTD in male rats (Hideo Mukai, 2007), while over expression of ER β is correlated with decreased spine formation (Szymczak et al., 2006). Additionally, exogenous PPT treatment, like E2 treatment, in OVX rats increases NMDAR specific binding (Morissette et al., 2008). However, a role of ER β cannot be completely excluded as the ER β knockout mice (ESR2 $^{-/-}$) show deficits in learning and in LTP magnitude (Day et al., 2005; Liu et al., 2008) and treatment of OVX rats with the ER β agonist DPN increases dendritic spine complexity (Day et al., 2005). Perhaps E2 utilizes both ER α and ER β to facilitate the increased spine density, NMDAR current, LTP magnitude and learning. Clearly, further studies are necessary to uncover which receptor is essential for the synaptic changes in hippocampal morphology, NMDAR current and plasticity.

Because LTP at CA3-CA1 synapses is a cellular model of learning and memory (Malenka and Bear, 2004; Whitlock et al., 2006) defining the mechanisms used by E2 to increase LTP are critical to advancing our understanding of how this hormone increases learning. Perhaps the E2-induced increase in synapse density and NMDAR current mediates the enhanced plasticity. On the other hand, the increased LTP magnitude may occur in spite of the change in synapse density and may require enhanced hippocampal excitability resulting from increased presynaptic glutamate release or decreased GABAergic inhibition. This paper intends to review what is known concerning the E2-induced enhancement in LTP magnitude at CA3-CA1 synapses and how this is related to the increase in spine density and NMDAR current.

1. E2 and LTP at CA3-CA1 synapses

The increase in LTP magnitude measured *in vivo* at CA3-CA1 synapses during proestrus in cycling rats can be mimicked by treating young adult OVX rats with exogenous E2. Therefore, studies of the cellular mechanisms that contribute to the heightened LTP magnitude at proestrus can be facilitated by using the OVX rat model where the timing of the increase in plasma E2 is controlled by the experimenter. Proestrous plasma levels of E2 (80-100 pg/ml) can be reached by injecting adult OVX rats subcutaneously with E2 (10 μ g/250g) twice at a 24 hour interval; the plasma E2 levels then slowly decline over a 5 day period (Woolley and McEwen, 1993). Using this model to define the time course during which E2 stimulates an increase in the LTP magnitude will provide a framework that can be used to determine the mediators of the

heightened plasticity, and thus learning. Therefore, we assessed the magnitude of tetanus-induced LTP at CA3-CA1 synapses using standard extracellular dendritic field potential recordings in acute slices at 24 hour intervals (i.e. E24, E48, E72, E120) following the second E2 (or oil vehicle) injection. In this model, the LTP magnitude is increased at E24 and E48, but by E72, the LTP magnitude is not different from that measured at E120 or in slices from vehicle treated OVX rats (Figure 1). The increase in LTP magnitude is not increased 24 hours after a single E2 injection (E24 Single; Figure 2A). Because the mechanisms required to increase the LTP magnitude could be slow to develop, we waited an additional 24 hours following a single E2 injection (E48 Single; Figure 2B) and still the LTP magnitude was not different from vehicle. These findings indicate E2 must be elevated for longer than 24 hours (as occurs in the 2 injection protocol) for the heightened LTP to occur.

The heightened LTP magnitude in slices from E2 treated animals compared to vehicle treated OVX animals could be interpreted as a rescue of a deficit caused by E2 depletion in OVX animals, rather than stimulation of de novo mechanisms (i.e. Increased NR2B subunit containing NMDA receptors, decreased inhibition, increased presynaptic glutamate release, increased intrinsic excitability, increased local estrogen synthesis within the hippocampus.) that increase the capacity of the circuit to undergo plastic changes. If the magnitude of LTP in slices from OVX rats is a consequence of E2 depletion, then the LTP magnitude should be less than that measured in slices from intact rats at diestrus (lowest E2 level during the estrous cycle). However, there is no significant difference in the magnitude of LTP in slices from OVX rats compared to slices from intact cycling rats at diestrus (where plasma E2 is approximately 10-30 pg/ml), which indicates E2 replacement at proestrous levels in OVX rats is not simply rescuing a deficit in LTP caused by E2 depletion (Figure 2C).

