ABSTRACT

Kaur, Paramjit <u>Cellular and Molecular Mechanisms That Distinguish the Effects of</u> <u>Progesterone and Medroxyprogesterone Acetate on Neuroprotection.</u>, Doctor of Philosophy, (Pharmacology and Neuroscience), July, 2006, 203 pp., 5 illustrations, 20 figures, and bibliography.

Women have a higher prevalence for Alzheimer's disease (AD) than men, suggesting that the precipitous decline in gonadal hormone levels following the menopause may contribute to the risk of developing AD. However, principal results from the Women's Health Initiative concluded that women taking conjugated equine estrogens combined with medroxyprogesterone acetate (MPA, tradename: Prempro) incurred more harmful than beneficial outcomes versus the placebo group (Rossouw et al., 2002). This dissertation was aimed at determing if the discrepancy between basic science reports and these clinical studies could have been due to the synthetic progestin, MPA. I hypothesized that P4 and MPA differed in their ability to protect against the excitotoxic/oxidative insult, glutamate. Further, I proposed that this difference in neuroprotective potential would be reflected in the difference in the ability of these hormones to elicit key effectors of two neuroprotection-associated signaling pathways, the ERK/MAPK and PI3-Kinase pathways. Finally, studies were initiated to evaluate the potential importance of BDNF (brain-derived neurotrophic factor) in mediating the protective effects of P4. I used organotypic explants of the cerebral cortex, and found that both P4 and MPA elicit the phosphorylation of ERK and Akt, two signaling pathways implicated in neuroprotection, with maximal phosphorylation occurring at a

concentration of 100 nM. Interestingly, P4 protected against glutamate- induced toxicity however, while an equimolar concentration of MPA (100 nM) did not. Further, P4 resulted in an increase in BDNF, while MPA did not. Our data bring into question the relevance of using MPA as a component of hormone therapies in postmenopausal women, and instead, argue that the relevant progestin for use in treating brain-related disorders is progesterone. Collectively, the data presented here suggest that P4 is protective via multiple, and potentially related mechanisms, and importantly, its neurobiology is different from the clinically used progestin, MPA.

CELLULAR AND MOLECULAR MECHANISMS THAT DISTINGUISH THE

EFFECTS OF PROGESTERONE AND MEDROXYPROGESTERONE

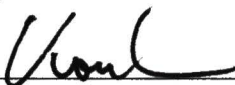# ACETATE ON NEUROPROTECTION

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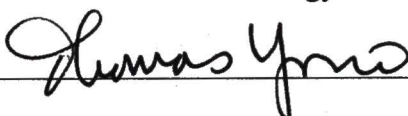
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CELLULAR AND MOLECULAR MECHANISMS THAT DISTINGUISH THE

EFFECTS OF PROGESTERONE AND MEDROXYPROGESTERONE

ACETATE ON NEUROPROTECTION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

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For the Degree of

DOCTOR OF PHILOSOPHY

By

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If I have seen farther than others, it is because I was standing on the shoulders of giants.

- Issac Newton.

During the course of my Master's work, I remember Dr. Ronald Goldfarb sitting me down in his office to say, "Pam, and this too shall pass". When I have closed my eyes in despair, I still hear the echo of his voice in my ears with his hand on my shoulders, "Pam, and this too shall pass". It was Dr. Goldfarb's strong faith in me that allowed me to put my foot forward to take the steps to achieve the goals I had set forth for myself. Thank you for believing in me, Dr. Goldfarb.

I would like to express my deepest gratitude to my advisor, Dr. Meharvan Singh, for his excellent guidance, care, patience, and providence of such a nurturing and focused environment during my Ph.D. studies. A Ph.D. to a large extent is an apprenticeship and requires a great deal of interaction between the professor and his or her student. This attainment of knowledge is so easily done when it is from someone whom one can respect not only for their intellect but also as a human being. I realized what a real scientific environment is when observing Drs. Singh and Simpkins. I am still standing in awe. It is a total honor to be in the scientific lineage of such decent people.

I would also like to acknowledge the support of all my committee members. Their critical eyes and constructive criticism helped to make this work one that I am proud of to present to others.

I would also like to acknowledge a number of people during this time in my life. Of course, there is "the family"- upon whose shoulders this work stands. And then there are my friends. I will not name each one of you – you know who you are. You put up with me, calmed me down, put a smile on my face, ate Szechuan's with me, and foremost inspired me to do more.

My dearest daughter – Sajal. I know that you will only understand when you are older of why I made the sacrifices with you to attain this. Thank you for motivating me to be the best that I could be my love.

