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## Estrogen receptor alpha and beta specific agonists regulate expression of synaptic proteins in rat hippocampus

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### Abstract

Changes in hippocampal CA1 dendritic spine density and synaptic number across the estrous cycle in female rats correlate with increased hippocampal-dependent cognitive performance in a manner that is dependent on estrogen receptors (ERs). Two isoforms of the estrogen receptor,  $\alpha$  and  $\beta$  are present in the rat hippocampus and distinct effects on cognitive behavior have been described for each receptor. The present study generated a profile of synaptic proteins altered by administration of estradiol benzoate, the ER $\alpha$  selective agonist PPT (1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole) and the ER $\beta$  selective agonist DPN (2,3-bis (4-hydroxyphenyl) propionitrile) alone and in combination in comparison to vehicle in the CA1 region of the dorsal hippocampus. In the stratum radiatum, estradiol, DPN, and PPT increased PSD-95 and AMPA-type glutamate receptor subunit GluR1. Only DPN administration regulated expression of AMPA receptor subunits GluR2 and GluR3, increasing and decreasing levels respectively. DPN also increased GluR2 expression in the other lamina of the CA1. These results support previous reports that estradiol and isoform specific agonists differentially activate ER $\alpha$  and ER $\beta$  to regulate protein expression. The distinct effects of DPN and PPT administration on synaptic proteins, suggest that the desired therapeutic outcome of estrogen may be accomplished by using specific estrogen receptor agonists. Moreover, the effects of estradiol treatment on PSD-95 expression are consistent with a growing body of evidence that this postsynaptic protein is a key marker of estrogen action related to spine synapse formation.

### Keywords

Hippocampus; Synaptic proteins; Estradiol benzoate; PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole); DPN (2,3-bis(4-hydroxyphenyl) propionitrile); Estrogen receptor agonists

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## 1. Introduction

Estradiol, derived from endogenous or exogenous sources, has many enhancing effects on neuronal plasticity and behavior related to cognition and mood (Korol, 2004; Hajszan and MacLusky, 2006). In addition, estradiol has neuroprotective as well as neurogenic actions in models of ischemic brain injury, neurodegeneration, and aging (Brann et al., 2007; De Nicola et al., 2009). Estrogen sensitivity has been described in the stratum radiatum of the CA1 region of the hippocampus, where high levels of estradiol result in increased dendritic spine density and synapse number (Gould et al., 1990; Woolley and McEwen, 1993), spine size and shape (Woolley et al., 1996; Li et al., 2004), and neurotransmission (Woolley et al., 1997; Scharfman et al., 2003; LeDoux et al., 2009) and modulate hippocampal-dependent learning and memory (Luine et al., 2003; Sandstrom and Williams, 2004).

In hippocampal neurons, estradiol mediates changes in synaptic protein expression in vitro (Lee et al., 2004b) and in vivo (Brake et al., 2001; Lee et al., 2004c) that parallel changes in synaptic number and efficacy (Woolley, 1998). Estradiol-induced synaptogenesis is dependent on a classical estrogen receptor (ER) (McEwen et al., 1999). Two ER isoforms have been identified in the hippocampus:  $\alpha$  and  $\beta$  (Milner et al., 2001; Milner et al., 2005) but their distribution does not completely overlap. ER $\alpha$  is present in nuclear and extranuclear sites in principal and inhibitory neurons (Milner et al., 2001). Extranuclear ER $\beta$  is present in principal cells and in a few inhibitory cells (Milner et al., 2005).

Activation of both ER $\alpha$  and  $\beta$  with estradiol or specific agonists has been implicated in learning and memory. In rats, both DPN and PPT treatment enhanced memory (Frye et al., 2007). In mice, EB and DPN improved performance on cognitive tests in wildtype but not ER $\beta$  knock-out (BERKO) mice (Walf et al., 2008). Loss of ER $\beta$  in BERKO mice results in deficits in CA1 LTP and hippocampal related memory (Day et al., 2005). However, over-expression of ER $\beta$ , which reduces spine formation in the mouse hippocampus (Szymczak et al., 2006), may also negatively impact memory. ER $\alpha$  also contributes to hippocampal plasticity as restoration of ER $\alpha$  expression in ER $\alpha$  knockout mice rescued both estrogen responsiveness and hippocampal related memory (Foster et al., 2008).

We compared the effects of administration of agonists selective for ER $\alpha$  (PPT) or ER $\beta$  (DPN) or estradiol benzoate (EB) to vehicle in ovariectomized female rats on synaptic protein levels. Synaptic proteins levels in the CA1 stratum radiatum were evaluated by quantitative densitometric immunohistochemistry (Pierce et al., 1999). Postsynaptic proteins examined include PSD-95, spinophilin, and AMPA-type glutamate receptor subunits GluR1-3 and presynaptic proteins include synaptophysin, vesicular GABA transporter (VGAT), and vesicular glutamate transporter 1 (VGluT1).

## 2. Results

### 2.1 EB, DPN and PPT differentially regulate expression levels of pre- and postsynaptic proteins

Pre- and postsynaptic protein expression levels were examined after administration of EB, and DPN or PPT alone and in combination and compared to vehicle in the CA1 stratum radiatum of the dorsal hippocampus (Fig. 1 A, B) of ovariectomized female rats. Each steroid was administered twice, separated by 24 hours, and tissue was collected 72 hours after the first dose. The vehicle group consists of animals administered DMSO or sesame oil; results from these animals were pooled because no differences in labeling were detected ( $p > 0.05$ ). Using quantitative densitometry, levels of presynaptic proteins, including synaptophysin, VGluT1, and VGAT, and postsynaptic proteins, including spinophilin, PSD-95 and the AMPA receptor subunits GluR1-3 (Fig. 1 B) were measured in all groups. We also measured GluR1-3 and

PSD-95 expression in the other layers of the CA1, the CA3, and the dentate gyrus. Interestingly, the CA1 stratum radiatum showed more changes in protein expression after steroid administration than the CA3 or dentate gyrus. This study demonstrated differential regulation of protein expression by specific ER agonists, which resulted in both increased and decreased labeling for synaptic proteins, depending on the subtype of ER activated.

## **2.2 PSD-95 levels, but not synaptophysin or spinophilin levels, change in response to EB, DPN, and PPT**

Synaptophysin is a membrane component of neuronal synaptic vesicles and is expressed in many of the terminals throughout the CA1 stratum radiatum of the dorsal hippocampus (Fig. 1 B) (Fykse et al., 1993). Neither EB nor the estrogen receptor agonists had a significant effect on synaptophysin levels in the stratum radiatum when compared to control ( $F(4, 20) = 0.9096$ ,  $p > 0.05$ ; Fig. 2A).