2. Mechanisms contributing to the E2-induced increase in LTP magnitude

Several potential mechanisms could contribute to the enhanced LTP magnitude induced by proestrous levels of E2. It has been assumed that the increase in dendritic spine density on CA1 pyramidal cells is a causal factor. Mechanistically, how an increase in spine density itself would cause an increase in LTP magnitude is not immediately clear. Perhaps more obvious mechanisms that exclude a role for the increase in spine density include: 1) an E2-induced increase in presynaptic glutamate release, since ER α is located at CA3 terminals (Adams et al., 2002; Hideo Mukai, 2007); 2) an E2-induced decrease in GABAergic inhibition (Murphy et al., 1998; Rudick and Woolley, 2001), causing disinhibition of pyramidal cells, and facilitating NMDAR activation required for LTP induction (Harris et al., 1984; Rudick and Woolley, 2001) or 3) an E2-induced increase in NMDAR expression and function could directly increase the LTP magnitude (Bi et al., 2001; Woolley et al., 1997).

2.1 Does an increase in presynaptic release probability contribute to the E2-induced heightened plasticity?

An E2-induced increase in the glutamate release probability at CA3-CA1 synapses could facilitate induction of LTP and lead to an increase in LTP magnitude. However, no differences in the paired pulse facilitation ratio (PPR) were found at any of the tested interstimulus intervals (Figure 3A), indicating that proestrous E2 levels do not cause a dramatic increase in release probability. It should be kept in mind however, that a lack of change in PPR does not rule out all potential presynaptic effects of E2. Interestingly, an increase in the steady-state dendritic depolarization occurs at E24, E48, and E72, but not at E120, during the high frequency stimulation (hfs) used to induce LTP (Figure 3B). Currently, the mechanism responsible for this increase in steady-state depolarization is unknown but could be due to activation of extrasynaptic NMDARs (Zamani et al., 2004). However, there appears to be less tetanus-induced depletion of neurotransmitter in slices from E2 treated animals, but this remains to be investigated further. Importantly, because the steady-state depolarization at E72 is significantly

increased compared to vehicle, but the magnitude of LTP is not different from vehicle at this time point (see Figure 1), indicates that the increase in dendritic depolarization achieved during hfs is not the major mechanism driving the heightened LTP magnitude.

2.2 Does a decrease in GABAergic inhibition contribute to the E2-induced heightened plasticity?

In separate studies, Woolley and Murphy and their colleagues suggested that the increase in spine density is caused by an E2-induced disinhibition of pyramidal cells resulting from decreased GABAergic inhibition (Murphy et al., 1998; Rudick and Woolley, 2001). This disinhibition, in theory, would facilitate activation of NMDARs and thus lead to heightened LTP magnitude. Rudick and Woolley (Rudick and Woolley, 2001) showed that proestrous levels of E2 depresses GABAergic inhibition and disinhibits pyramidal cells as early as 24 hours following a single injection of E2. Therefore, if the hormone-induced increase in LTP magnitude is a direct consequence of decreased inhibitory drive onto pyramidal cells, then the LTP magnitude should be heightened by 24 hours following a single injection of E2. However, as mentioned above, the LTP magnitude is not increased 24 or 48 hours following a single E2 injection (Figure 2A,B). These findings indicate that the E2-induced decrease in GABAergic inhibition is not likely responsible for the increase in LTP.

In assessing a potential role of decreased GABAergic inhibition, we reasoned that if disinhibition is responsible for the difference in LTP magnitude between E24 (following 2 E2 injections as in Figure 1) and vehicle treated control, then pharmacologically blocking GABA_ARs with picrotoxin (100 μ M) should prevent this difference. We found that the LTP magnitude generated in slices from vehicle treated animals in the presence of picrotoxin was still significantly different from that generated in E24 slices (Figure 4A), indicating that disinhibition is likely not responsible for the enhanced LTP magnitude.

Finally, we also wondered whether the reason the LTP magnitude at E72 is no longer significantly elevated above vehicle results from the re-establishment of GABAergic inhibition to normal levels that occurs by this time point (Woolley et al., 1990b). If inhibition is more potent at E72 than at E24, then it is possible that the same high frequency tetanus would not activate NMDARs as effectively at E72, thus less effectively inducing LTP at E72 compared to E24. In this case, pharmacological inhibition of GABA_A receptors in slices from animals at E72 should permit an increase in LTP magnitude near that observed at E24. However, this was not the case (Figure 4B). Together these findings show that a decrease in GABAergic inhibition as shown by Rudick and Woolley (2001) does not underlie the increase in LTP at E24 or the return of LTP at E72.