I foremost would like to thank God. I look back and am in surprise of what "I" have achieved. For I only know that the Lord has carried me through this journey. Before I perceived I could do no more, He sent someone to literally carry me to my goals. It was only through His hand that I was allowed to achieve this. "Lakh khusee-aa paatisaahee-aa jay satgur nadir karay-i" translated hundreds of thousands of princely pleasures are enjoyed, if the true Guru bestows His glance of grace.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AF	Activation function
Akt	Protein Kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenosine triphosphate
BAD	BCL- associated death promoter
BCL-2	B-Cell Leukemia/Lymphoma - 2
BDNF	Brain derived neurotrophic factor
BFF	Basic Final Feed
Ca^{2+}	Calcium
CCI	Cortical contusion injury
CDK2	CyclinC-dependent kinase 2
CEE	Conjugated Equine Estrogens
CHD	Coronary Heart Disease
CNS	Central Nervous System
C_T	Critical threshold cycle
5-DHP	5-alpha Dihydroprogesterone
DMSO	Dimethylsulfoxide
E2	17-β Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay

ERK	Extracellular Signal-Regulated Kinases
Gey's BSS	Gey's Balanced Salt Solution
GFAP	Glial fibrillary acidic protein
GSK	Glycogen synthase kinase
HDS-R	Revised Hasegawa's Dementia Scale
HRT	Hormone Replacement Therapy
Hsp	Heat Shock Protein
HT	Hormone Therapy
LY294002	2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
MFC	Medial Frontal Cortex
MMS	Mini- mental state
MPA	Medroxyprogesterone acetate
mPR	Membrane Progesterone Receptor
mRNA	Messenger RNA
NES	Nuclear Export Signal
NFκ-B	Nuclear Factor Kappa B
NMDA	N-methyl-D-asparate
NSD	New screening test for Dementia
P3	Postnatal Day 3

P4	Progesterone (4-pregnen-3, 20 - dione)
P75 NTR	p75 Neurotrophin Receptor
PARP	Poly (ADP) polymerase
PBR	Peripheral Type Benzodiazepine Receptor
PI-3K	Phosphoinositide-3 kinase
PP2A	Protein phosphatase - 2A
PR	Progesterone Receptor
PRα	Progesterone Receptor - A
PRβ	Progesterone Receptor - B
PRE	Progesterone Response Element
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription polymerase chain reaction
RU486	Mifepristone
StAR	Steroidogenic Acute Regulatory Protein
TBI	Traumatic Brain Injury
TBS-T	Tris-Buffered Saline Tween - 20
3α, 5α-THP	3 - alpha Hydroxyl 5-alpha-Pregan 20 - one
UO126	1,4 – diamino - 2,3 – dicyano - 1,4 – bis [2 - aminophenyalthio] butadiene
WHI	Women's Health Initiative
WHIMS	Women's Health Initiative Memory Study
Δ	Change

PREFACE

Considerable evidence now exists, from *in vitro* and *in vivo* studies, describing the neuroprotective effects of estrogen (E2) and supports the potential therapeutic merit for estrogen in staving off cellular dysfunction seen with age and age-associated diseases like Alzheimer's disease. More recently, we and others have begun investigating the potential role of another major ovarian steroid hormone, progesterone (P4), in cytoprotection. In fact, P4 can reduce vulnerability of cells to toxic insult. Given that both estrogen and P4 have been shown to be protective in various *in vitro* and *in vivo* model systems, it came as quite a surprise that the results of the Women's Health Initiative Memory Study (WHIMS) revealed a negative influence of hormone therapy on markers of cognitive function and dementia. Among the proposed explanations for this discrepancy included the possibility that the progestin used may have antagonized estrogen's beneficial actions. We have modified this argument by suggesting that it is the specific type of progestin used (medroxyprogesterone acetate) that may be at fault, and that this progestin, though effective at reducing the uterotrophic effects of unopposed estrogen, may not be beneficial for the brain. As such, this dissertation was aimed at distinguishing the mechanisms by which medroxyprogesterone acetate (MPA) and the natural progestin, P4, elicited their protective actions. We hypothesized that P4 and MPA differed in their capacity to activate signal transduction pathways, which in turn, reflected their relative differences in modulating cellular vulnerability. This hypothesis was evaluated within the confines of the following specific aims:

1. **Characterization and comparison of progesterone (P4) - and medroxyprogesterone acetate (MPA) - induced MAPK and the PI-3K pathways.** The temporal pattern of ERK and Akt, key effectors of the MAPK and PI-3K pathways, respectively, by P4 and MPA was assessed in organotypic explants of the cerebral cortex. Concentration-response curves for MPA and P4 were also assessed at the time point of maximal phosphorylation of ERK and Akt.

2. **Evaluation of progesterone and MPA's ability to protect against glutamate toxicity.** Using the glutamate model of cytotoxicity, we evaluated if progesterone and MPA differed in their ability to reduce cell damage/death. Assessment of this cellular damage was accomplished by measuring lactate dehydrogenase (LDH) release. Concentration-response curves were generated in addition to defining the duration of hormone pre-treatment that was optimal for promoting cytoprotection.

3. **Determination of whether cytoprotection by progesterone against L-glutamate cytotoxicity is dependent upon the MAPK/ PI-3K pathway.** Once the optimal duration and concentration of P4 was determined with respect to its effects on signaling (Aim 1) and protection (Aim 2), we evaluated whether the effect of P4 on signaling (Aim 1) was required for its effects on protection (Aim 2). This was accomplished with the aid of pharmacological inhibitors of the MAPK and/or PI-3K pathways, respectively to determine if P4 could still protect in the presence of these inhibitors.

