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Critical Considerations for Hormone Replacement Therapy:

The Mechanism of Estrogen-Progesterone Interactions Unraveled

ABSTRACT

Alzheimer Disease (AD) is more prevalent in women than in men, suggesting that the depletion of female sex hormones, such as progesterone (P4) and estrogen, in post-menopausal women increases the risk of developing AD. Based on several in vitro and in vivo experiments, 17 β -estradiol (E2) and progesterone (P4) have been found to be neuroprotective against a variety of insults (Aguirre & Baudry, 2009; Nilsen & Brinton, 2002). However, when E2 and P4 were combined, P4 generally reversed E2-mediated neuroprotection (Rosario *et al*, 2006; Aguirre & Baudry, 2009). Recent studies indicated that administering E2 for 20 h to cultured hippocampal slices and then adding P4 for 4 h eliminated the neuroprotective effects of E2 against N-methyl-D-aspartate (NMDA) toxicity (Aguirre & Baudry, 2009). In this study, we examined the role of the two major subtypes of estrogen receptors, ER α and ER β in E2-mediated neuroprotection against NMDA neurotoxicity in organotypic hippocampal slices. Treatment of cultured hippocampal slices with E2 did not modify ER α mRNA levels but significantly increased ER β mRNA levels. Further, treatment with P4 reversed E2-induced increases in ER β mRNA and protein levels. In addition, P4 treatment reversed E2-mediated increase in ER α protein levels. Experiments with an ER α agonist, PPT, or an ER β agonist, DPN, indicated that E2-mediated neuroprotection against NMDA toxicity was mediated by the activation of ER β but not ER α receptors. Understanding estrogen-progesterone interactions is important in order to develop a safe and effective treatment for postmenopausal women at risk for developing neurodegenerative diseases.

INTRODUCTION

According to the *2009 Alzheimer's Disease Facts and Figures* report, 5.3 million people in the United States are living with Alzheimer Disease (AD), which is the 6th leading cause of death. In addition to the large death rate among America's oldest age groups due to this disease, the costs of the disease to society are estimated to be over \$148 billion per year. Currently, there exists neither a cure nor an effective treatment for this neurodegenerative disease.

On average, menopause begins when women are approximately 54 years of age (Arias and Smith, 2003). One prominent characteristic of menopause, or the ceasing of menstruation, is a depletion of female sex hormones, such as estrogen and progesterone. Several studies have shown that women over the age of 65 have a much higher prevalence of AD than men (Henderson, 1997; Gao *et al.*, 1998). Ergo, since AD is most commonly diagnosed in postmenopausal women, it has been hypothesized that a deficiency of primary female sex hormones may increase the likelihood of these women developing neurodegenerative diseases.

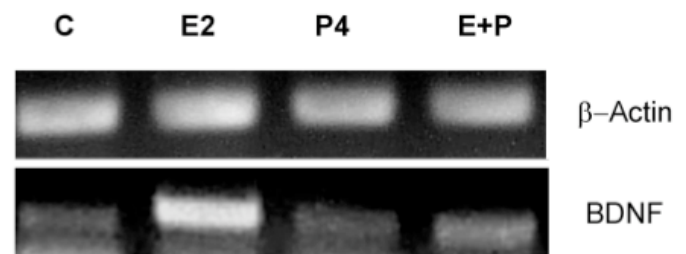
In order to counteract the decline in levels of ovarian hormones, physicians have prescribed hormone replacement therapy (HRT). In 1999, approximately 37.6% of all post-menopausal women in the United States received HRT (Keating *et al.*, 1999). However, researchers from the Women's Health Initiative have concluded that women taking conjugated equine estrogens combined with medroxyprogesterone acetate, a synthetic progestin, not only were not protected against AD but had increased risks for several diseases as compared to the placebo group (Rossouw *et al.*, 2002). This finding is quite paradoxical since both estrogen and progesterone have been found to be neuroprotective in a variety of experimental models of neuronal damage (Aguirre & Baudry, 2009; Nilsen & Brinton, 2002). Thus, it is still critical to develop an effective treatment to counteract the depletion of female sex hormones. In order to do so, we must first understand the interactions between estrogen and progesterone in experimental models of neurodegeneration.

REVIEW OF LITERATURE

Gonadal Hormones and BDNF

A large body of evidence indicates that gonadal hormones, such as estrogen and progesterone, directly target multiple regions within the central nervous system (i.e. the hippocampus and the cortex) (Baulieu *et al.*, 1996). Based on several *in vitro* and *in vivo* experiments on neurodegeneration, 17 β -estradiol (E2), a main form of estrogen, has been shown to have neuroprotective properties against neurotoxicity (Aguirre & Baudry, 2009). In addition, several experiments have also demonstrated potent neuroprotective effects of P4 against glutamate-induced cell death (Nilsen & Brinton, 2002) and traumatic brain injury (Roof *et al.*, 1997). However, when E2 and P4 are combined, P4 has been shown to reverse E2-mediated neuroprotection (Aguirre & Baudry, 2009; Rosario *et al.*, 2006).

In addition to the neuroprotective effects of estrogen, estrogen has been found to stimulate the production of the neurotrophin, brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family of growth factors and is abundant in hippocampus, cortex, and basal forebrain, areas critical to learning and memory. Among other functions, BDNF supports the survival of existing neurons and participates in growth, differentiation, and synaptic plasticity at existing synapses (Hofer *et al.*, 1990). It has been proposed that E2-mediated neuroprotection is mediated by increased production and release of BDNF (**Figure 1**) (Aguirre & Baudry, 2009).



Aguirre, CC, Baudry, M., 2009.

Figure 1: Estrogen leads to an increase in BDNF

Estrogen Receptors and Agonists

Since progesterone and estrogen are both neuroprotective, it is critical to understand why, under certain conditions, progesterone can reverse the neuroprotective effects of estrogen. This

prompted us to investigate the differential roles of the two major types of estrogen receptors – ER α and ER β – in E2-mediated neuroprotection. This literature review section will focus mainly on these receptors and their respective functions in relation to plasticity. In order to study the effects of different receptors, specific agonists binding to each type of receptors are generally used. An agonist is a ligand that binds to a receptor and mimics the effects of the endogenous molecule that activates this receptor. 17 β -estradiol is an agonist that binds equally well to both estrogen receptors. However, in order to differentiate between the effects of ER α and ER β , two different agonists that bind selectively to the two receptors have been identified – DPN, an agonist for ER β , and PPT, an agonist for ER α .

Estrogen Receptors α and β

Estrogen Receptor α (ER α)

Several studies indicate the importance of ER α in memory function, suggesting a critical role of this receptor for age-related cognitive decline or neurodegenerative diseases, such as AD. In particular, spatial learning deficit was observed in ER α KO mice (i.e., mice lacking ER α); this deficit was reversed by reintroducing ER α by using lentiviral transduction of a plasmid coding for ER α . The study was limited to female ER α KO mice because they exhibit learning and memory deficits compared to wild type (WT), unlike male ER α KO mice (Foster *et al.*, 2008). These results suggest that the learning deficits found in female ER α KO mice are not only due to the lack of ER α during development, an organizational effect, but also due to the disruption in ER α function in adults. However, it is still unclear whether the beneficial effects of adding ER α to these KO animals is occurring through the natural ER α pathways or through another compensatory pathway.

Other studies have examined the roles of estrogen receptors on cognition using the specific agonists DPN and PPT. For instance, a recent study found an effect of ER α agonist but not of ER β agonist on the modulation of NMDA receptors (NMDAR) in hippocampus (Morissette *et al.*, 2008).