3. Spines, NMDARs, and LTP

Reports showing that spines on CA1 dendrites and LTP at CA3-CA1 synapses are elevated at proestrus suggest that the increase in spines could be required for the heightened LTP magnitude (Cooke and Woolley, 2005). However, missing from the literature is a direct demonstration that this is actually the case. Mechanistically speaking, how would an increase in synapse density alone cause an increase in LTP magnitude? This is not immediately obvious. It can be imagined that the magnitude of LTP at saturation (i.e., the maximum LTP achieved after multiple rounds of the LTP inducing stimulus) should be increased when the synapse density is increased simply because there are more synapses available that can eventually undergo potentiation. However, it is not clear why a single LTP inducing stimulus should elicit LTP of greater magnitude when spine density is increased at proestrus in vivo or in slices from E2 treated OVX rats compared to diestrus and vehicle treated OVX rats, which have a lower spine density.

E2 does not alter the input/output response at CA3-CA1 synapses (Woolley et al., 1997) therefore, for accurate comparison of the LTP magnitude between vehicle and E2 treated experimental groups, baseline transmission should be set at the same strength based upon fiber volley amplitude (a measure of action potentials generated in axons) and/or fEPSP slope (a measure of the postsynaptic depolarization), regardless of the E2 status. In this case, an LTP inducing stimulus theoretically should elicit the same magnitude postsynaptic depolarization and subsequent NMDAR activation required for LTP induction, and elicit expression of LTP at the same magnitude, unless there is something distinct about the synapses or intrinsic excitability when plasma E2 is elevated to proestrous levels. In fact, in difficult electron microscopy studies, Woolley and coworkers showed that E2 increases the density of multiple synapse boutons which preferentially make synapses with spines on separate CA1 pyramidal cells (Woolley et al., 1996) causing an overall increase in synapse density (Woolley and McEwen, 1992). As a result, innervation of new spines occurs without the growth of additional presynaptic axon terminals. Therefore, the E2-induced increase in spine density not only increases overall innervation of individual CA1 pyramidal cells, but also increases the ability of CA3 pyramidal cells to synchronize the activity of their CA1 cell targets (Woolley et al., 1996; Yankova et al., 2001). If the synapses formed between the pre-existing boutons and the new spines are silent (NMDAR-only) synapses, these synapses would not contribute to baseline transmission (fEPSP slope). However, conversion of these newly formed silent synapses to active synapses via insertion of AMPARs following the tetanus used to induce LTP (Isaac et al., 1995; Liao et al., 1995; Malinow and Malenka, 2002; Montgomery et al., 2001) could be responsible for the heightened LTP magnitude observed at proestrus or in slices from E2 treated OVX animals. This potential scenario is supported by the work of Woolley et al (1997) and Smith and McMahon (2005) where a selective increase in NMDAR transmission is correlated with an increase in spine density in E2 treated OVX rats.

A further consideration in the potential role of the increase in spine density to the heightened LTP magnitude is that in early development, immature “silent” synapses primarily express NMDARs with NR2B subunits (Law et al., 2003; Owen et al., 2004; Sans et al., 2000). Importantly, E2 increases NR2B subunit mRNA, the number of NR2B binding sites, and the synaptic localization of NR2B-containing receptors (Adams et al., 2004; Cyr et al., 2001). Moreover, a recent report shows that overexpression of NR2B subunits increases hippocampal memory (Tang et al., 1999). Collectively these data lead to the hypothesis that E2 uses developmental mechanisms to increase plasticity in a mature synaptic circuit resulting in enhanced learning.

Thus, if the increases in spine density and NMDAR transmission are required for the E2-induced increase in LTP magnitude, then blocking the increase in spines should also prevent the increase in NMDAR transmission and LTP. Additionally, the time course of the increase in LTP magnitude should be mirrored by the increase in spines and NMDAR transmission. Finally, if E2 stimulates mechanisms used in development to heighten plasticity, then the increase in NMDAR transmission could be a consequence of an increase in receptors containing NR2B subunits.