CHAPTER I

INTRODUCTION

Women's Health Initiative

Menopause, or cessation of the menses, is characterized by a precipitous decline in the levels of circulating ovarian hormones (such as estrogen and progesterone). Given that the average age of the menopause is approximately 54 years, and that the average lifespan of women is now approximately 80 years of age (Arias and Smith, 2003), a substantial portion of a woman's lifetime is spent in a hormone-deprived state. Women over the age of 65 have a two to three time higher prevalence of Alzheimer's disease (AD) than men (Henderson, 1997; Gao et al., 1998; Andersen et al., 1999; Sherwin, 1999). Since AD is diagnosed in women typically after the menopause, it has been postulated that hormone deficits may increase the vulnerability of these women to the development of neurodegenerative diseases.

In order to alleviate the symptoms of the menopause, clinicians have widely prescribed hormone therapy; an estimated 37.6% of all post-menopausal women in the United States used hormone therapy in 1999 (Keating et al., 1999). Basic research in this area also supports the beneficial effects of estrogen replacement against a wide variety of injuries in the brain (Wise, 2002; Simpkins et al., 2004; Simpkins et al., 2005). However recently, principal results from the Women's Health Initiative concluded that women taking conjugated equine estrogens combined with medroxyprogesterone acetate (MPA,

tradename: Prempro) incurred more harmful than beneficial outcomes versus the placebo group (Rossouw et al., 2002). In fact, they concluded that women in the treatment group experienced an increased risk of invasive breast cancer, coronary heart disease (CHD) including myocardial infarction, stroke, pulmonary emboli, and deep vein thrombosis. This data, in addition to other trials, was the basis for the American Heart Association's recommendations against the initiation, and for the discontinuation, of postmenopausal hormone treatments in menopausal women for the prevention of cardiovascular disease.

More recently, three additional studies were published from the Women's Health Initiative (WHI) investigating the effect of treatment with 0.625 mg of conjugated estrogen and 2.5 mg of MPA on central nervous system (CNS) outcomes (Shumaker et al., 2003; Espeland et al., 2004; Shumaker et al., 2004). Overall, they concluded that women who took hormones for an average of more than four years had double the risk of developing Alzheimer's disease (AD) or other forms of dementia, compared with those on placebo.

As a result of these studies, the prescribing patterns of hormone therapy have drastically changed. In 2004, there was a 46% and 28% decline in the initiation of estrogen therapy and hormone therapy, respectively (Buist et al., 2004).

The discrepancies between the beneficial effects of hormone therapies seen in basic research studies and earlier clinical trials, and the more recent WHI studies, may be attributed to many aspects of the trial. The women enrolled in the WHI trials had a mean age of 63 and had no prior history of HT use; given that the average age of the

menopause is approximately 54 years, these women were hormone deficient for approximately a decade. In addition, this trial only tested one drug regimen, conjugated equine estrogens (0.625mg/d) plus MPA (2.5 mg/d), in postmenopausal women with an intact uterus. These results did not test the lower dosages of these drugs and other formulations of either oral or transdermal estrogens and progestins. Further, these reports also stated within the limitations section of the discussion that the trial could not distinguish the effects of estrogen from those of the progestin (Shumaker et al., 2003; Espeland et al., 2004; Shumaker et al., 2004).

As an important consideration with regard to the failure of the WHI studies to reveal beneficial effects with hormone therapy, I hypothesize that the choice of progestin included in this study was a major flaw resulting in the disparity between the basic science and clinical trials. Therefore, in order to help resolve this discrepancy, I addressed the mechanistic differences between the natural P4 and the synthetic progestin MPA, with particular emphasis on protection-related signal transduction cascades, particularly the MAPK and PI-3K/ Akt pathways. This analysis was done in the cerebral cortex, an important target of gonadal steroid hormones, and also a principal region affected by neurodegenerative disorders, including Alzheimer's disease (AD).

Progesterone

Steroid hormones found in the periphery are produced either in the gonads (the ovary (corpus luteum in the female and testes in the male), or the adrenal cortex. Males

secrete 1-5 mg of progesterone daily resulting in plasma levels of 0.03 μg/dL. Progesterone levels are only slightly higher in the female during the follicular phase of the menstrual cycle (0.10 μg/dL). However, during the luteal phase of the menstrual cycle, progesterone levels increase by 10 fold (Porterfield, 2001). The half life of progesterone in the plasma is approximately 12 hours where it is found to be bound mainly to albumin and to a lesser extent cortisol - binding globulin (transcortin). With regards to pharmacokinetics, there is a wide variation in the absorption and bioavailability of progesterone in individuals (de Lignieres et al., 1995; Fotherby, 1996). Oral administration of progesterone results in an extensive first-pass effect of metabolism by the liver whereupon it is converted to pregnanediol and pregnanolones (Schindler et al., 2003). These metabolites have also been demonstrated to exert neuroprotective effects (Hoffman et al., 2003; Ciriza et al., 2004; Ciriza et al., 2006).