NMDA receptors are a class of glutamate receptors that has been implicated in increased spine density and cognition. The researchers saw a decrease in the NMDAR subunit, NR2B, in hippocampus following ovariectomy. Estradiol and PPT, but not DPN treatment reversed this effect. In cortex, decrease in NR2B was observed following E2 treatment but not with either DPN or PPT (Morissette *et al.*, 2008). Another study focused on ER subtypes and α -Ca²⁺/calmodulin-dependent kinase II (α CaMKII) signaling to discover the mechanisms underlying estrogenic effects on cognitive function (O'Neill *et al.*, 2008). α CaMKII plays an important role in neuronal differentiation and cognitive processes and is highly responsive to Ca²⁺ levels. Under normal conditions, this kinase is inhibited until Ca²⁺/CaM binds to its autophosphorylation domain and permits kinase activity. In this study, E2 rapidly induced α CaMKII autophosphorylation in an ER α - and Ca²⁺ influx-dependent manner. In addition, PPT induced autophosphorylation in a dose-dependent manner, while DPN treatment did not modify CamKII phosphorylation. Together, these data propose a central role for ER α in E2-mediated effects on learning and cognitive processes.

Estrogen Receptor β (ER β)

In contrast to these studies supporting a role for ER α in cognition, other laboratories have favored ER β as being the more important subtype of the two receptors for synaptic plasticity. There has been a handful of recent evidence relating ER β to plasticity, and to learning and memory. To begin in a broader sense, estrogen, acting through both receptors, plays a key role in the activation of neural circuits involving the ventromedial nucleus of the hypothalamus (Sá *et al.*, 2009), and more generally in both neuroprotection and neurodegeneration altogether (Aguirre & Baudry, 2009). At the University of Albany, researchers found that administering E2 into the hippocampus of rats 10 minutes after training enhanced performance in a hippocampus-dependent task, indicating the critical role estrogen plays in mediating some hippocampal functions (Walf & Frye, 2008).

More specifically, the rapid effects of ER β activation in hippocampus were found to mediate some of the functional effects of estrogen. Administering agonists specific for ER β , rather than ER α , resulted in enhancing effects on hippocampal processes similar to those of E2. These effects were eliminated when ER β expression was knocked out in transgenic models. Thus, E2's actions appear to be mediated through rapid, membrane-mediated effects and intracellular estrogen receptors in hippocampus (Walf & Frye, 2008). In a recent study, Liu *et al.*, (2008) reported that ER β was critical for hippocampal synaptic plasticity and learning and memory by using specific agonists for ER β and examining both electrophysiological models and behavioral models of synaptic plasticity and learning and memory.

By increasing cell firing in hippocampus, a brain structure that plays an important role for cognition and affective behavior, E2 has been shown to enhance cognitive performance. Researchers have speculated that the β isoform of estrogen receptors may be important targets for E2's effects on hippocampus-mediated processes. Another study has linked ER β to synaptic plasticity against acoustic trauma in mice (Meltser *et al.*, 2008). In particular, the role of ER β in auditory trauma was studied by utilizing agonists of ER β to test for its effects on BDNF production. Activating ER β by adding DPN to wild type (WT) mice, not only led to a slight increase in BDNF protein, but also led to a significant increase in BDNF mRNA. Adding DPN to mice deficient in ER α (ER α KO) and aromatase (ARKO) led to a relatively larger increase in the production of BDNF protein as compared to WT mice. Thus, these researchers concluded that ER β protects the auditory system against acoustic trauma, presenting the first evidence that directly correlates ER β expression to the protection of auditory function. Although this research was done in the auditory system, this evidence provides support for the role of ER β in plasticity and BDNF activation (Meltser *et al.*, 2008).

Progesterone's effects on estrogenic neuroprotection

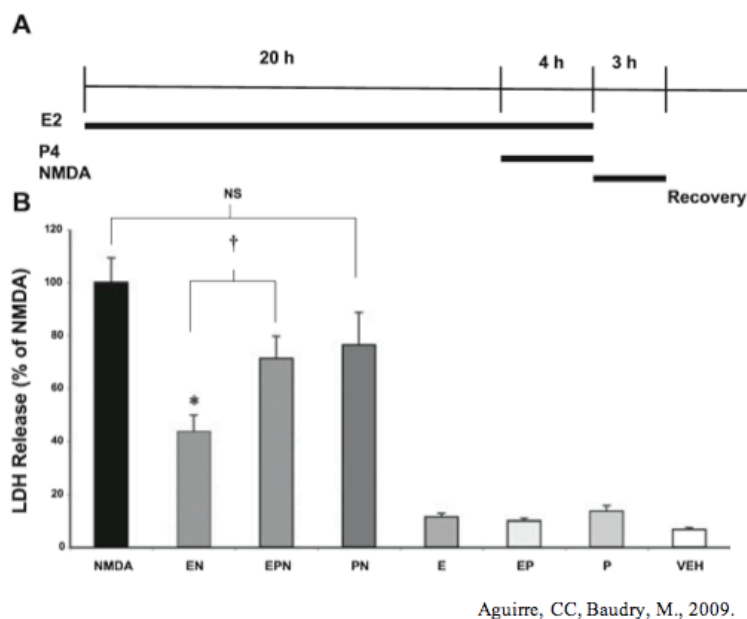


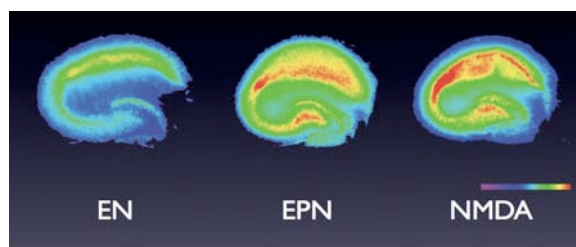
Figure 2: P4 mediated reversal of E2-induced neuroprotection against NMDA toxicity in OHSC

- (A) Treatment protocol timeline for hormones and NMDA
 (B) Lactate dehydrogenase (LDH) release in the medium measured 24 h after NMDA treatment (Aguirre & Baudry, 2009)

A substantial amount of information has been acquired regarding the mechanisms underlying estrogen's protective effects, mainly through the use of ovariectomized animal models. However, in humans, menopause is characterized by the loss of both estrogen and progesterone, implicating the loss of both estrogen and progesterone as a possible cause of increased risk for developing AD. Much less research has focused on progesterone and its relationship with estrogen in neurodegenerative models. Nevertheless, progesterone has been found to possess neuroprotective effects in

various experimental models. For instance, in hippocampal neurons, progesterone reduced neuronal vulnerability to glutamate, FeSO₄, and Aβ toxicity (Singh, 2006).

However, further studies have shown that administration of progesterone reverses estrogenic neuroprotection. In particular, while E2 was neuroprotective against N-methyl-D-aspartate (NMDA) toxicity in cultured rat hippocampal slices, adding P4 for 4 h after pretreatment of E2 for 20 h led to a reversal of E2-mediated neuroprotection against toxicity (Aguirre & Baudry, 2009; [Figure 2](#); [Figure 3](#)).



Aguirre, CC, Baudry, M. 2009.

Figure 3: Pseudo-colored propidium iodide (PI) uptake data

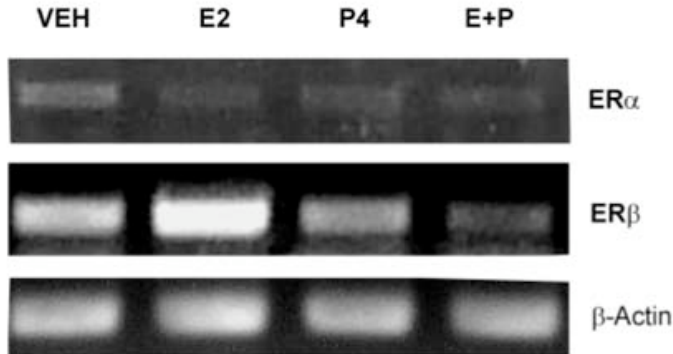
Effects of E2 and P4 on NMDA-induced neuronal damage assessed with PI uptake: NMDA treatment resulted in increased levels of red and yellow fluorescence intensity, and estrogen attenuated this increase. P4 reversed the protective effects of E2 (Aguirre & Baudry, 2009).