Spinophilin is a dendritic, actin-binding protein present primarily in excitatory neurons (Feng et al., 2000). It is expressed throughout the CA1 stratum radiatum of the dorsal hippocampus (Fig. 1B). Neither EB, DPN or PPT administration alone or in combination resulted in a significant change in spinophilin levels relative to vehicle treatment ( $F(4, 20) = 2.47$ ,  $p > 0.05$ , Fig. 2B). However, when an additional post hoc test was used to compare DPN, PPT and DPN plus PPT to EB treatment, DPN plus PPT treatment was significantly reduced in comparison to EB ( $F(4,20) = 2.47$ ,  $p < 0.05$ ).

PSD-95 is a scaffolding protein involved in the organization glutamate receptors and other constituents of the postsynaptic density (Kim and Sheng, 2004). PSD-95 labeling was diffusely distributed throughout the CA1 but also outlined dendritic profiles in stratum radiatum (Fig. 1 B). Comparison of EB, PPT alone or DPN plus PPT to control showed increased levels of PSD-95 in all groups ( $F(4, 19) = 3.646$ ,  $p < 0.05$ , Fig. 2C). Post hoc analysis confirmed that PSD-95 labeling in the EB, PPT and DPN plus PPT was significantly increased compared to vehicle ( $p < 0.05$  for all comparisons). In addition, there was a trend ( $p = 0.061$ ) for DPN alone to increase PSD-95.

## **2.3 Expression of neurotransmitter transporters, VGluT1 and VGaT, were not altered by steroid replacement**

VGluT1 and VGaT were examined to assess changes in the relative number of excitatory and inhibitory terminals, respectively. VGluT1 is the predominant form of glutamate transporter found in the postnatal brain (Nakamura et al., 2005) and is expressed exclusively in terminals of glutamatergic neurons (Herzog et al., 2001). VGaT expression is restricted to terminals and cell bodies of GABAergic interneurons (Chaudhry et al., 1998). Punctate staining for both VGaT and VGluT1, presumed to represent axon terminals, outlining dendrite profiles was apparent in the CA1 stratum radiatum of the dorsal hippocampus (Fig. 1 B). Steroid administration had no effect of the levels of VGluT1 or VGaT in the CA1 stratum radiatum (VGluT1:  $F(4, 20) = 0.7749$ ,  $p > 0.05$ ; VGaT:  $F(4,20) = 1.493$ ; Fig. 3).

## **2.4 AMPA receptor subunits, GluR1-3 were differentially regulated by EB, PPT and DPN**

GluR1, 2, 3 have different roles in the properties of AMPA receptors and their movement between synaptic and extrasynaptic sites during synaptic transmission and changes in their expression alter subunit composition of AMPA receptors and consequently synaptic efficacy (Newpher and Ehlers, 2008). In the CA1 stratum radiatum of the dorsal hippocampus, GluR1, 2, and 3 immunoreactivity appeared primarily as diffuse peroxidase labeling (Fig 1 B), representative of their widespread expression. There was a small increase in GluR1 levels with DPN or PPT administration alone compared to vehicle ( $F(4,19) = 2.723$ ,  $p > 0.05$ ). Post hoc analysis confirmed that DPN and PPT significantly increased GluR1 expression ( $p < 0.05$  for

both comparisons) but DPN plus PPT administration did not ( $p > 0.05$ ) (Fig. 4A). There was a trend for EB administration to increase GluR1 levels ( $p = 0.054$ ). In contrast, DPN but not PPT increased GluR2 and GluR3 expression levels. GluR2 labeling significantly increased after DPN administration but did not change after EB, PPT or DPN plus PPT in comparison to vehicle administration ( $F(4,18) = 3.591$ ,  $p < 0.05$ , Fig. 4B). Post hoc analysis confirmed that DPN caused a significant decrease in GluR2 ( $p < 0.05$ ) while EB, PPT alone or DPN plus PPT had no effect on GluR2 levels ( $p > 0.05$ ). The reverse effect of DPN was detected for GluR3 expression, DPN decreased GluR3 labeling ( $F(4, 20) = 2.696$ ,  $p > 0.05$ ) and post hoc analysis confirmed there was a significant decrease ( $p < 0.05$ ). Similar to GluR2, GluR3 expression was not altered by EB, PPT alone or DPN plus PPT ( $p > 0.05$ , Fig. 4C).

### 2.5 PSD-95 and GluR2 expression in other hippocampal areas differed in their steroid sensitivity

We also examined PSD-95 and GluR2 expression in the other lamina of the CA1 in addition to the dentate gyrus and CA3, because of the profound effects that these proteins have on synaptic physiology (ref). In addition, synaptic protein expression fluctuates with estrogen levels in mice in all hippocampal regions (Li et al., 2004; Spencer et al., 2008) and estrogens opioid peptide expression in the dentate gyrus and CA3 regions (Torres-Reveron et al., 2008; Torres-Reveron et al., 2009). Although GluR1 and GluR3 expression was also not altered by any of the treatments in any of the other hippocampal subregions (data not shown), DPN administration had wide spread effects on GluR2 levels in the lamina of the CA1 in the dorsal hippocampus. DPN increased GluR2 expression significantly in the stratum pyramidale ( $F(4,18) = 4.488$ ,  $p < 0.05$ ; post hoc test  $p < 0.05$ ) and the stratum oriens ( $F(4,19) = 2.202$ ,  $p > 0.05$ , post hoc test  $p < 0.05$ ) (Fig. 5A). Examination of PSD-95 expression in the other lamina of the CA1 and the CA3 did not reveal any significant changes due to the effects of EB, DPN or PPT. However, in the dentate gyrus of the dorsal hippocampus post hoc comparisons suggest there was a significant increase in PSD-95 expression in the inner and middle molecular layers (IML:  $F(4,20) = 1.787$ ,  $p > 0.05$ , post hoc  $p < 0.05$ ; MML:  $F(4,20) = 1.89$ ,  $p > 0.05$ , post hoc  $p < 0.05$ ) (Fig. 5B).

## 3. Discussion

We examined the ability of ER $\alpha$  and ER $\beta$  activation to mimic estradiol induced changes in synaptic proteins and identified some novel effects of individual ER activation the levels of AMPA-type glutamate receptor subunits. Quantitative immunohistochemistry revealed differential effects of ER $\alpha$  and ER $\beta$  activation on the expression of synaptic proteins. The alterations in the profile of synaptic proteins present in the CA1, particularly PSD-95, GluR2 and GluR3, along with previous reports of EB regulation of NMDA-type glutamate receptor subunits (Cyr et al., 2001) suggest the widespread potential of ER actions to affect glutamatergic transmission in the CA1 region of the hippocampus.