Progesterone, the Neuroactive Steroid

As one is doing research in the area of ovarian hormones and their effects on the brain, it is also important to recognize that the brain itself has the machinery and ability to produce steroids itself (Le Goascogne et al., 1987; Schumacher et al., 2003; Garcia-Ovejero et al., 2005). For example, though the source of steroid hormones - estrogen, progesterone and testosterone – has generally been thought to originate only in the gonads, numerous reports have shown that the brain expresses biosynthetic enzymes that can synthesize steroids *de novo* from cholesterol (Baulieu, 1991; Mellon et al., 2001;

Sinchak et al., 2003; Tsutsui et al., 2003). The biosynthetic pathway of steroids from cholesterol in the brain is depicted in Illustration 4, Appendix. Equal amounts of the biosynthetic enzymes are not present throughout the brain and instead are distributed in a region specific manner as summarized in Illustration 4, Appendix. Due to the localization of enzymes to specific areas of the brain, research has been furthered to define the different neurosteroids produced preferentially by differing cell types (Sinchak et al., 2003). The neurosteroidogenic pathway specific to each cell type in the brain is summarized schematically in Figure 1. The heterogenous distribution of the biosynthetic enzymes and steroidal receptors in the brain are in part responsible for the specificity of effects demonstrated by neurosteroids (Zwain and Yen, 1999; Mellon and Griffin, 2002). As evidenced by the literature, gonadal biosynthetic enzymes and gonadal hormone receptors are also modulated by the fluctuations of peripheral steroids over the estrous cycle in a region specific manner in the brain (Jung-Testas et al., 1991; Guerra-Araiza et al., 2003). As steroids decrease within the periphery, the brain, perhaps as a compensatory mechanism, may regulate their synthesis locally. For example, Bixo *et al.* described that brain concentrations of gonadal steroid hormones were elevated following the menopause, despite a decline in peripheral, circulating hormones, suggesting that something to produce this elevation of hormones had occurred - either accumulation or *de novo* synthesis (Bixo et al., 1997).

The brain may also produce steroids *de novo* as a compensatory response to insult. Not only are the steroids themselves regulated but so are their steroidogenic

machinery. The steroidogenic proteins peripheral-type benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR) are up-regulated in the nervous system after injury (Sierra et al., 2003). PBR and StAR protein allow the passage of cholesterol from the external to the internal mitochondrial membrane making cholesterol available to the first enzyme of the steroidogenic pathway, p450 side-chain cleavage (p450 scc), which transforms cholesterol into pregnenolone, the precursor for glucocorticoids, mineralocorticoids, and sex steroids. Thus as a natural consequence to the upregulation of the steroidogenic enzymes, there is also a marked increase in the neurosteroids themselves (Sierra et al., 2003).

StAR undergoes marked changes with development, aging and injury (Sierra, 2004; Lavaque et al., 2006). PBR is an enzyme more predominantly found in the glia whereas StAR is localized to neurons. Glia surrounding a traumatic brain lesion , but not in the focal site, has been shown to produce the steroids pregnenolone, pregnenolone - sulfate, P4 and its reduced metabolites 3alpha-hydroxy-5alpha-pregnan-20-one ($3\alpha5\alpha$- THP, allopregnanolone) and 5alpha-dihydroprogesterone (5α-DHP) (di Michele et al., 2000). The perifocal area has glia hyperplasia and the increase in neurosteroids observed is attributed to the activation of these cells. In addition, steroidogenesis in neurodegenerative conditions are altered (Garcia-Ovejero et al., 2005). StAR is upregulated in response to excitotoxicity in the hippocampus and cerebral cortex and in aged rats (Sierra et al., 2003) perhaps in a compensatory manner to save the area from damage.

Another important aspect of brain neurosteroidogenesis is that different cell types in the cortex express differing types of steroidogenic enzymes involved in neurosteroidogenesis (Zwain and Yen, 1999). As a consequence, the production of one hormone by one cell type may influence an adjacent cell (paracrine mechanism) to produce yet another steroid hormone. This possibility is supported by the work of Sinchak *et al.* who demonstrated that estrogen induces *de novo* synthesis of P4 in astrocytes (Sinchak et al., 2003). By inference, this also argues that in addition to the compensatory upregulation of neurosteroidogenesis described in the paragraphs above, age-dependent reductions in ovarian function (i.e., after the menopause) that result in reduced circulating hormone levels, may modulate the levels of hormones produced locally in the brain.

Mechanisms of Progesterone (P4) Action in the Brain

Classically, the lipophilic nature of P4 has been thought to enable its translocationion across the plasma membrane, where it can then bind to the intracellular P4 receptors. These receptors act as nuclear transcription factors and bind to P4 response elements (PRE) within the promoter region of target genes to regulate transcription. In addition, P4 can elicit its effects in the cell through the ability to recruit signal transduction systems including the MAPK and PI-3K/Akt pathway. It is still unclear, however, whether the receptor that mediates the effects of P4 on gene transcription and cell signaling are identical. Given that novel receptors for P4 have recently been identify,

it is indeed possible that the "classical" effects of P4 and the non-genomic effects of P4 may be mediated by different receptor mechanisms. This is the subject of ongoing analysis in the Singh laboratory.