A similar conclusion about progesterone's effects can be drawn from another experiment in which researchers discovered that progesterone blocks estrogenic neuroprotection against kainate neurotoxicity in middle-aged female rats. The effects of estrogen and progesterone, both alone and combined, on hippocampal neuron survival following kainate lesion were studied in 14-month-old female rats entering the end of their reproductive stages. The results showed that adding estrogen to ovariectomized rats led to a reduction of neuron loss (Carroll *et al*, 2008). However, this reduction was blocked by co-administration of continuous progesterone. Both of the preceding experiments yield crucial data that provide new insight into factors that regulate estrogen neuroprotection. On a larger scale, estrogen neuroprotection has clear implications for hormone replacement therapy in postmenopausal women.

Qualitative Meta-analysis

While it has been shown that progesterone may reverse estrogenic neuroprotection, the exact biological mechanism underlying this interaction is unknown. We do not have enough data to conclude which receptor, ER α or ER β , is responsible for mediating neuroprotection by estrogen. While some reports indicated the importance of ER α splice variants for estrogen therapy, others disregarded them and focused on the role of ER β in neuroprotection. The mechanism underlying the reversal of estrogenic neuroprotection by P4 treatment is similarly unknown. One speculation was that P4 treatment results in down-regulation of ER β mRNA and protein, but there has not been solid published evidence yet. Therefore, it is important to understand the underlying mechanisms of estrogen-progesterone interactions in order to develop a safe and effective treatment for postmenopausal women at risk for developing neurodegenerative diseases such as Alzheimer disease.

Progesterone Reverses Estrogen-Mediated Increase in ER β mRNA



Aguirre, CC, Baudry, M., 2009.

The following polymerase chain reaction (PCR) data are currently in the process of publication by Claudia Aguirre and Dr. Michel Baudry (Figure 4). Based on this experiment, it can be concluded that progesterone reverses estrogen-mediated increase in ER β mRNA, although ER α mRNA levels are unaltered by hormone treatments (Aguirre & Baudry, 2009).

Figure 4: P4 reverses E2-mediated neuroprotection against NMDA toxicity by down-regulating ER β mRNA.

This result was seen in parallel with the reversal of estrogenic neuroprotection against NMDA toxicity by progesterone, suggesting that this effect may be due to the down-regulation of ER β .

SPECIFIC AIMS

The objective of this project was to investigate the roles of the two main subtypes of estrogen receptors – ER α and ER β in E2-mediated neuroprotection against NMDA toxicity in cultured hippocampal slices. In order to differentiate between the effects of estrogen receptor α (ER α) and estrogen receptor β (ER β), we used specific agonists for each type of receptor. The ER β receptor agonist used was diarylpropionitrile (DPN) and the ER α receptor agonist used was Propyl Pyrazole Triol (PPT).

It had been concluded, based on our previous research, that progesterone (P4) reverses estrogen-mediated neuroprotection against NMDA toxicity. Our lab has also obtained data showing that adding P4 reverses E2-induced ER β activation. Therefore, we investigated whether there exists a link between neuroprotection and E2-induced ER β activation. In short, our research question was: Is ER β necessary for neuroprotection?

The hypothesis of this research project is that E2-mediated neuroprotection against NMDA toxicity is caused by activation of ER β receptors. To further validate this claim, we also speculated that activating ER β would increase BDNF protein levels. The ultimate goal of this project is to provide more insight into the mechanism for P4-mediated suppression of E2-mediated neuroprotection. Moreover, the discovery of such a mechanism will bolster our current understandings of the interactions between progesterone and estrogen, thereby aiding in the development of an effective HRT for postmenopausal women at risk of developing neurodegenerative diseases.

METHODOLOGY

The following materials and methods were based on those utilized in the laboratory of Claudia Aguirre and Dr. Michel Baudry at the University of Southern California (Aguirre & Baudry, 2009).

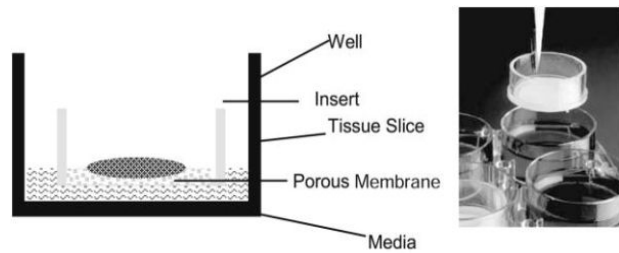
Animals

Laboratory rats were handled and treated according to the principles and procedures set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of Southern California approved all protocols performed. Timed pregnant Sprague-Dawley rats were acquired from Charles River Laboratories in Wilmington, Massachusetts and kept in the vivarium in a temperature-and light-controlled environment with a 12 h light to dark cycle. This project required the use of rat pups, ranging from 8 to 10 days old.

Preparation of cultured hippocampal slices

Organotypic hippocampal slice cultures were prepared from postnatal day 8-10 Sprague-Dawley rat pups (Stoppini *et al.*, 1991; **Figure 5**). Immediately after decapitation, brains were removed from the cranium and placed into chilled cutting medium [Earle's minimum essential medium (in

mM), HEPES, 25; Tris-base, 10; D-glucose, 10; MgCl₂, 3; pH 7.2]. The cutting medium was placed in four small Petri dishes, which were secured on a bucket of ice to keep the media chilled. After isolation of the hippocampus from each hemisphere of the brain, 400- μ m-thick transversal slices were cut using a McIlwain tissue



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Figure 5: Stoppini Method

The tissue is placed on an insert and positioned in a media-containing well without direct contact by the tissue to the media.

chopper. Slices were placed into chilled cutting medium and separated under a microscope. Six slices from the midsection of the hippocampus were then plated onto a 0.4- μ m culture plate insert (Millipore, Billerica, MA, USA) in six-well flat bottom tissue culture plates (BD Falcon, San Jose, CA, USA). Cultures were maintained in 1mL of steroid-deficient maintenance medium containing 20% heat-inactivated charcoal stripped horse serum (Sigma, St. Louis, MO, USA), Earle's balanced salt solution, Basal Medium Eagle (Sigma), (in mM): NaCl, 20; CaCl₂, 0.2; MgSO₄, 1.7; L-glutamine, 2.7; HEPES 27; NaHCO₃, 5; D-glucose, 48; ascorbic acid, 0.5; 0.5% penicillin/streptomycin; 0.01% insulin (Sigma); pH 7.4. The hippocampal slices were kept in an incubator at 35°C with 5% CO₂, and cultured for 14 days with complete medium exchange every 3 days prior to experimentation.

Treatment of cultured hippocampal slices

In order to allow for recovery from sectioning damage, cultured hippocampal slices were maintained in an incubator, *in vitro*, for 14 days prior to treatments. After those 14 days of incubation in fresh media, appropriate treatments (vehicle, hormones, agonists, and NMDA) were administered to the slices in serum-free medium for the indicated duration of time. 17- β Estradiol (E2) was diluted in dimethyl sulfoxide (DMSO) to a stock solution of 10 μ M, and was further diluted in culture medium to a final concentration of 10 nM and applied for 24 h. Progesterone (P4) was diluted in the same way as

E2 and was administered at a final concentration of 10nM. Control samples received vehicle (dimethylsulfoxide, DMSO) treatments in similar hormone-deprived conditions because the hormones were all diluted in DMSO to begin with. Using DMSO as a control was necessary to exclude the possibility that the observed effects could be due to this solubilizing compound. Estrogen receptor agonists DPN (ER β agonist) and PPT (ER α agonist) were diluted in phosphate buffered saline (PBS) to produce different concentrations. PPT was diluted to a final concentration of 1 μ M, 100 nM and 10 nM, while DPN was diluted to a final concentration of 100 nM, 10 nM and 1 nM. P4 was administered to slices at a final concentration of 10 nM for 4 h after treating slices with E2, DPN, or PPT. NMDA (50 μ M) was applied for 3 h following hormone treatments. Vehicle controls were conducted parallel to the treatments and received 0.1% DMSO in serum-free medium.