These results along with previous reports demonstrate that expression of a large number of postsynaptic proteins is regulated by steroids. Although fewer presynaptic proteins have been studied, in general protein expression in terminals appears to be less sensitive to steroid regulation. Interestingly, estrogen has been reported to increase the number of multisynaptic boutons (Woolley et al., 1996), suggesting that rearrangements of presynaptic structures occurs while postsynaptic increases may require new protein expression or decreased protein turnover.

### Methodological considerations

The steroid replacement paradigm has previously been shown to increase spine density and synaptic number in the medial portion of the apical dendrites in the CA1 stratum radiatum. In addition, the doses of DPN and PPT have been demonstrated to be in the effective range of

DPN and PPT (Lund et al., 2005; Gonzales et al., 2008; Morissette et al., 2008); however, the DPN plus PPT treated animals may have experienced pharmacological effects due to the higher levels of steroids administered with the combined treatment (Gonzales et al., 2008). As expected, uterine weights were increased by EB, PPT and DPN plus PPT administration; however, uterine weights after PPT were not as large as after EB, suggesting that in this steroid replacement paradigm, PPT does not fully reproduce EB effects on ER $\alpha$  in the periphery as previously reported (Harris et al., 2002). This may be due to dose or decreased length of the treatment period. Previously we have shown that estrogen receptors mediate estrogen-induced increases in spines and synapses using this estradiol replacement paradigm (McEwen et al., 1999).

### **ER selective agonists can mimic select actions of EB**

ER $\alpha$  or ER $\beta$  activation differentially regulated protein expression when agonists were administered separately or in tandem. Previous reports suggest that estradiol replacement can increase spinophilin protein in vitro (Lee et al., 2004b) and in vivo (Brake et al., 2001; Lee et al., 2004c). Synaptophysin is also upregulated long term estradiol replacement in ovariectomized female rats (Sharma et al., 2007) and decreased by inhibition of estradiol synthesis in hippocampal cultures (Prange-Kiel et al., 2006). However, synaptophysin and spinophilin expression were not altered by steroid replacements compared to vehicle in this study, which may be due to the timing or the dose of steroid administered. In contrast, all steroid regimens resulted in increased expression of PSD-95 protein. Similar findings have been reported in rat neuronal cell line, where rapid actions of estradiol increase translation of PSD-95 (Akama and McEwen, 2003), while long-term estradiol administration increases PSD-95 transcription (Akama K and McEwen BS personal observation). In mice, increased levels of PSD-95 are detected during proestrus and after ER $\beta$  activation (Liu et al., 2008; Spencer et al., 2008). In total, estradiol may regulate the balance between transcription, translation, and degradation but this remains to be tested in future studies.

Interestingly, alterations in PSD-95 were also detected in the dentate gyrus. Here estradiol alone increased PSD-95 in the inner two thirds of the molecular layer. Interestingly DPN plus PPT did not increase PSD-95, perhaps due to the different binding affinities of each steroid for its receptor and inability to equally activate both receptors at the same time as EB can. The molecular layers receive excitatory inputs from a number of brain areas. Notably, processes from pyramidal basket cells, mossy cell axons and projections from the entorhinal cortex are among the substrates that form synapses with granule cells dendrites in the molecular layer. The inner third of the molecular layer receives excitatory inputs from the mossy cells in the hilus (Soriano and Frotscher, 1994; Buckmaster et al., 1996; Wenzel et al., 1997) and cholinergic projections from the septal nuclei (Wheal and Miller, 1980). The middle third of the molecular layer contain projections from the entorhinal cortex that form asymmetric synapses with the dendritic spines of granule cells (Witter, 1993). The mossy cells, cholinergic systems and entorhinal cortex are also estrogen sensitive brain areas, multiplying the number of pathways through which estrogen can alter the function of the hippocampal formation.

Estradiol has effects, neuroprotective and cognitive, related to its ability to modulate excitatory and inhibitory tone. We examined whether estrogens could alter the expression of vesicular transporters that would parallel the estradiol mediated increase in synapse number. However, we did not see any significant changes in the expression of VGluT1 or VGaT after steroid replacement. It is possible the protein levels are not upregulated to accommodate changes in dendritic spines and synapses, rather a redistribution of protein due to alterations in protein synthesis and degradation or transformation from single to multi-synaptic boutons that shares the same protein pool may occur. Estradiol also drives movement of vesicles into the readily



releasable pool (Hart et al., 2007), which may contribute to its effects on plasticity without altering vesicle number or content.

### ER agonists differentially alter the expression of AMPA receptor subunits

Estradiol regulates the number of asymmetric or excitatory synapses in the CA1 stratum radiatum. While estradiol mediated synaptogenesis is dependent on NMDA receptors *in vivo* (Woolley and McEwen, 1994) and *in vitro* (Murphy and Segal, 1996) and inhibition of AMPA receptors has no effect (Woolley and McEwen, 1994), we nonetheless wondered if specific actions of ER $\beta$  or ER $\alpha$ , since both have been implicated in memory, (Frye et al., 2007; Walf et al., 2008), could regulate the expression of AMPA receptor subunits. Activation of ER $\beta$  with DPN treatment did alter the expression of AMPA receptor subunits increasing GluR2 and decreasing GluR3. DPN also had more widespread effects in the CA1 region as it also increased GluR2 in the stratum oriens and stratum pyramidale.

Previously, estradiol has been shown to increase NMDA receptor transmission (Woolley et al., 1997; Gureviciene et al., 2003) and expression of the subunits NR2B and NR1 but has no effect on NR2A (Cyr et al., 2001). In addition, PPT but not DPN mimicked the estrogen induced increase in NR2A/B binding and expression (Morissette et al., 2008). Interestingly, estrogen has been reported to increase binding for NMDA receptor but had no overall effect on binding for AMPA receptors (Woolley et al., 1997; Cyr et al., 2001). One explanation suggests that estrogen mediated synaptogenesis results in silent synapses that contain NMDA but not AMPA (Woolley, 1998). Estrogen also enhances hippocampal LTP (Warren et al., 1995; Cordoba Montoya and Carrer, 1997; Foy et al., 1999; Smith and McMahon, 2005) and LTP requires both NMDA receptors for induction and AMPA receptors for continuation.