Receptors for Progesterone

The Classical Progesterone Receptor (PR)

The PR exists as either an A- (97 kDA) or B- (120 kDa) isoform created from the same gene by the use of two different promoters. Each isoform contains a C-terminal hormone-binding domain (HBD), a DNA binding domain (DBD), a hinge region (H), and at least two transcriptional activation function (AF) domains, located within the HBD (AF-1) and N-terminus (AF-2); PR-B contains an additional AF (AF-3) within the unique 164 amino-acid B-upstream segment (Figure 2). After ligand binding in reproductive and brain tissues, the progesterone receptor (PR) undergoes rapid down-regulation of its expression (Nardulli and Katzenellenbogen, 1988; Savouret et al., 1989). In the presence of estrogen, however, the rate at which the progesterone receptor is produced is increased and the degradation remains unaltered (Nardulli et al., 1988). This occurs in areas such as the hypothalamus and limbic structures. However, in the cerebral cortex, septum, and cerebellum, estrogen stimulation does not appear to affect PR expression (MacLusky and McEwen, 1978).

In the absence of hormone, the PR is associated with several chaperone molecules including heat shock protein (Hsp) 90, Hsp70, Hsp40, Hop and p23; these interactions are

required for proper protein folding and assembly of stable steroid receptor - Hsp90 heterocomplexes that are competent to bind ligand (Pratt, 1998). Upon exposure to P4, the ligand-activated receptor undergoes a conformational change, dissociates from Hsps, dimerizes, and directly interacts with specific P4 response elements (PREs) in the promoter regions of target genes, including *c-myc , fatty acid synthetase*, and the *MMTV* promoter. There are three configurations of the receptor resulting from dimerization: either homodimers: A-A, B-B, or the heterodimer A-B. The dimer complex binds to hormone responsive elements on DNA at the promoter regions of target genes (Lange, 2004).

The two isoforms of the classical receptor, PR-A and PR-B, have been demonstrated to have different functional properties. PR-A is a trans-dominant repressor of PR-B (Vegeto et al., 1993). The repressor function is cell and target gene specific. The isoform expression is dependent upon the developmental and hormonal levels in the brain. PR-A thus can diminish the P4 responsiveness specific to target genes. This ability of PR-A is not only inhibitory towards PR-B but also towards the estrogen, glucocorticoid, mineralocorticoid receptor-dependent gene activation (Ozawa, 2005).

Like other steroid hormone receptor family members, PR isoforms are heavily phosphorylated by multiple protein kinases; phosphorylation occurs primarily on serine residues throughout each molecule, but is concentrated within the amino-termini. The progesterone receptor contains a total of 14 known phosphorylation sites. Serines at positions 81, 162, 190 and 400 are defined as "basal" sites constitutively phosphorylated

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in the absence of hormone (Zhang et al., 1997). Serines 102, 294 and 345 are sites maximally phosphorylated 1-2 hours following progestin treatment (Zhang et al., 1995). Kinases responsible for the phosphorylation of selected sites have also been identified. For example, the serines at positions 81 and 294 have been demonstrated to be phosphorylated by casein kinase II and mitogen-activated protein kinase (MAPK), respectively; progestins can also stimulate Ser294 phosphorylation independently of MAPKs by activation of an unknown kinase(s). Eight of the total 14 sites (Serines 25, 162, 190, 213, 400, Thr430, 554, 676) have been demonstrated to be phosphorylated by cyclin A/cyclin-dependent protein kinase 2 (CDK2) complexes *in vitro* (Zhang et al., 1997). Five of these sites (Serines 162, 190, 213, 400, 676) have been confirmed as authentic *in vivo* phosphorylation sites (Zhang et al., 1997).

Sex differences in the regulation of the isoforms of the progesterone receptor expression in the rat brain have been reported (Guerra-Araiza et al., 2002; Quadros et al., 2002). For example, higher levels of progesterone receptor immunoreactivity in the preoptic area of males were observed when compared to females at around birth (Quadros et al., 2002). Although this is not the area emphasized within my body of work, it demonstrates that sex differences in progesterone responsivity could occur and exist as early as the time of birth, and could explain, in part, the sex difference I observed with respect to P4's ability to protect against glutamate-toxicity.

The effects of E_2 and P4 on progesterone isoforms have also been studied in ovariectomized females and gonadectomized male rats (Guerra-Araiza et al., 2002). Both

PR isoforms were induced by E2 and downregulated by P4 in the hypothalamus in female rats. In the hippocampus of females, PR-A was induced by E2, whereas P4 had no effects. In the cerebellum and the frontal cortex, however, neither E2 nor P4 modified PR expression. However in the male rat population studied, only the cerebellum was modified by treatments, where PR-A was induced by E2.