3.1 Are spines required?

Previous studies have shown that the E2-induced increase in spine density is blocked by the ER modulator tamoxifen and the NMDAR antagonist, MK-801 (Murphy and Segal, 1996; Woolley and McEwen, 1994). If the increase in spines is required for the increase in NMDAR transmission and LTP (Bi et al., 2001; Cordoba Montoya and Carrer, 1997; Smith and McMahon, 2005; Woolley et al., 1997) then tamoxifen or MK-801 should also block the increase NMDAR transmission and LTP. To test this we concurrently treated OVX animals with E2 and tamoxifen (T; 2mg/kg) or E2 and MK-801 (0.2mg/kg), a non-competitive

NMDAR antagonist using the 2 injection protocol as in Figure 1; NMDAR transmission and LTP were assessed at E24. As predicted, tamoxifen prevented the increase in spine density, NMDAR transmission and LTP magnitude (Figure 5). The sensitivity of these effects of E2 to tamoxifen suggests not only that the increase in spines, NMDAR transmission, and LTP are mechanistically tied together, but also indicates that they require activation of classical genomic ERs, rather than activation of a membrane associated ER that mediates acute rapid effects of E2 (Foy et al., 1999; Fugger et al., 2001). While rapid effects of estrogen cannot be excluded as they are likely occurring synergistically with activation of classical genomic receptors, a more prominent role of classical ERs in mediating these *in vivo* effects of proestrous levels of E2 is supported by the finding that an increase in NMDAR transmission induced by acute application of E2 (1nM) to naïve slices could persist in the presence of tamoxifen (100nM) (Figure 5D). Although a role for classical ERs appears to be clear, what is not apparent is whether these effects of E2 are mediated by a single cellular pathway that induces sequential changes in spine density, NMDAR transmission and heightened LTP or if they are mediated through independent but parallel pathways that all require activation of classical ERs for their initiation.

Similar to the studies with tamoxifen, when OVX rats were treated concurrently with E2 and MK-801, the increase in spine density, NMDAR transmission and LTP measured at E24 was prevented (Figure 6). Thus, the simultaneous blockade of the increase in spines, NMDAR transmission, and LTP magnitude by NMDAR inhibition suggests that the morphological and functional changes are tightly coupled to one another mechanistically. However, similar to the caveat with the interpretation of the tamoxifen results, it is not clear whether blockade of NMDARs with MK-801 blocks the initiation of a single cellular pathway that induces sequential changes in spine density, NMDAR transmission and LTP, or whether separate parallel pathways all of which require NMDARs, are inhibited.

As mentioned above, the finding that blocking the E2 induced increase in spine density also prevents the increase in NMDAR transmission and LTP magnitude suggests a mechanism whereby the increase in spine density and NMDAR function are required for the increase in LTP magnitude. However, this potential mechanism would necessitate that the increase in spine density and NMDAR transmission occur precisely when the heightened LTP also occurs following an increase in plasma E2 to proestrous levels. Therefore, because the LTP magnitude is increased at E24 and E48, but not at E72 (Figure 1), the increase in spine density and NMDAR transmission should mirror this time course, if they are causal to the heightened LTP magnitude. When the temporal relationship of the increase in spine density was assessed, spines were found to be increased at E24, E48, and E72, with a return to vehicle levels at E120 (Figure 7) (Smith and McMahon, 2005). Thus, this time course does not perfectly overlap the time course of the increase in LTP magnitude. The maintained increase in spine density at E72, while the LTP magnitude at this time point is not different from that in slices from vehicle treated animals suggests that the increase in LTP is not clearly dependent upon the increase in synapse density.