Excitatory synapses also contain a concentration of AMPA-receptors, which mediate fast synaptic transmission and likely neuron-neuron interactions related to learning and memory. GluR1, 2, 3 are co-expressed with ERs in the hypothalamus, amygdala, and septum although estrogen regulates AMPA receptor expression only in the hypothalamus (Diano et al., 1997). Although colocalization of ERs and AMPA-receptor subunits has not been demonstrated in the hippocampus, our findings, particularly the effects of ER $\beta$  activation, suggest another mechanism for estradiol to alter calcium dynamics and excitability and alter synaptic plasticity involved in cognition and neuroprotection (Zhao et al., 2004). Specific activation of ER $\beta$  by DPN can regulate GluR2 and GluR3 expression while activation of ER $\alpha$  by estradiol or PPT does not regulate these subunits and may block ER $\beta$  regulation as shown by co-administration of DPN and PPT. The ability of ER $\alpha$  and  $\beta$  to antagonize the other's effects when coexpressed has been reported (Hall and McDonnell, 1999; Matthews et al., 2006). In contrast, EB, DPN, and PPT administration alone modestly increased GluR1 expression.

In the adult hippocampus, most AMPA receptors exist as heteromers of GluR1/GluR2 and GluR2/GluR3 subunits (Craig et al., 1993) and, since the expression of GluR3 is relatively low, more than 70% of GluR2 is associated with GluR1 (Wenthold et al., 1996). Another AMPA receptor subunit GluR4, forms complexes with both GluR1 and GluR2 or 3 but is expressed at lower levels in principal cells in the CA1 and was not examined here (Wenthold et al., 1996). GluR1 regulates activity dependent delivery of AMPA receptors to the synapse (Shi et al., 1999). GluR2 regulates Ca<sup>2+</sup> permeability (Hume et al., 1991; Sommer et al., 1991) and glutamate-induced redistribution of surface AMPA receptors to internal pools (Lee et al., 2004a). A large increase in GluR2 at the same time that GluR1 is only modestly increasing and GluR3 in decreasing suggests several possible outcomes for AMPA receptor composition and function. Decreased GluR3 could reduce the available pool of GluR2/3 for constitutive insertion in the synapse and increased GluR2 could enhance activity dependent insertion of GluR1/2, shifting the complement of synaptic AMPA receptors. The formation of GluR2 homomers may also increase. Together, the effects of increased GluR2 could dramatically

reduce Ca<sup>2+</sup> influx and decrease the potential of excitatory events, such as increased glutamate release, to have adverse effects. Increased GluR2 could also delay a restoration in the balance of NMDA and AMPA receptor activity as an increase in AMPA receptor activity is associated with the termination of estrogen enhancement of LTP (Smith and McMahon, 2005).

In comparing the actions of DPN and PPT to EB, we found that DPN affected the expression of more synaptic proteins than PPT or even EB. Although both ER $\alpha$  and ER $\beta$  have been identified at similar extranuclear sites in hippocampal pyramidal cells (Milner et al., 2001; Milner et al., 2005), it has not been established that ER $\alpha$  and  $\beta$  are colocalized in the same compartments. ER $\alpha$  and ER $\beta$  bind estradiol with similar affinity despite having only moderately similar ligand binding domains (AF-2). Their DNA binding domains are highly conserved but their N-terminal domains (AF-1) share poor homology (Kuiper et al., 1996). Because hippocampal ERs are extranuclear, it is likely that these differences in the N-terminal domains, which mediate receptor interactions with signaling pathways, result in receptor specific effects on protein expression through alterations in gene transcription and protein translation or degradation. Downstream convergence of signaling pathways could mediate protein effects that are similar for each receptor. It has also been reported that ER $\beta$  actions can antagonize effects of ER $\alpha$  (Hall and McDonnell, 1999; Matthews et al., 2006; Gonzales et al., 2008). In these reports and our experiments, low doses of DPN or PPT were used to avoid nonspecific activation of the other receptor. PPT is 410 fold more selective for ER $\alpha$  than ER $\beta$ , whereas DPN is 72 fold more selective for ER $\beta$  than ER $\alpha$ ; however, at high doses both DPN and PPT can have effects on either ER (Stauffer et al., 2000; Meyers et al., 2001). Simultaneous activation of ER $\beta$  and ER $\alpha$  may result in no net change in the expression of some proteins. The reverse may also be true, when estradiol activates ER $\alpha$  and ER $\beta$  in tandem effects can include synaptogenesis and potentiation of LTP. Our findings further suggest that other effects of the ER $\alpha$  and  $\beta$  subtypes can be unmasked by treatment with specific agonists and these effects may have potential for clinical understanding. The effects of estradiol, DPN and PPT may also be related to their ability to regulate neurotrophins such as brain-derived neurotrophic factor, which has also been shown regulate GluR1 and 2 (Cyr et al., 2001; Caldeira et al., 2007a; Caldeira et al., 2007b).

While the relative contributions of ER $\beta$  versus ER $\alpha$  on gene transcription versus protein translation or degradation awaits further study, reports on the differential effects of ER $\alpha$  and  $\beta$  activation on intracellular Ca<sup>2+</sup> dynamics, phosphorylation of signaling molecules and neuroprotection suggest each receptor has differential contributions to neuronal physiology, synaptic plasticity, and hippocampal-dependent behavior (Day et al., 2005; Szymczak et al., 2006; Zhao and Brinton, 2007; Foster et al., 2008; Walf and Frye, 2008; Walf et al., 2008). Although selective agonists of ER $\alpha$  and  $\beta$  did not completely mimic the actions of EB examined here, both individual and simultaneous actions of ER $\alpha$  and  $\beta$  may contribute to estrogen's effects on hippocampal plasticity and memory. These reports, along with our results suggest that targeted estrogen replacement that selectively activates ER $\alpha$  and  $\beta$  may offer therapeutic opportunities to specifically enhance the beneficial effects of estrogen.

## 4. Experimental Procedures

### 4.1 Animals

Adult female Sprague Dawley rats (250–275g) from Charles River Laboratories (Wilmington, MA) were housed three to a cage with ad libitum access to food and water and with 12:12 light/dark cycles. Ovariectomy was performed following previously published protocols (Becker et al., 2005). All procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines.