At birth, males express a higher level of progesterone receptor immunoreactivity than females in the preoptic area (Quadros et al., 2002). In the cortex, however, there appeared to be no statistically significant difference in the amount of PR mRNA relative to that seen in males, although an age-dependent increase was noted. PR-B mRNA was predominant in 2 day-old female rats and these levels increased through day 8 within the cortex. There was a slight decrease in the amount of mRNA at day 10 (Kato et al., 1993). Interestingly, despite the absence of a statistical difference in basal expression of the PR, P4 treatment resulted in a greater accumulation of PR in females than males (Kato et al., 1984). These studies clearly demonstrate a sexual dimorphism of the progesterone receptor within the rat brain.

Membrane-Associated Progesterone Receptors

Differential binding of P4 to various proteins within the cell produces several diverse physiological effects. P4 has been documented to interact with the classical steroid receptors PR-A, PR-B, and more recently the membrane associated progesterone receptors, mPR, and 25-Dx.

Iodinated P4 that was conjugated to bovine serum albumin (BSA), a membrane impermeable radioligand, was observed to bind to several regions within the brain (cerebral cortex, brainstem, cerebellum, corpus striatum, and hypothalamus) suggesting the presence of a membrane P4 binding sites in various parts of the brain. In contrast, there was little or no binding of this compound in the uterus, ovary, liver, and spleen (Ke and Ramirez, 1990). Upon further characterization of the P4 binding protein, the protein was described as 28-kDa protein in mouse (Meyer et al., 1996) and its rat analogue was termed 25-Dx (Krebs et al., 2000; Sakamoto et al., 2004). 25-Dx is up-regulated in neurons and astrocytes after TBI (Meffre et al., 2005). Using immunohistochemical analysis, 25-Dx was found to be abundant in areas of the brain important in CSF production and in osmoregulation. Double immunofluorescence also demonstrated that 25-Dx was co-expressed with vasopressin. The relative levels of 25- Dx were higher in pseudopregnant females (which had higher levels of endogenous P4) as compared to males (Meffre et al., 2005). Also, the same pseudopregnant females had better functional outcomes and less edema formation after traumatic injury. In male rats after complete spinal cord transection, levels of PR were significantly decreased whereas 25-Dx levels remain unchanged compared to controls. When spinal cord injury animals were treated for 72 hours with P4, PR levels were unaffected whereas the 25-Dx mRNA levels were significantly increased. The PR was also localized in this study to both neurons and glia whereas the 25-Dx were localized to membranes of neurons (Labombarda et al., 2003). The ability of progesterone to protect against CNS injury and the observation of a

concurrent increase of the binding protein prior to exposure to progesterone, particularly the 25-Dx receptor, suggests a potential role of this receptor in protection in response to injurious events.

RU486 (Mifepristone), a PR antagonist, protected Purkinje cells from developmental cell death (Ghoumari et al., 2003) in mice lacking either the PR or the glucocorticoid receptor (GR). As such, it was postulated that RU486's protective effects in PR knockout mice, could have been mediated by 25-Dx (Sakamoto et al., 2004), or some other membrane associated progesterone receptor, such as the mPR (see below). Subsequent reports which confirmed the expression of 25-Dx in Purkinje cells further supported the possibility that RU486 may exert its protective actions via a membrane receptor. Interestingly, Singh demonstrated that RU486 was able to increase the phosphorylation of Akt and yet not alter the phosphorylation of ERK even in the absence of P4 (Singh, 2001). Therefore, a plausible mechanism for this observation is that RU486 is an agonist of 25-Dx membrane progesterone receptor.

As alluded to in the preceding paragraph, other membrane progesterone receptors have also been described. For example, a membrane-associated PR was recently cloned and found to exist in human, mouse and teleost, the latter being the first species in which this membrane progesterone receptor was identified. (Zhu et al., 2003; Zhu et al., 2003). Interestingly, unlike 25-Dx which, based on hydrophobicity plots (Kyte-Doolittle), is believed to have only a single transmembrane domain, the mPR has seven, and exhibits characteristics that would suggest that the mPR is a G-protein coupled receptor.

Changes in Progesterone Receptors Across the Menstrual/Estrous Cycle

During the estrous cycle of the rat, progesterone receptor isoforms are expressed differentially. The PR-B is the predominant isoform in the hypothalamus and the frontal cerebral cortex. Both of the isoforms are expressed similarly in the hippocampus. The highest amount of PR-B is found on proestrous in the hypothalamus and metaestrus in the preoptic area and diestrus in the frontal cortex. No changes in the isoforms with the menstrual cycle were observed in the hippocampus (Guerra-Araiza et al., 2000). While these data are consistent with the synaptic remodeling that is known to take place even within the small time frame, such as over the course of the menstrual (or estrous) cycle, it is possible that more permanent changes in brain structure might occur when hormone levels are altered for longer periods of time, such as in post-menopausal women, or in chronically ovariectomized rodents, or in the reproductively senescent animal. Thus, the failure of the WHI to show a beneficial effect of hormone treatment in older post-menopausal women may be related to significant alterations in brain structure, consequent to long term hormone deprivation, that in turn, does not favor the protective effects of progesterone (or estrogen) during the perimenopause.