Cell viability assessment

At the end of the series of treatments, hippocampal slices were collected to determine extent of cellular death. The two methods utilized to gauge the amount of cell death were propidium-iodide uptake, using whole tissue slices, and lactate dehydrogenase release in the medium surrounding the tissue. Slices used for neuroprotection assays were washed with serum-free medium and underwent a 24-h recovery period in serum-free medium containing propidium iodide (PI; 4 μ M; Calbiochem, San

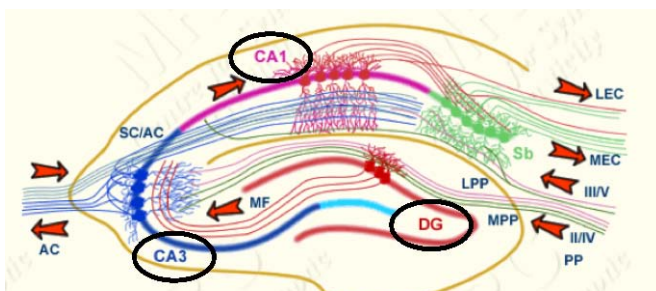


Figure 6: Hippocampus (CA1, CA3, and DG)

<http://www.bristol.ac.uk/synaptic/info/pathway/hippocampal.htm>

Diego, CA, USA). Propidium iodide is a molecule that binds to the nuclei of dead cells, and intercalates between DNA bases. Once the dye is bound to nucleic acids, the molecule fluoresces, allowing us to measure the amount of cell death. After fluorescent microscopy, the medium was collected to measure the

amount of lactate dehydrogenase (LDH) released using a spectrophotometric assay. An elevated level of the enzyme, LDH, can be used as a marker of cell membrane damage.

Quantification of the raw PI images was performed on Photoshop CS2 by manually selecting regions of interest corresponding to CA1, CA3, and dentate gyrus (DG) of the hippocampus (**Figure 6**). Pixel intensity values were then recorded on Microsoft Excel. The absolute intensity was calculated using total pixel intensity in the analyzed regions. Values were recorded as a percentage of the values found in slices treated with NMDA (50 μ M) for 3 h to induce maximal cell death.

Western Blot Protocol and Antibodies

After the treatments and cell death measurements, the slices were collected and homogenized by sonication. An aliquot of the homogenate was then taken to determine the protein concentration by the Bradford method (BioRad, Hercules, CA, USA). Samples were prepared for immunoblotting by dilution 1:1 in Laemmli buffer with 5% β -mercaptoethanol (BioRad). Twenty to forty micrograms of proteins were loaded onto 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels along with both Precision unstained and stained molecular weight markers to approximate protein molecular weights (BioRad). Following electrophoresis, proteins were electroblotted onto NitroPure nitrocellulose membranes (Osmonics, Minnetonka, MN, USA). Western blot membranes were washed with TBS-Tween (0.05%) and blocked with 5% non-fat milk. Two primary antibodies were used to detect proteins– 1:500 anti-BDNF and 1:10,000 anti- β -actin antibodies. After washing several times with TBS-Tween, secondary antibodies were used – anti-rabbit for BDNF and anti-mouse for β -actin. Immunoblots were then visualized autoradiographically using enhanced chemiluminescence.

Analysis of data

To quantify the images obtained from the propidium iodide (PI) uptake assay and Western blot film, optic density analysis was performed using Image J software. The three regions of the hippocampus quantified for the PI assay were CA1, CA3, and dentate gyrus (DG) (**Figure 6**). For the PI assay, results were reported as a percentage of NMDA-induced cell death. For the Western blot, results were reported as a percentage of vehicle control (i.e. BDNF production). The final calculations and graphs were obtained using Microsoft Excel.

RESULTS

E2-mediated neuroprotection against NMDA toxicity is due to the activation of ER β .

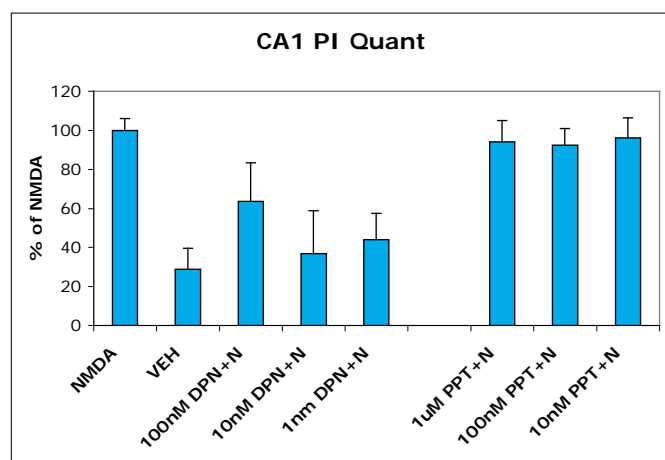


Figure 7: CA1 Propidium Iodide (PI) Quantification

The three major regions of the hippocampus are CA1, CA3 and dentate gyrus (DG) and were analyzed in the study (**Figure 6**).

Figure 7 illustrates the quantification of Propidium Iodide (PI) uptake in CA1. The results for this region of the hippocampus were the most

In order to determine whether activation of different estrogen receptors resulted in neuroprotection against NMDA toxicity, organotypic hippocampal slices were treated with each respective agonist (DPN for ER β , PPT for ER α) for 24 h before being treated with NMDA for 3 h. Analysis of PI uptake in slices treated under various conditions is shown in figures 7 through 9.

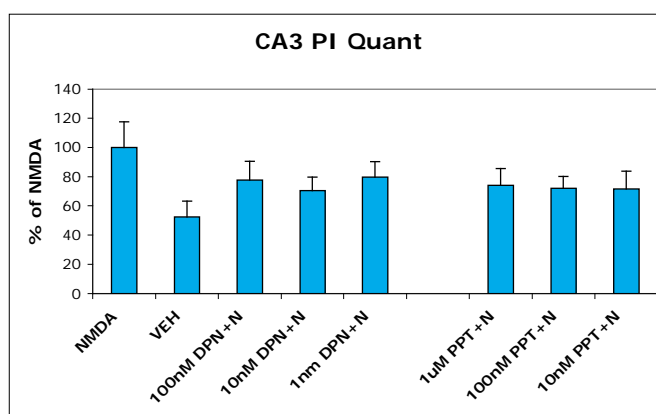


Figure 8: CA3 Propidium Iodide (PI) Quantification

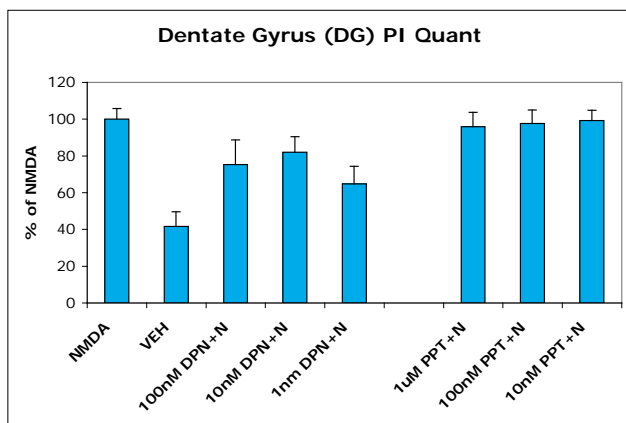


Figure 9: DG Propidium Iodide (PI) Quantification

statistically significant and robust in comparison to the other regions. In comparison to NMDA treatment, which produces maximum cell death, DPN was shown to protect slices most effectively compared to PPT. In fact, 10 nM DPN, the ER β agonist, completely prevented NMDA-mediated neurotoxicity. This illustrates that 10 nM is the optimal concentration of the

three tested – 100 nM, 10 nM, and 1 nM. **Figure 8** shows the quantification for the CA3 region. However, we cannot draw a clear conclusion from the results, since both agonists do not seem to be neuroprotective. **Figure 9** shows the quantification for the dentate gyrus (DG) region. The results are quite similar to those in Figure 6, and DPN was shown to protect slices most effectively. PPT, the ER α agonist did not provide any protection against NMDA-induced cell death. In fact, results indicate even more cell death than in NMDA-treated slices.