3.2 What is the role of NMDARs?

Interestingly, the role for NMDARs in mediating the E2-induced increase in synaptic plasticity is clear when the time course of the increase in NMDAR transmission, the increase in spines and the increase in LTP magnitude are evaluated together. Measurements of the input/output relationship of the E2 induced changes in total glutamate transmission and pharmacologically isolated AMPAR and NMDAR transmission revealed a selective increase in NMDAR transmission with no change in AMPAR transmission at E24 (Figure 8), confirming earlier results of (Woolley et al., 1997). At this time, both spine density and the LTP magnitude are increased. At E48, a delayed increase in AMPAR transmission accompanies a maintained increase in NMDAR transmission, spine density and LTP magnitude. At E72, transmission

mediated by both NMDAR and AMPAR are significantly elevated. Importantly, at this time, as indicated above, spine density remains elevated, but the LTP magnitude is not different from vehicle control. By E120, the maximum transmission mediated by both NMDARs and AMPARs has now returned to the level measured from vehicle treated control slices, thereby resetting transmission in the circuit. Importantly, the re-establishment of the NMDAR and AMPAR mediated transmission to control levels occurs precisely when spine density has also returned to the density measured in vehicle treated control slices, indicating that the density of functional synapses at E120 and in vehicle treated-control slices is similar. Analysis of the NMDAR:AMPA ratio in whole-cell recordings from CA1 pyramidal cells shows that at E24 and E48 there is a significant increase in this ratio (Figure 9B), precisely when LTP and spines are also increased. However at E72, even though transmission mediated by both AMPARs and NMDARs is significantly increased (Figure 8), the NMDAR:AMPA ratio is reset (Figure 9B), indicating that the relative contribution of each receptor is normalized, which is correlated with the return of the LTP magnitude to that in vehicle control (Figure 1). Thus, spine density may be required, but clearly the estrogen-induced heightened plasticity is coupled to increased NMDAR transmission.

3.3 Does a developmental mechanism have a role?

An E2 induced increase in the NMDAR:AMPA ratio at E24 and E48, when the LTP magnitude is increased (as well as spine density) is consistent with an increase in the density of silent synapses at these time points. Because both the silent synapse density and expression of NMDARs containing NR2B subunits are greater early in development, together with the fact that E2 increases NR2B subunit mRNA, the number of NR2B binding sites, and the synaptic localization of NR2B-containing receptors (Adams et al., 2004; Cyr et al., 2001), suggests the hypothesis that the increase in NMDAR:AMPA ratio at E24 and E48 results from a selective increase in NMDA current carried by NR2B containing receptors at these time points. Indeed, at E24 and E48 transmission mediated by NR2B containing receptors is significantly increased (Figure 9B,C), and this increase in NR2B mediated transmission is entirely responsible for the increase in NMDAR:AMPA ratio at these time points (Figure 9B). Moreover, this increase in NR2B transmission is causal to the heightened LTP magnitude (Figure 10). Finally, this increase in NR2B transmission could be responsible for the increase in steady-state depolarization during hfs (Figure 2) because there is a trend toward less depolarization in the presence of the NR2B subunit antagonist, RO25-6981, although this must be investigated further.

4. Model

Collectively, our findings combined with the results from other laboratories suggest the following model (Figure 11). Circulating E2 at proestrous levels increases NR2B-containing silent synapses to heighten plasticity, a notion supported by the selective increase in transmission mediated by NR2B containing NMDAR receptors at E24, with no change in AMPAR transmission. With time, excitability within the circuit *in vivo* stimulates homeostatic mechanisms (Rudick and Woolley, 2001; Scharfman et al., 2003; Smith and Woolley, 2004) leading to insertion of AMPARs into the silent synapses (AMPA and NMDAR-containing), hence the delayed increase in AMPAR transmission that begins at E48 (Smith and McMahon, 2005). As E2 continues to decline, the NMDAR:AMPA ratio is reestablished to control (and presumably the ratio of silent to active synapses) and transmission mediated by NR2B containing receptors declines, thereby resetting the level of plasticity back to control levels. This occurs despite a continued increase in spine density and excitatory transmission overall. It is important to note NMDAR transmission remains elevated at E72 suggesting a replacement of NR2B containing receptors with those containing NR2A subunits. Finally, after plasma E2 levels have decreased to near diestrous levels, the system has entirely reset: spine density,

transmission mediated by both NMDARs and AMPARs, and the LTP magnitude are not different from control (E120). Whether the alterations in glutamate transmission are a result of receptor insertion into new or existing spines is not clear. However, the simultaneous return of spine density and evoked glutamate transmission to control levels at E120 argues that the enhanced transmission is due to receptor insertion into the new synapses.