Link between hormone decline and disease pathology

The precipitous decline in hormone levels associated with the menopause is correlated with an increased incidence of many disease processes including:

cardiovascular disease, osteoporosis, and dementia, particularly that caused by Alzheimer's disease (AD) (Watson et al., 1996; Moffat et al., 2004; Rosario et al., 2004). This correlation suggests that the decrease in the levels of gonadal hormones following the menopause predisposes women to AD and other disease pathologies. However, not all post-menopausal women develop AD. This may not be surprising given that the loss of gonadal hormones is a risk factor, and not a causative factor in the development of AD. Still, even the risk for developing AD may be different among post-menopausal women. This could be attributed to the differences in "residual" hormone levels (due to local brain production or adrenal production of hormones) such that those with lower levels may be at higher risk for developing neurodegenerative diseases. This premise is supported by the work of Weill-Engerer *et al.* 2002 who illustrated that relatively lower levels of basal (residual) steroid hormone levels exist in the AD brain relative to age-matched controls. Using gas chromatography- mass spectrometry and high pressure liquid chromatography, a general trend toward decreased levels of all steroids, including P4, was observed in all of the AD patients' six brain regions as compared with controls (Weill-Engerer et al., 2002). Weill-Engerer and colleagues also observed that the abundance of paired helical filaments of the tau protein were significantly and negatively correlated with dehydroepiandrosterone levels (another protective hormone) in the hypothalamus (Weill-Engerer et al., 2002). Additionally, levels of cortical Aβ were significantly and negatively correlated to pregnenolone sulfate levels and dehydroepiandrosterone levels in striatum and cerebellum (Weill-Engerer et al., 2002).

In yet another study performed in 2005 by Tsolaki, M. *et al.,* a statistically significant decrease in the amount of estradiol in Alzheimer's disease patients was found when compared to non-demented control patients, as measured by radioimmunoassay. No differences were seen in other steroids using the RIA technique however the sensitivity of gas chromatography-mass spectrometry with high pressure liquid chromatography allows for greater sensitivity of detection (in the femtomolar range) and also has the ability to discriminate free versus conjugated steroids, as done by Weill-Engerer (Schumacher et al., 2003). This methodological difference could thus explain the inability of prior studies to distinguish the differences between steroidal levels in AD patients when compared to age-matched controls.

Progesterone and Protection

As stated in previous sections, P4 has been demonstrated to be protective in various models (Goodman et al., 1996; Nilsen and Brinton, 2002; Nilsen and Brinton, 2002; Nilsen and Brinton, 2003). For example, P4 (100 nM – 10 μM) significantly attenuated glutamate and glucose deprivation–induced toxicity. P4 was also effective against $FeSO_4$ toxicity, and amyloid β-peptide toxicity in primary hippocampal cultures (Goodman et al., 1996).

Hall et al. (1991) observed that necrosis due to ischemia was less pronounced in females when compared to male gerbils (Hall et al., 1991). This observation prompted the authors to infer that the feminizing hormones played a role in the protection against

ischemia. P4 also enhanced recovery after stroke and spinal cord injury. Jiang *et al.* illustrated that the administration of P4 before middle cerebral artery occlusion (MCAO) resulted in a marked reduction in cerebral infarction and reduced impairments that resulted from the occlusion (Jiang et al., 1996). Others have demonstrated that post-ischemic administration of P4 was also effective in reducing the lesion size, and impairments resulting from the ischemia (Kumon et al., 2000; Morali et al., 2005). Another group demonstrated the effectiveness of P4 in the post-ischemic model on a variety of functional measures, including the rotarod test, adhesive-backed somatosensory and neurological scores (Chen et al., 1999).

P4 has also been demonstrated to be effective in the treatment of traumatic brain injury (TBI). The administration of P4 has been able to reduce cerebral edema for up to 24 hours after injury. In a rodent model of medial frontal cortex (MFC) impact injury, P4 reduced complement factor C3, glial fibrillary acidic protein (GFAP), and nuclear factor kappa beta (NFκB) (Pettus et al., 2005), all of which can be interpreted as serving as protective mechanisms. The increased GFAP indicates reactive gliosis due to inflammation, cytotoxic edema, and elevated intracranial pressure because of impact injury. Thus, the reduction in GFAP in P4 treated animals suggests that there is a reduction in the amount of injury taking place and therefore less reactive gliosis compared to control animals. As observed in males gerbils that were found to be more prone to necrotic death after ischemia, human males also exhibited a higher amount of lipid peroxidation accrued after TBI relative to females as assessed in the cerebrospinal

fluid (two–fold higher than females) (Bayir et al., 2004). P4 decreased the levels of lipid peroxidation in male rats when administered after traumatic brain injury (Roof and Hall, 2000). Following TBI, females were observed to have fewer spatial learning impairments when compared to their male counterparts. The lesions were still similar in size, however there was less ventricular dilation indicating lower edema and water retention (Attella et al., 1987). Male and normal females were given P4 after TBI and then at 6, 24, and 48 hours after damage. Animals treated with P4 showed almost no edema whereas controls had high levels of edema three days post-injury (Roof et al., 1996). In studying the effects of P4 on spinal cord contusion injuries, Thomas *et al.* found that there was a marked reduction in the size of the lesion and a prevention of secondary neuronal loss with one P4 injection per day for five days (Thomas et al., 1999). Interestingly when P4 was compared to MPA, MPA required a larger dose than P4 to accomplish a comparable reduction in cerebral edema. However regardless of the dose of MPA, MPA did not favor a better behavioral recovery than progesterone (Stein, 2005). This suggests that behavioral protection occurs via mechanisms that are specific to P4 and are divergent from MPA.