Activating ER β increases BDNF protein levels (Preliminary Results)

In order to further validate the role of ER β in neuroprotection, we conducted a Western Blot analysis to determine the levels of BDNF induced by each agonist. The data shown in Figure 9 are preliminary, for they are based on only one experiment. We are currently working on repeating the experiment at least two more times to be able to draw a solid conclusion. According

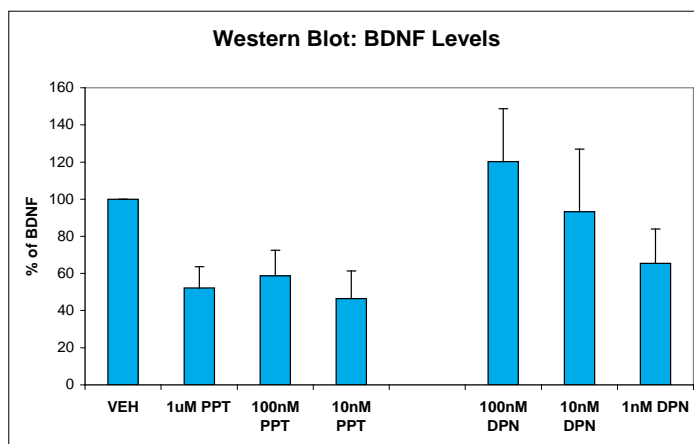


Figure 10: Western Blot: BDNF Levels (Preliminary)

to **Figure 10**, adding DPN, the ER β agonist, led to an increase in the amount of BDNF produced in a

dose-dependent manner. Specifically, 100 nM DPN seemed to increase the amount of BDNF in comparison to vehicle. However, adding PPT, the ER α agonist, seemed to decrease the production of BDNF. Once again, these results are preliminary for this dataset is based on one experiment.

P4 reverses ER β -mediated neuroprotection against NMDA toxicity

In order to determine whether P4 reverses the effects of ER α or ER β on NMDA toxicity, organotypic hippocampal slices were treated with either DPN or PPT for 24 h, and P4 was added to each respective medium during the final 4 h of the 24 h treatment period. Analysis of PI uptake in slices treated under those conditions is shown in figures 11 to 13. Similar to the previous analysis of PI uptake, the three major regions of the hippocampus (CA1, CA3, and DG) were examined. Unlike the previous PI uptake data from figures 7 to 9, only the optimal concentration was used for each respective agonist (i.e. 10 nM for DPN and 100 nM for PPT).

Figure 11 shows the quantification for the CA1 region, indicating the neuroprotective effects of 10 nM DPN in comparison to 100% cell death, produced by NMDA. Interestingly, DPN provided a complete protection against NMDA toxicity. However, when P4 was added after DPN, the neuroprotection induced by DPN was completely

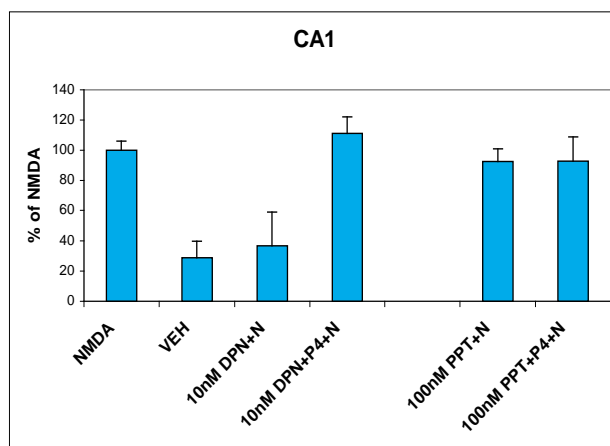


Figure 11: P4 and agonists in CA1

eliminated. When 100 nM PPT was added, not much neuroprotection was induced and adding P4 had no effect.

Figure 12 shows the quantification for the CA3 region. The results for the treatment with DPN were similar to those in the CA1 region, except that the level of

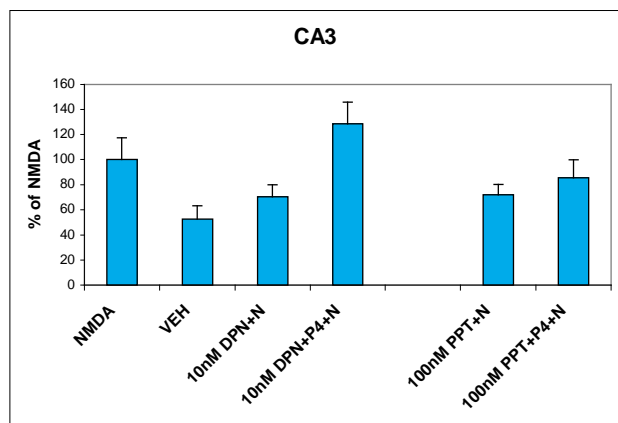


Figure 12: P4 and agonists in CA3

neuroprotection and the level of reversal of neuroprotection by progesterone were not as drastic.

However, contrary to the results from the CA1 region, 100 nM PPT was slightly neuroprotective, and P4 also slightly reduced these neuroprotective effects. **Figure 13** shows the quantification for the DG region. Treatment with 10 nM DPN produced a small amount of neuroprotection, and P4 treatment had no significant effect on DPN-induced protection. PPT treatment did not provide

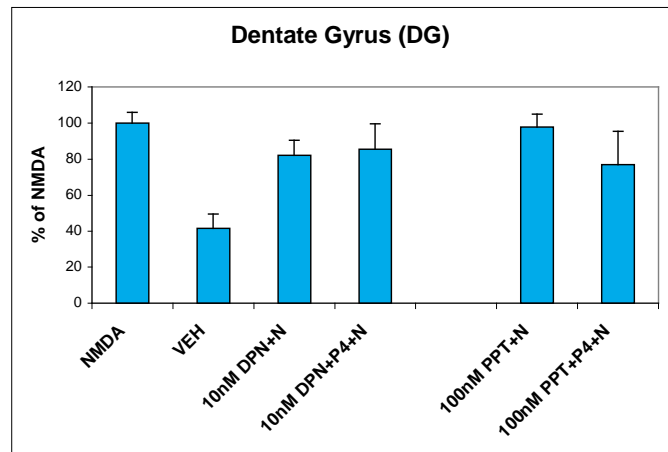


Figure 13: P4 and agonists in DG

any protection and there was no significant effect following the addition of P4.

DISCUSSION

One of the major characteristics of menopause is a precipitous decline in female gonadal hormones as well as cognitive deficits, suggesting a possible link between age-related hormonal deficiency and cognitive changes. More specifically, estrogen and progesterone (P4) have been deemed as the most prominent of the female sex hormones that become depleted during this stage in a woman's life cycle. An extensive amount of studies has shown that the two hormones are neuroprotective against a variety of insults, when administered separately. However, when these two hormones are combined, progesterone has been shown to drastically affect estrogen-mediated effects. For example, while administering P4 has no effect on spine density, co-administering it with E2 prevents E2-induced increases in spine density (Murphy & Segal, 2000). Likewise, while E2 treatment led to neuroprotection against glutamate toxicity, adding progestins to E2 led to an increase or decrease in neuroprotection, depending on which type of progestins was used (Nilsen & Brinton, 2002).