## 4.2 Steroid replacement

Steroid administration regimens followed a protocol previously used by our laboratory to reproduce the modifications of dendritic spines in CA1 normally produced during the estrous cycle (Woolley and McEwen, 1993). Three days after ovariectomy, rats received 100  $\mu$ l s.c. injection of either 10  $\mu$ g of estradiol benzoate (Sigma, St. Louis, MO), ER $\alpha$  specific agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) at 1 mg/kg (Tocris, Ellisville, MO), ER $\beta$  specific agonist 2,3-bis(4-hydroxyphenyl) propionitrile (DPN) at 1 mg/kg (Tocris, Ellisville, MO), a combination of both agonists in DMSO, oil or DMSO. These doses of PPT and DPN have been reported to alter behavior and glutamate receptor binding (Lund et al., 2005; Morissette et al., 2008). Rats received 2 injections of vehicle or drug 24 hrs apart and were perfused 48 hrs after the last injection. Biological availability of the steroids was monitored through changes in uterine weights. The average weight of uteri from oil or DMSO-administered animals was 0.233 $\pm$ 0.022 grams. After the various steroid regimens the uteri weighed 0.563 $\pm$ 0.036 grams for EB, 0.333 $\pm$ 0.009 grams for PPT alone, and 0.312 $\pm$ 0.079 grams for DPN alone and 0.290 $\pm$ 0.008 grams for PPT plus DPN (N = 5 per group). EB, PPT and DPN plus PPT administration resulted in a significant increase in uterine weight compared to vehicle (F(5, 20) = 109.1, p < 0.0001; post hoc test p < 0.05), while DPN did not (p > 0.05).

## 4.3 Immunohistochemistry

For quantitative light microscopic localization of synaptic proteins, serial dilutions for each antibody were established and a linear function of antibody concentration against labeling intensity was obtained using densitometry, as previously described (Chang et al., 2000; Torres-Reveron et al., 2008). The dilution that produced slightly less than half-maximal labeling was chosen for use to allow for variations in labeling intensity (Chang et al., 2000). To insure identical labeling conditions during immunocytochemistry (Pierce et al., 1999), sections of each treatment group were rinsed in PB, coded with hole punches in the cortex and pooled into single containers. The tissue was processed according to the avidin-biotin complex (ABC) method (Hsu et al., 1981). Briefly, sections were incubated in: (1) PB to remove cryoprotectant; (2) 1% sodium borohydride in PB, 30 min to neutralize free aldehydes; (3); 0.5% bovine serum albumin (BSA) in Tris-saline solution (TS; 0.9% NaCl in 0.1 M Tris, pH 7.6) to block non-specific antibody binding, 30 min; (4) the primary antiserum in 0.1% BSA/TS either without Triton (GluR1-3) or with 0.1% Triton (PSD-95, synaptophysin, spinophilin, VGaT, VGluT1, 1 day at room temperature (~23 °C) followed by 1–4 days at 4°C; (5) 1:400 of anti-mouse or rabbit biotinylated-IgG, 30 min; (6) a 1:100 dilution of peroxidase-avidin complex (Vectastain Elite Kit), 30 min; and (7) 3,3'-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub> in TS, 6 min. All incubations were separated by washes of TS. Sections were mounted on gelatin coated slides, dehydrated in ascending concentrations of alcohols, and cover-slipped with D.P.X. neutral mounting medium (Sigma).

## 4.4 Primary Antiserum

The antibody to synaptophysin (Mouse, 1:300,000, Sigma) was generated using clone SVP-38 (Wiedenmann and Franke, 1985) and detects a single band of 38 kD by western blot (Brake et al., 2001). The antibody to spinophilin (Rabbit, 1:500,000, Patrick Allen, Yale University) has been characterized by western blotting (Allen et al., 1997) and in knock out mice (Stafstrom-Davis et al., 2001). Characterization of the antibody to VGluT1 (Guinea Pig, 1: 20,000, Chemicon) has been described by Boulland (Boulland et al., 2007). Specificity of the VGaT antibody (Rabbit, 1:15,000, Synaptic Systems, Gottingen, Germany) has been described by Takamori (Takamori et al., 2000). The antibody to PSD-95 (Mouse, 1:15,000, Sigma) recognized a single band of 95 kD by western blot (manufacturer's technical information). Antibodies to GluR1 (Rabbit, 1:1000, Calbiochem, La Jolla, CA), GluR2 (Mouse, 1:500,



Chemicon), and GluR3 (Mouse, 1:1000, Chemicon) have been characterized by western blot and in knock-out mice (Wenthold et al., 1992; Molnar et al., 1993; Medvedev et al., 2008).

#### 4.5 Analysis

Sections were analyzed and photographed on a Nikon Eclipse 80i light microscope equipped with bright-field and differential interference optics and a Micropublisher digital camera (Q imaging, Barnaby, BC). Photomicrographs were prepared by adjusting levels, brightness and contrast in Adobe Photoshop CS and final figures were assembled in Adobe PowerPoint 11.3.

For quantitative densitometry, images of regions of interest (R.O.I.) were captured using a Dage MTI CCD-72 camera and NIH Image 1.50 software. The mean gray value (of 256 gray levels) for each selected R.O.I. was determined as previously described (Pierce et al., 1999; Torres-Reveron et al., 2008; Torres-Reveron et al., 2009). To compensate for background staining and control for variations in illumination level between images, the average pixel density for 3 regions within the corpus callosum was subtracted. Tissue from control and experimental animals were processed together in the same crucibles. Sections from each animal were selected from the dorsal midseptotemporal level of the hippocampal formation (AP -3.90 to -4.20 Bregma; Swanson approximately level 32 (Swanson, 2000) and matched sections were selected processing with each antibody. A single hippocampi from each animal with the best morphology and consistent peroxidase labeling was included in the analysis. Optical density values were measured using NIH image. Net optical density values obtained after subtracting background values were converted to a percentage scale of 256 preset gray values ranging from 0 to 100%. A planned comparison was performed to compare steroid administration to vehicle treatment. A one-way ANOVA followed by Bonferroni's Multiple Comparison Test with a 95% confidence level using Prism 5 for Mac Os X (Graphpad Software, Inc., La Jolla, CA). For this analysis, p values of < 0.05 in the ANOVA and planned comparison test were considered significant. ANOVA results with p values greater than 0.05 also were examined by post-hoc for significant differences. Post hoc p values between 0.05 and 0.065 were considered to be a trend towards significance.

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#### Abbreviations

GABA, ( $\gamma$ -Aminobutyric acid); AMPA, (alpha-amino-3-(3-hydroxy-5-methylisoxazole-4-yl) propionate); PPT, (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole); DPN, (2,3-bis(4-hydroxyphenyl) propionitrile); ER $\alpha$  and  $\beta$ , (estrogen receptor alpha and beta); PSD-95, (post-synaptic density protein); VGluT1, (vesicular glutamate transporter 1); VGaT, (vesicular GABA transporter).