5. Future Directions

Given the above model there are several questions that need to be addressed. First, it is critical to determine whether activation of NR2B containing receptors is causal to the E2-induced improvements in learning and memory. This is likely given that overexpression of NR2B subunits in transgenic mice mediates enhanced cognitive performance (Tang et al., 1999). It is also critical that we determine precisely how E2 increases NR2B current. It is possible the E2-induced increased NR2B current results from an increase in synaptic receptor expression. On the other hand, it could be due an E2 induced increase in tyrosine phosphorylation (Xu and Zhang, 2007) of NR2B subunits, which is known to increase NMDAR current amplitude and be associated with heightened learning (Caputi et al., 1999; Mizuno et al., 2003; Xu et al., 2006). E2 stimulates Src kinases, increasing NR2 subunit phosphorylation (Bi et al., 2000); recently E2 specifically was shown to increase phosphorylation of NR2B subunits (Xu and Zhang, 2007). Perhaps, E2 utilizes both mechanisms to increase current.

It is also important to examine how input to hippocampus from other brain regions impacts the E2-induced increase in synaptic function and learning. E2 increases cholinergic function by increasing expression of choline acetyltransferase (ChAT) and the choline transporter (Gibbs et al., 2002). Further, a collection of studies have shown that cholinergic function is required for the E2-induced increase in NMDAR expression (assayed in binding studies), and heightened spatial and working memory (Daniel and Dohanich, 2001; Gibbs, 1999a; Leraneth et al., 2000). Thus, it is probable cholinergic innervation of hippocampus is necessary for the E2-induced alterations in synaptic function, morphology and heightened learning.

Additionally, it is necessary to examine whether E2 reverses the decline in learning and memory observed following prolonged hormone deprivation. Growing evidence indicates that a “window of opportunity” exists within which E2 replacement is most effective for maintaining cardiovascular and cognitive health. After this time, E2 replacement is ineffective and may be detrimental (Morrison et al., 2006). It is assumed that the ineffectiveness of E2 replacement results from decreased ability of cells to respond to E2 due to decreased expression of estrogen receptors (Thakur and Sharma, 2006). However, changes in estrogen receptor expression may not be the problem. Potentially, the reason E2 replacement in women following surgical menopause appears more effective than in reproductive senescence (Savonenko and Markowska, 2003) is probably due to age related changes independent of hormone loss. Unfortunately, very little is known concerning the impact of prolonged hormone loss on the ability of E2 replacement to induce an increase in synaptic function and plasticity. Thus, well controlled studies investigating the consequences of prolonged hormone loss on the ability of E2 to induce the synaptic changes necessary to increase cognitive function are greatly needed.

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Figure 1. Time course of the E2-induced increase in LTP magnitude

LTP magnitude recorded in slices from E24, E48, E72, E120 and vehicle treated control animals. LTP was induced with a high frequency tetanus (100 Hz, 0.5 s duration, repeated 4x, 20 s interval). LTP magnitude is significantly increased compared to vehicle control at E24 and E48 but not at E72 or E120. Dotted line represents the magnitude of LTP measured at E24. LTP from vehicle control is replotted on each graph for comparison. Error bars represent SEM. Adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and reprinted with permission.

Figure 2. Effects of short-term exogenous and diestrus E2 on LTP

(A, B) The LTP magnitude is not increased 24 (A) or 48 (B) hours following a single E2 injection (vehicle control replotted from Figure 1). Dotted line represents LTP measured at E24 from Figure 1. (C) The LTP magnitude in slices from vehicle treated animals or animals at diestrus is not significantly different. A and B are adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and are reprinted with permission.

Figure 3. E2 effects on the PPF ratio

(A) Top, Overlaid averaged paired pulse fEPSP waveforms from vehicle and E24 slices elicited at increasing interstimulus intervals. Bottom, Bar chart shows no significant differences between the PPF ratios at any interstimulus interval. Error bars represent SEM. (B) Representative fEPSP waveforms during high frequency stimulation (hfs) from E24, E48, E72 and E120 overlaid with vehicle. The steady-state dendritic depolarization during hfs is elevated at E24, E48, and E72 but returns to vehicle control by E120. Reprinted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 with permission.

Figure 4. Effect of GABA_AR inhibition on E2-induced increase in LTP magnitude

(A) Blockade of GABA_ARs with 100 μ M picrotoxin (picro) does not prevent the E2-induced increase in LTP magnitude in slices at E24. (B) Blockade of GABA_ARs with picro does not permit an increase in LTP magnitude recorded in slices at E72 with respect to vehicle treated control slices. LTP from V+picro in A is replotted in B for comparison. Error bars represent SEM. Adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and reprinted with permission.