In order to investigate the mechanism underlying these phenomena, researchers have begun studying the roles of specific subtypes of estrogen receptors, such as ER α and ER β in synaptic plasticity, learning and memory and neuroprotection. While ER α has been shown to be necessary for some cognitive processes, there is a wealth of data indicating ER β as being critical as well. Since there still exist discrepancies in literature regarding the relative contribution of these receptors, we cannot rule out one or the other as being the most critical receptor regarding enhancement of learning and memory.

In our study, we utilized agonists (DPN for ER β , PPT for ER α) for the two receptors in order to discover which receptor is essential for neuroprotection. Since the hippocampus is one of the first areas of the brain to suffer damage in patients with AD and is essential for learning and memory, we chose to use organotypic hippocampal slice cultures (OHSC) as our experimental model. The OHSC model maintains the cytoarchitecture and synaptic circuitry of the *in vivo* situation, but also allows us to accessibly administer soluble drugs as treatments for a functional analysis of hippocampal properties. The three main regions (CA1, CA3, and DG) of the hippocampus were analyzed. Neuronal degeneration was produced by the neurotoxin NMDA. In hippocampus, each region responded differently to NMDA toxicity: CA1 was the most sensitive, followed by CA3, and DG (CA1>CA3>DG) (Davolio & Greenamyre, 1995). This high sensitivity in CA1 can account for the differences we observed in each region. Although results were inconsistent for CA3 and DG, results for CA1 have been consistent in clearly showing substantial modifications in cell death and neuroprotection.

The primary conclusion that can be drawn from the PI assay results obtained with ER agonists is that E2-mediated neuroprotection against NMDA toxicity is due to the activation of ER β (**Figure 7-9**). To further validate this conclusion, we used Western blot to investigate the effect of agonists on BDNF production. By analyzing BDNF levels, we are able to indirectly measure the amount of

neuroprotection induced by each respective treatment, assuming that an increase in BDNF levels results in neuroprotection. So far, we can speculate that activating ER β increases BDNF protein levels, while activating ER α may decrease BDNF protein levels (**Figure 10**). Unfortunately, because our results for the Western blots are from one experiment, it is too early to draw a solid conclusion. When we added P4 with the optimal concentrations of each respective agonist, we found that P4 reversed ER β -mediated neuroprotection against NMDA toxicity (**Figure 11-13**). Thus, we can speculate that progesterone may counteract estrogen-mediated neuroprotection by decreasing levels of ER β receptors and down-regulating ER β -induced BDNF production.

Since we understand that progesterone counteracts estrogenic neuroprotective effects under certain conditions, such as against NMDA toxicity, it is crucial to inquire deeper into the mechanism behind such a phenomenon. By doing so, we can begin to develop a safe and effective means of HRT for postmenopausal women who are prone to neurodegenerative diseases.

CONCLUSION

Based on previous studies indicating that P4 reverses E2-mediated neuroprotection against NMDA toxicity, and P4 down-regulates ER β mRNA and protein levels, this study aimed to understand the interaction between activation of ER β and estrogenic neuroprotection by using specific agonists for ER α and ER β . Our results show that activating ER β leads to neuroprotection against NMDA-induced toxicity. In addition, because it was previously shown that increased BDNF is correlated with E2-mediated neuroprotection, preliminary data suggests that activating ER β increases BDNF protein levels. This further validates the primary conclusion that ER β is indeed necessary for neuroprotection. When P4 was added following treatments with ER agonists, results from the PI assay showed that P4 reversed ER β -mediated neuroprotection against NMDA toxicity, further bolstering the role of ER β as opposed to ER α . By unraveling the mechanism underlying the interactions between estrogen and

progesterone, the primary female gonadal hormones, it is our hope that it will be possible to develop an effective hormone replacement therapy for postmenopausal women at risk for developing neurodegenerative diseases.

Abbreviations:

AD: Alzheimer Disease

α CaMKII: α -Ca²⁺/calmodulin-dependent kinase II

BDNF: Brain-derived Neurotrophic Factor

DG: Dentate Gyrus

DPN: diethylpropionitrile

E2: 17 β -estradiol

ER α : Estrogen Receptor α

ER β : Estrogen Receptor β

HRT: Hormone replacement therapy

KO: Knock Out

NMDA: N-methyl-D-aspartate

NMDAR: NMDA receptors

OHSC: Organotypic hippocampal slice cultures

P4: Progesterone

PCR: Polymerase chain reaction

PI: Propidium iodide

PPT: Propyl Pyrazole Triol

WT: Wild type

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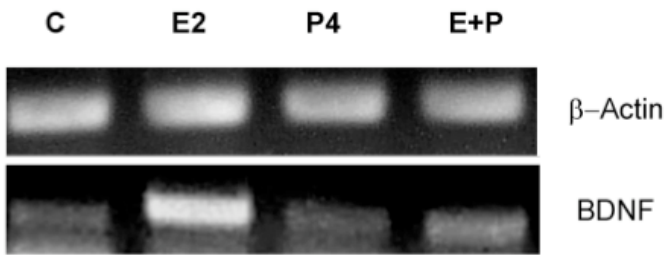
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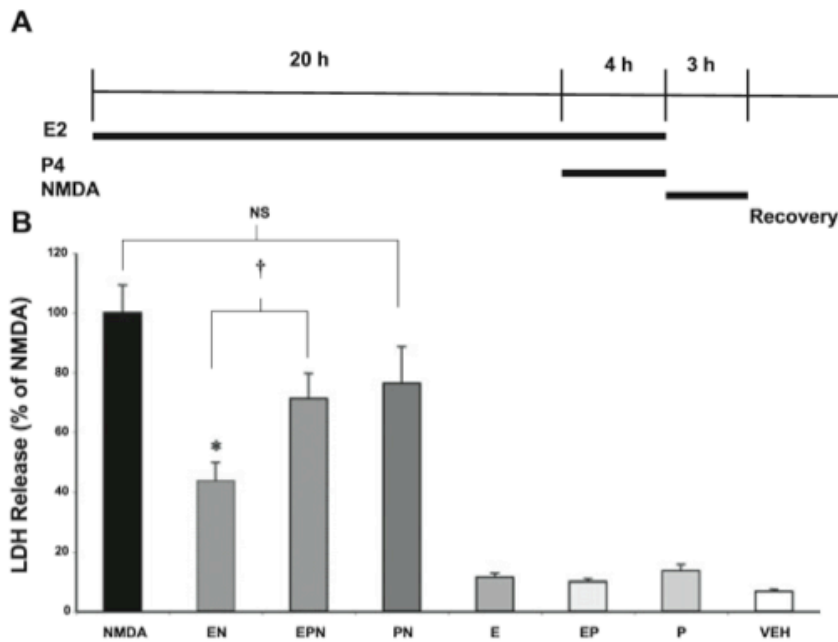
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Figure 1: Estrogen led to an increase in brain-derived neurotrophic factor (BDNF)



Aguirre, CC, Baudry, M., 2009.

Figure 2: P4 mediated reversal of E2-induced neuroprotection against NMDA toxicity in OHSC

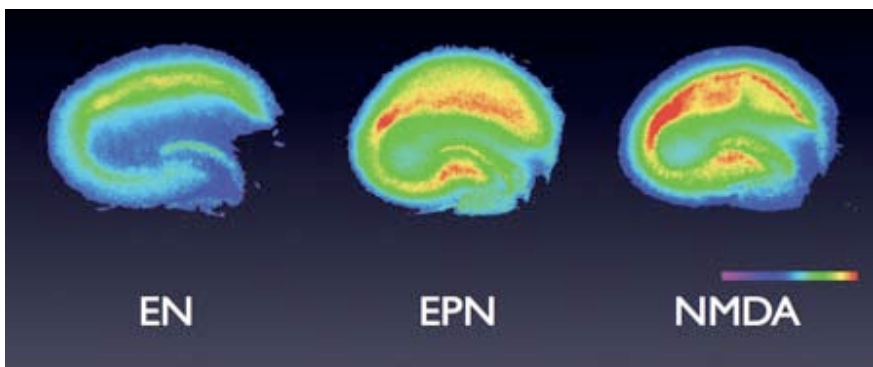


Aguirre, CC, Baudry, M., 2009.