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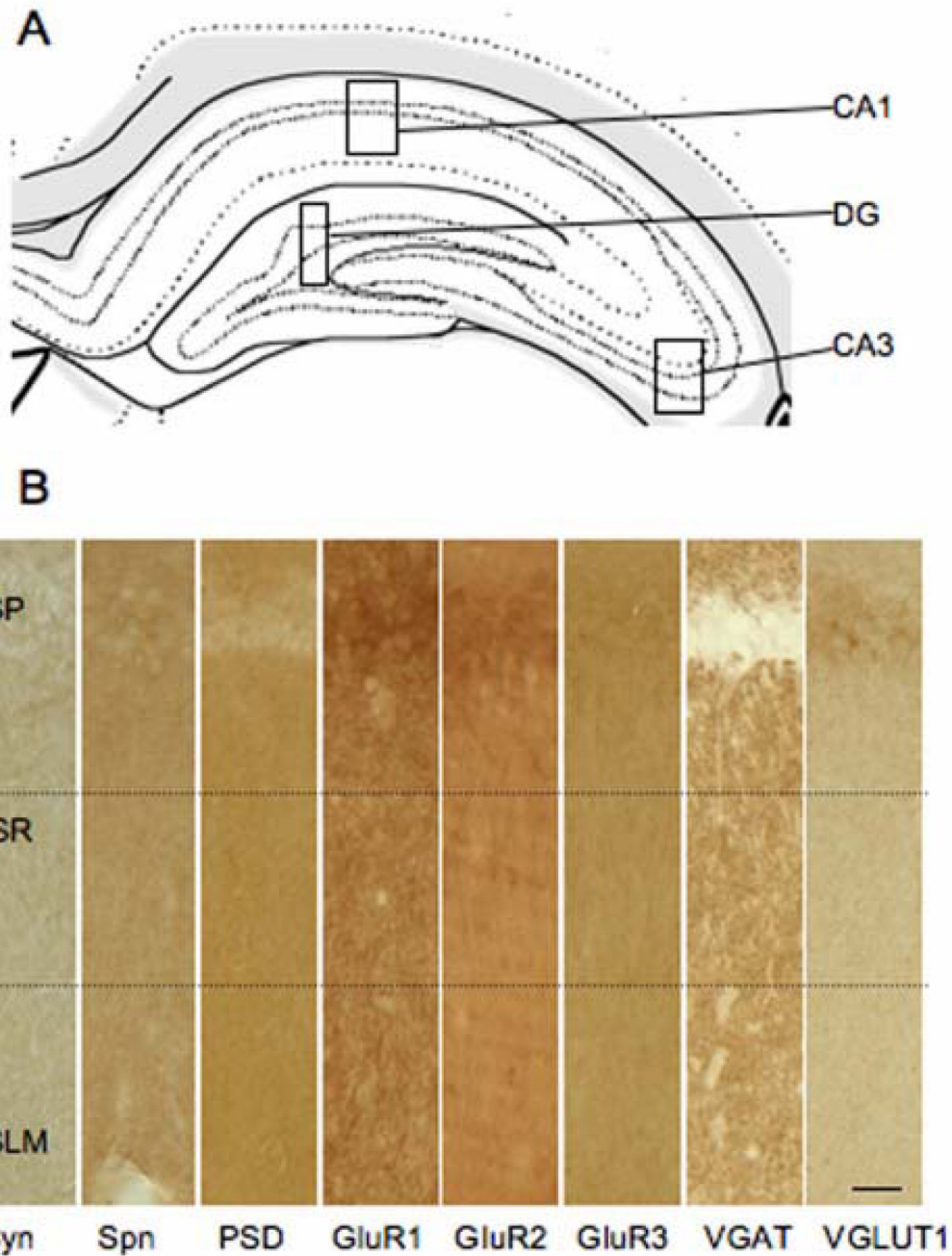
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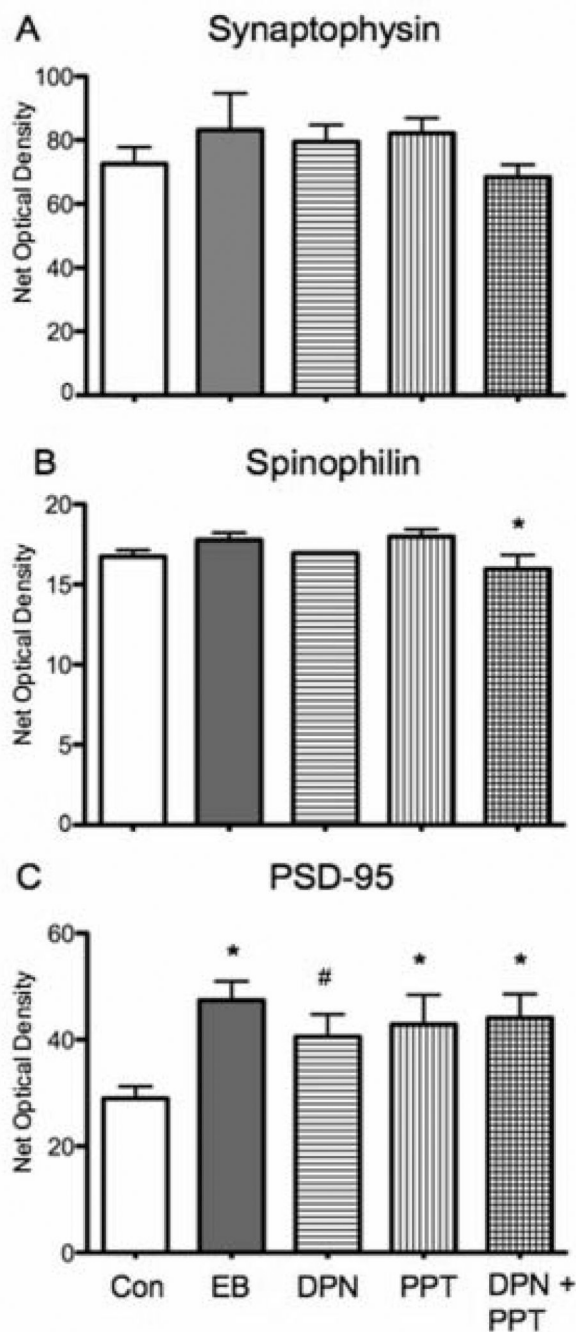
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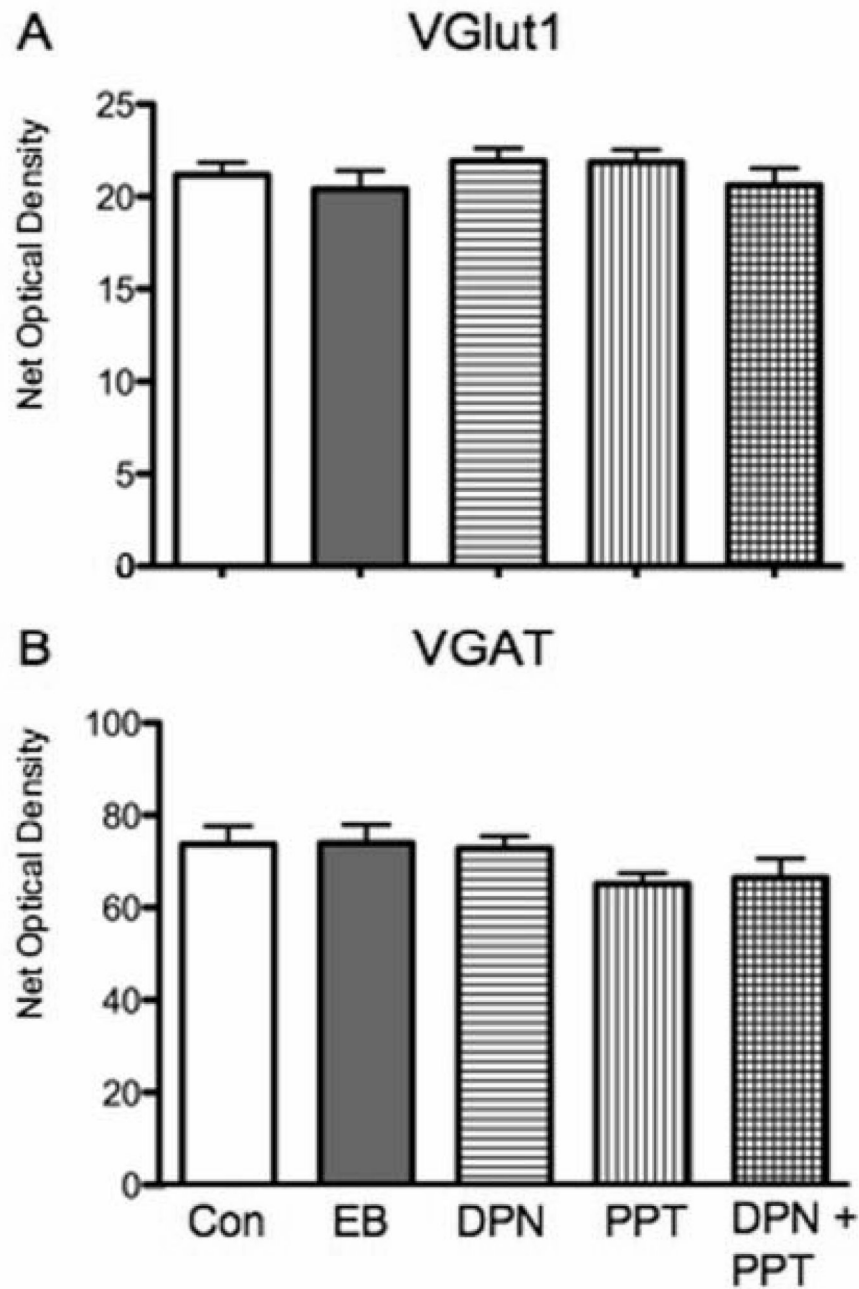