Figure 5. The E2-induced increase in spine density, NMDAR transmission, and LTP magnitude requires activation of classic estrogen receptors

(A) Plot shows that tamoxifen blocks the increase in LTP magnitude. Vehicle and E24 are replotted from Figure 1 for comparison. (B) Bar chart shows density of dendritic spines/10μm of apical dendrite from each experimental group. Both vehicle treated and E24 are replotted from Figure 1 for comparison. (C) Stimulus response curve of pharmacologically isolated NMDAR transmission from each experimental group. (D) Stimulus response curves of pharmacologically isolated NMDAR transmission show that the increase in NMDAR transmission from acute application of estradiol (1nM) applied to hippocampal slices from OVX rats is not blocked by tamoxifen (100nM). Asterisk denotes significance as indicated. Error bars represent SEM. Adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and reprinted with permission.

Figure 6. MK-801 blocks the E2-induced increase in spine density, the selective increase in NMDAR transmission and LTP magnitude

(A) MK-801 blocks the E2-induced increase in LTP magnitude. Vehicle and E24 are replotted from Figure 1 for comparison. (B) Bar chart shows the density of dendritic spines/10µm of apical dendrite from each experimental group. Both vehicle and E24 are replotted from Figure 1 for comparison. (C) Stimulus response curves of pharmacologically isolated NMDAR transmission from each experimental group. Asterisk denotes significance as indicated. Error bars represent SEM. Reprinted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 with permission.

Figure 7. Time course of E2-induced modulation of spine density

(A) Representative confocal images of Golgi stained apical dendrites from vehicle treated control, E24, E48, E72 and E120. Scale bar represents 5µm. (B) Bar chart shows average number of dendritic spines/10 µm of apical dendrite from each experimental group. Asterisks denote significance as indicated. Error bars represent SEM. Adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and reprinted with permission.

Figure 8. Time dependent modulation of glutamate transmission by estradiol

Stimulus response curves of glutamate (A), AMPAR (B), or NMDAR (C) transmission. (A) Glutamate transmission is unchanged at E24, is increased at E48 and at E72, but is normalized by E120. (B) AMPAR-mediated transmission is unchanged at E24, is increased at E48 and E72, but is normalized by E120. (C) NMDAR-mediated transmission is selectively increased at E24, is increased at E48 and at E72, but is normalized by E120. Asterisks denote significance from vehicle. Scale bars represent 0.5mV/5msec for glutamate and AMPAR transmission and 0.4mV/10msec for NMDAR transmission. Error bars represent SEM. Adapted from Smith and McMahon J. Neuroscience 2005; 25: 7780-7791 and reprinted with permission.

Figure 9. E2-induced increase in NMDAR/AMPA ratio is prevented when NR2B subunits are blocked

(A) Averaged evoked glutamate-, NMDAR-, and AMPAR-mediated EPSCs at each time point. (B) The NMDAR/AMPA ratio is significantly increased at E24 and E48, but not at E72 or E120. The NMDAR/AMPA ratio is the same in all experimental groups when NR2B-containing receptors are blocked with the NR2B subunit selective antagonist RO25-6981 (0.5μM) indicating a causal role of NR2B containing receptors in mediating the increased NMDAR current at E24 and E48. (C) Graph shows a 5 fold increase in NR2B-containing EPSC amplitude compared to vehicle at E24 and E48. Error bars represent SEM. A, B, and C are adapted from Smith and McMahon J. Neuroscience 2006; 26: 8517-8522 and are reprinted with permission.

Figure 10. E2-induced increase in LTP magnitude is prevented when NR2B subunits are blocked (A) LTP induced in slices from E24, E24+RO, V, and V+RO (RO; 0.5 μ M RO 25-6981, a NR2B subunit selective antagonist). Error bars represent SEM. (B) Waveforms of depolarization during hfs in slices from experimental groups. Adapted from Smith and McMahon J. Neuroscience 2006; 26: 8517-8522 and reprinted with permission.

Figure 11.
Model of increase in spine density, NMDARs and LTP magnitude with E2 replacement.