(A) Treatment protocol timeline for hormones and NMDA.

(B) Lactate dehydrogenase (LDH) release in the medium measured 24 h after NMDA treatment. *(Aguirre & Baudry, 2009)

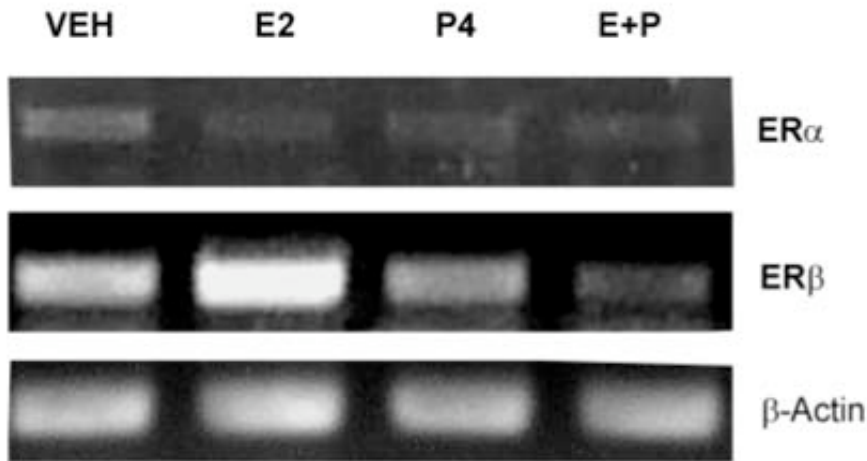
Figure 3: Pseudo-colored propidium iodide (PI) uptake data



Effects of E2 and P4 on NMDA-induced neuronal damage in cultured hippocampal slices, assessed with PI uptake: NMDA treatment resulted in increased levels of fluorescence intensity, and estrogen attenuated this increase. P4 reversed the protective effects of E2 (Aguirre & Baudry, 2009).

Aguirre, CC, Baudry, M., 2009.

Figure 4: Progesterone reverses estrogen-mediated neuroprotection against NMDA toxicity by down-regulating ER β mRNA.



Aguirre, CC, Baudry, M., 2009.

Figure 5: Stoppini method

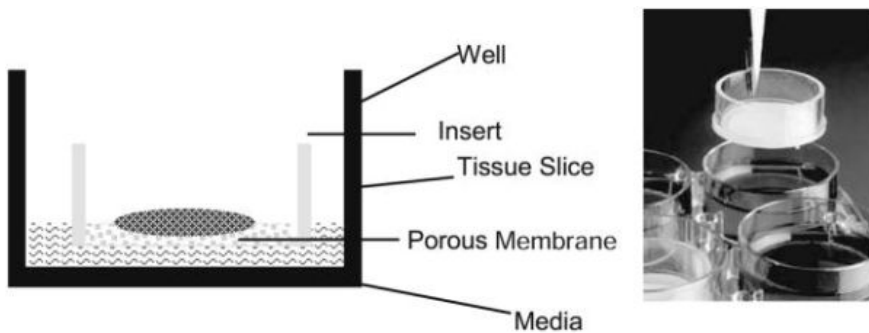
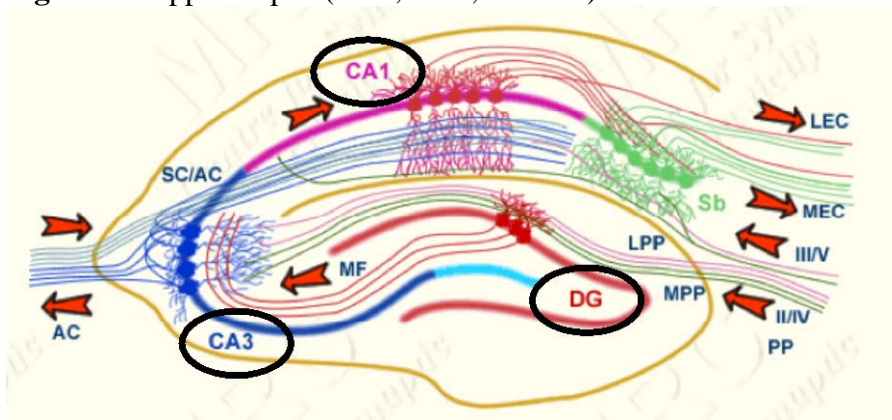


Diagram and image of the Stoppini method for culturing tissue slices. The tissue is placed on an insert and positioned in a media-containing well without direct contact by the tissue to the media.

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Figure 6: Hippocampus (CA1, CA3, and DG)