**Figure 1.** Examination of synaptic protein expression in the hippocampal formation. **A:** Schematic representation of hippocampal formation with boxes to demonstrate the areas of the CA1, CA3 and dentate gyrus (DG) analyzed [59]. **B:** Distribution of synaptic proteins in the CA1 region of the hippocampus. Representative light photomicrographs of synaptophysin (Syn), spinophilin (Spn), PSD-95, GluR1, GluR2, GluR3, VGAT, and VGLUT1 peroxidase labeling. Dotted lines demonstrate the medial portion of the stratum radiatum (SR) relative to the strata pyramidale (SP) and lacunosum-moleculare (SLM). Scale bar = 20  $\mu$ m.

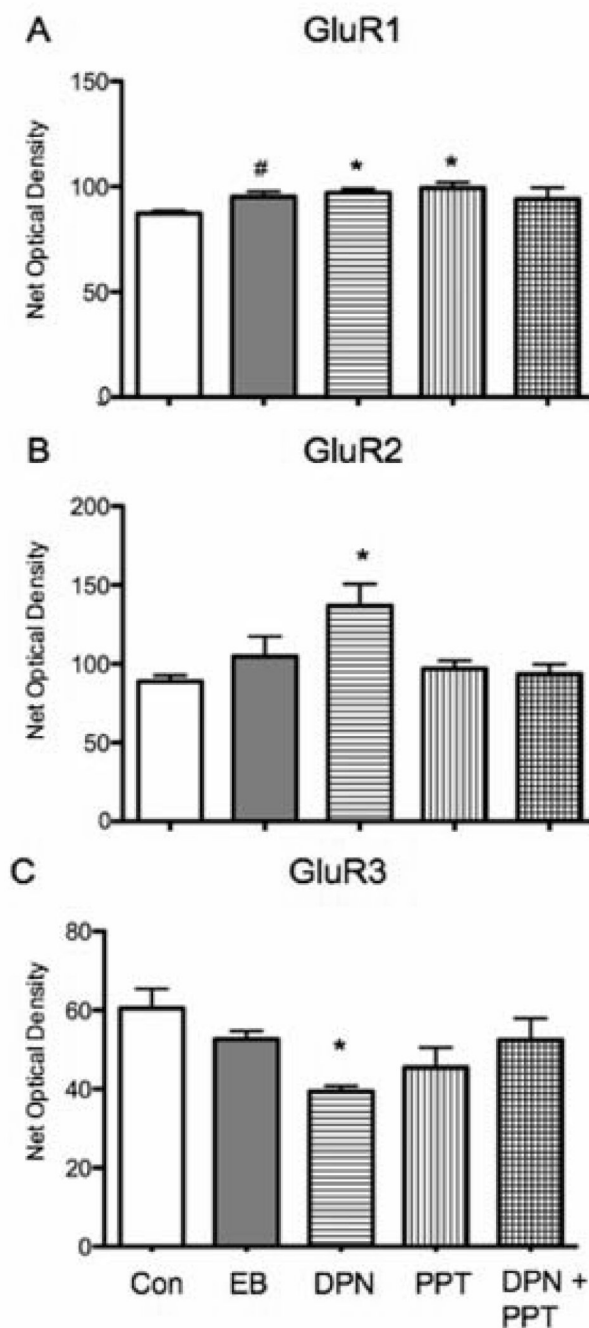


**Figure 2.**

Effects of selective ER agonist administration on synaptophysin, spinophilin, and PSD-95 immunoreactivity in the CA1 stratum radiatum of the dorsal hippocampus. **A:** Synaptophysin expression was not altered by any of the steroid regimens when compared to vehicle (Veh). **B:** Labeling for spinophilin was not altered in any treatment group compared to vehicle. In comparison to EB treatment, DPN plus PPT significantly reduced synaptophysin levels (post hoc test \*  $p < 0.05$ ). **C:** PSD-95 immunoreactivity was significantly higher in EB, PPT alone and DPN + PPT treated hippocampi compared to vehicle (post hoc test \*  $p < 0.05$ ). In the DPN alone group there was a trend for increased PSD-95 expression (post hoc test #  $p = 0.061$ ). For this and all subsequent figures error bars represent SEM.