<http://www.bristol.ac.uk/synaptic/info/pathway/hippocampal.htm>

Figure 7: CA1 Propidium Iodide (PI) Quantification

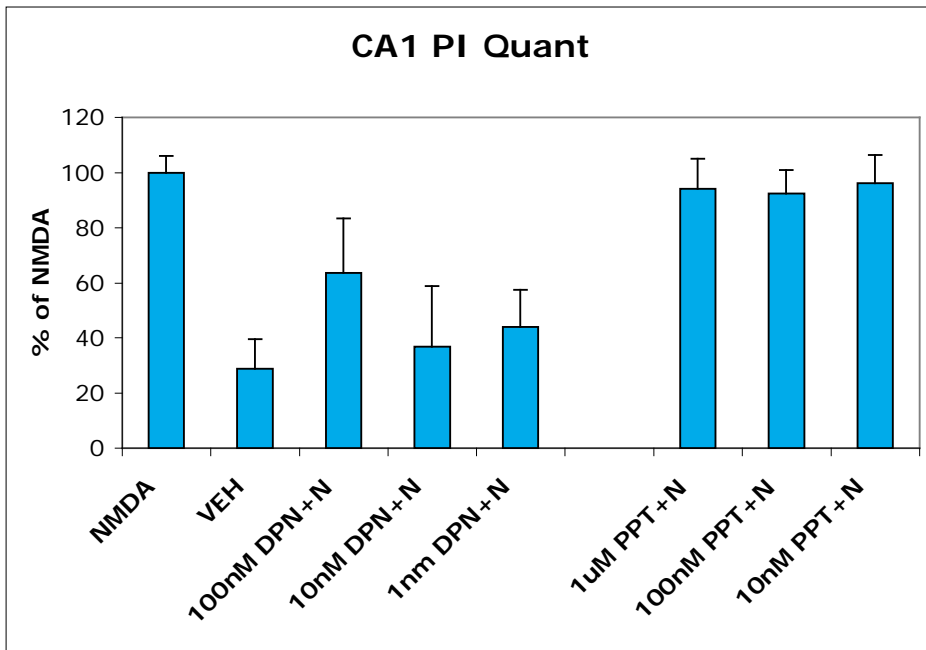


Figure 8: CA3 Propidium Iodide (PI) Quantification

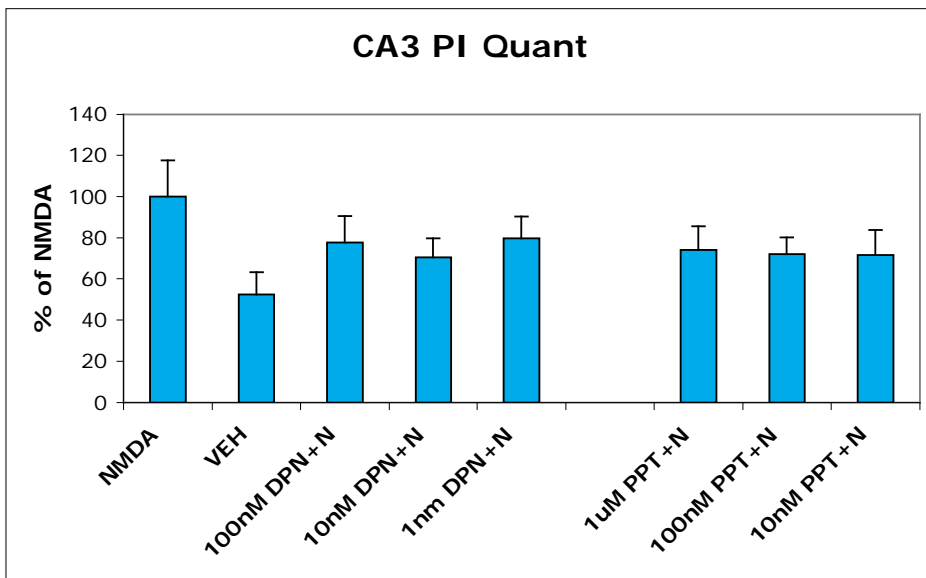


Figure 9: DG Propidium Iodide (PI) Quantification

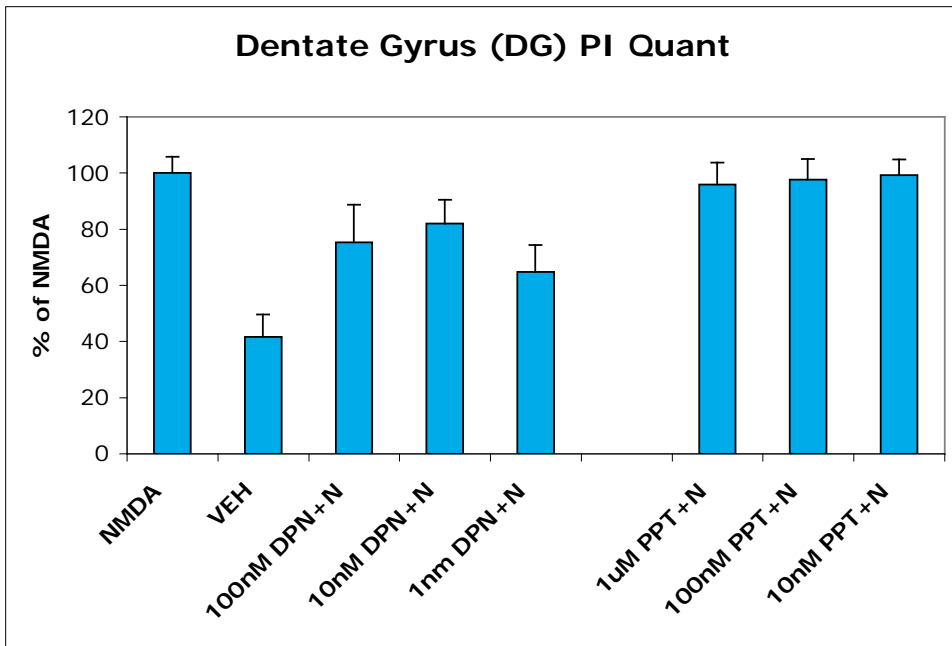


Figure 10: Western Blot: BDNF Levels (Preliminary)

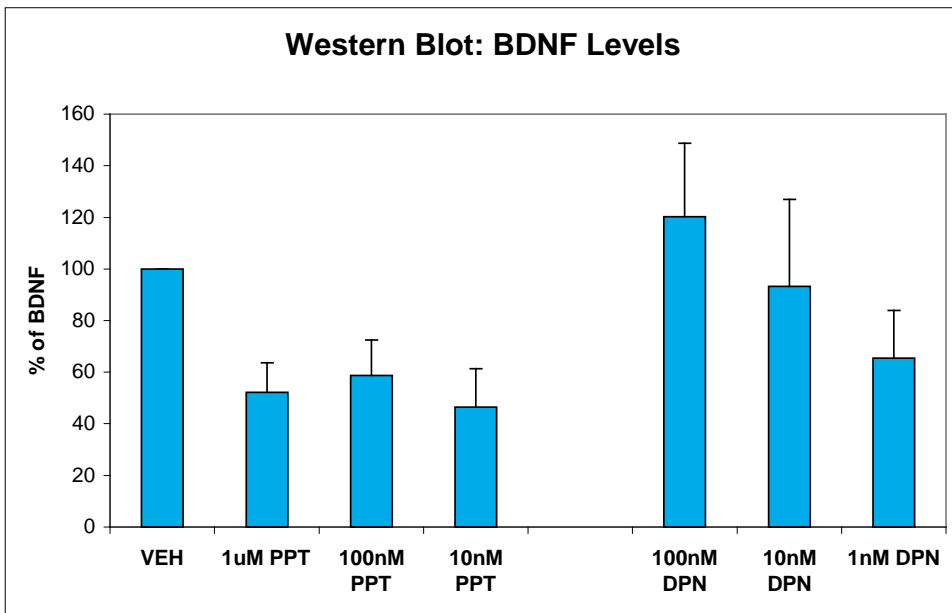


Figure 11: P4 and agonists in CA1

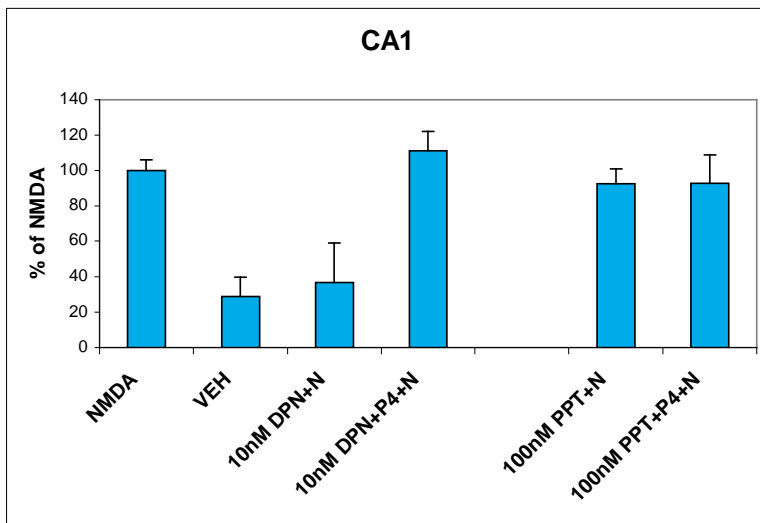


Figure 11: P4 and agonists in CA1

Figure 12: P4 and agonists in CA3

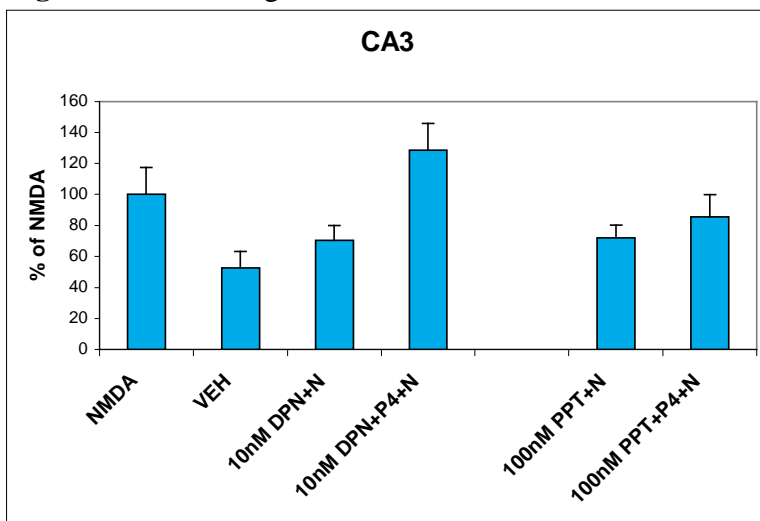


Figure 13: Progesterone and agonists in DG

Dentate Gyrus (DG)