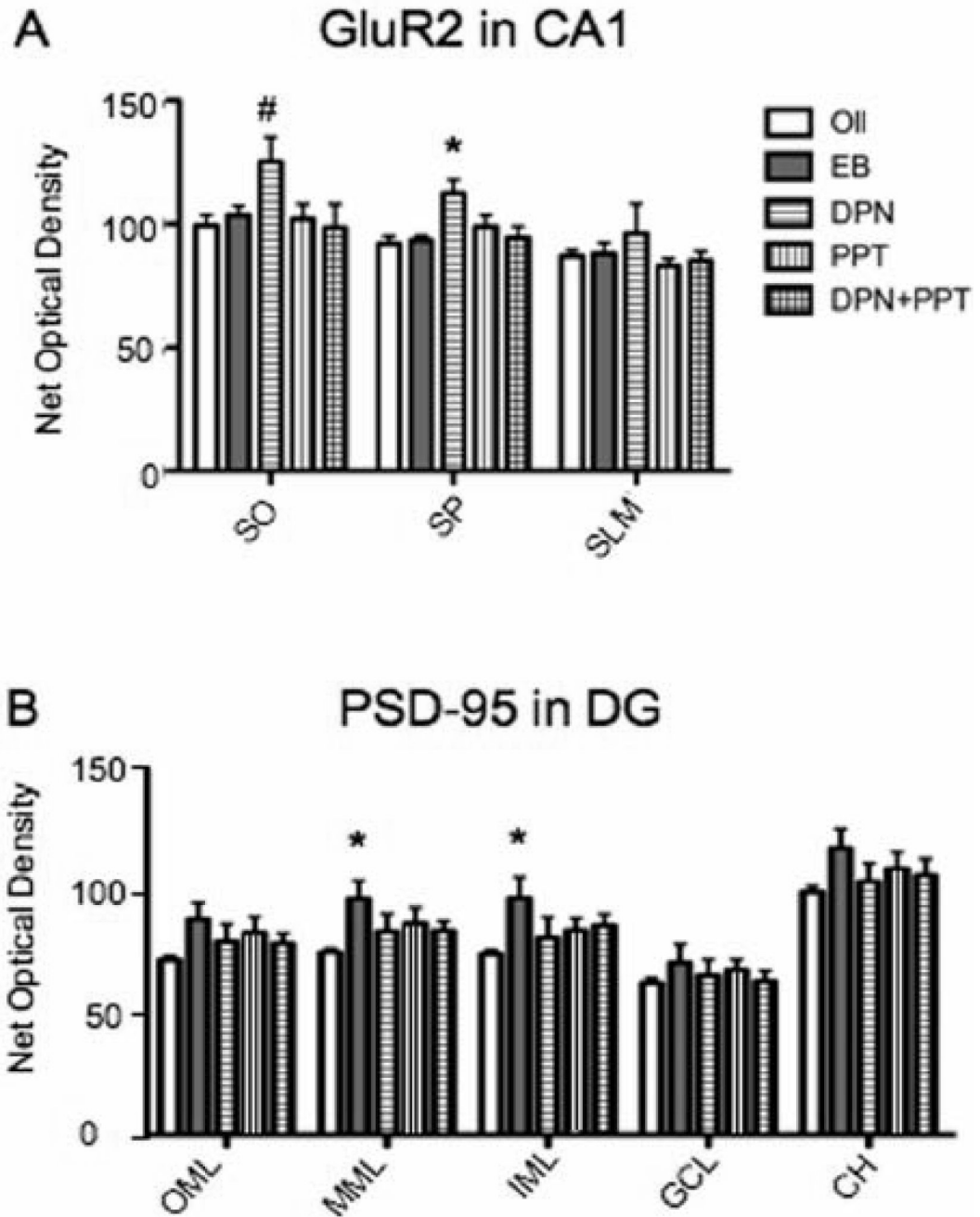
**Figure 3.** Expression of vesicular transporters in stratum radiatum of CA1 did not change with any of the steroid regimens. EB, PPT alone, DPN alone and PPT + DPN did not significantly alter VGlut1 or VGAT expression levels in comparison to vehicle.



**Figure 4.**

Estradiol and ER selective agonists differentially regulated AMPA receptor subunits GluR1-3 in CA1 stratum radiatum. **A:** The level of GluR1 immunoreactivity was significantly increased relative to vehicle by PPT alone and DPN alone (post hoc test \*  $p < 0.05$ ). There was a trend for EB to increase the levels of GluR1 immunoreactivity (post hoc test #  $p = 0.054$ ). **B:** GluR2 labeling was significantly increased by DPN alone (post hoc test \*  $p < 0.05$ ) but not by EB, PPT alone or DPN + PPT when compared to vehicle. **C:** A significant decrease in the levels of GluR3 immunoreactivity was observed with DPN alone (post-hoc test \*  $p < 0.05$ ) but not administration of EB or PPT alone.





**Figure 5.**

Steroid replacement alters GluR2 and PSD-95 labeling selectively in other CA1 layers and in the dentate gyrus. **A:** DPN administration significantly increased GluR2 immunoreactivity in the CA1 stratum pyramidale (SP) and stratum oriens (SO) (post hoc test \*  $p < 0.05$ ). There was no effect of DPN in the stratum lacunosum-moleculare (SLM) and the other steroid regimens had no effect in any of the layers. **B:** In the dentate gyrus, compared to vehicle, EB increased PSD-95 levels in the inner molecular layer (IML) and in the middle molecular layer (MML) (post hoc test \*  $p < 0.05$ ). There was no effect of any treatment on the outer molecular layer (OML), granule cell layer (GCL) or central hilus (CH).