While all the above mentioned studies support the ability of P4 to protect against various injuries to the brain, the cellular/molecular mechanisms underlying this protective effect remain unclear.

The MAPK Pathway

The exact mechanism by which P4 elicits protection is unknown. One possibility is through the activation of signal transduction pathways such as the MAPK pathway. The mitogen-activated protein kinases (MAPK) are a family of serine/threonine protein kinases that transduce signals from the cell surface to the nucleus. Extracellular signal-regulated protein kinases (ERKs), downstream effectors of the MAPK family, have historically been associated with signals initiated by growth factors that lead to differentiation and growth (Marshall, 1994).

Different temporal patterns of ERK phosphorylation mediate distinct effects within a cell. A rapid onset yet limited duration of ERK phosphorylation may bring about differentiation and/or survival whereas a more delayed and prolonged activation is associated with cellular death (Marshall, 1995). Singh et al., observed that treatment of cerebral cortical explants with P4 resulted in the phosphorylation of ERK in a manner that was consistent with the pattern of phosphorylation associated with cell survival (i.e., was rapid in onset and limited in duration) (Singh, 2001). This particular pattern of ERK phosphorylation is also elicited by E_2, which is protective against various insults (Singh, 2001; Simpkins et al., 2005). These observations lead us to the formulation of the hypothesis that P4 is protective through activation of the ERK / MAPK pathway.

The persistent activation of ERK1/2, on the other hand, as is observed during excitotoxicity and oxidative stress, results in cell death. UO126 inhibits both unphosphorylated and the already phosphorylated MEK1/2 to prevent the phosphorylation of the downstream element ERK (Favata et al., 1998). Thus by

inhibiting ERK pharmacologically, the temporal pattern of phosphorylation changes – leading to different signals interpreted by the cell. Therefore in theory, by inhibiting the chronic activation of ERK - protection should result. This hypothesis was supported in neuronal mouse hippocampal cells and rat primary cortical cultures. Within this model UO126 protected against oxidative stress induced by glutamate (Satoh et al., 2000). Chronic ERK activity has been reported in organotypic explants derived from transgenic animal models of AD overexpressing Aβ. Therefore the chronic activation of ERK may be a plausible mechanism leading to the pathology of Alzheimer's Disease. In fact, the distribution of phospho - ERK1/2 was found to correspond to the neurofibrillary changes in AD (Pei et al., 2002). In accordance to the *in vitro* data, Alzheimer's brains have also been illustrated to have a higher amount of phospho- ERK 1/2 when compared to control cases (Russo et al., 2002).

ERK phosphorylation seen at a certain time point (i.e. western blot, immunocytochemistry etc) is resultant of not only the accumulation of the phosphorylation of the protein but also by the action of phosphatases which de-phosphorylate ERK (Hunter, 1995). It is hypothesized that the accumulation of phosphorylated ERK is due to the decreased activity of protein phosphatase-2A (PP2A)(Gong et al., 1993; Gong et al., 1995). This hypothesis is reinforced by the fact that okadaic acid, an inhibitor of PP2A, produced activation of ERK1/2 similar to that seen in AD (Pei et al., 2003). In agreement, the expression of mRNA of PP2A in the AD

brain has also been illustrated as lower than that in the normal brain (Vogelsberg-Ragaglia et al., 2001)

As an added level of complexity by which ERK can elicit its effects, ERK translocates to the nucleus and promotes the transcription of genes leading to either the proliferation, differentiation or apoptosis of a cell. The mechanisms of ERK import/export are not completely understood, and controversy exists in regards to its mechanism of sequestration to the cytoplasm or translocation to the nucleus. This compartmentalization and duration of ERK phosphorylation has been hypothesized to be critical for the transmission of pro- vs. anti-apoptotic signaling. Recently a review by Callaway *et al.* (2005) described various hypotheses to explain the passage of ERK through the nuclear membrane. ERK could translocate by directly binding to the nuclear pore complex. The translocation of ERK2 is thought to occur in this manner due to the net change of protein phosphorylation in both the cytosol and nucleus. In addition to the phosphorylation state that might affect the ability of ERK to traverse into the nucleus, the monomeric and dimeric forms of MAPK have also been postulated to enter the nucleus by passive diffusion and active transport mechanisms, respectively (Adachi et al., 1999). Transport of monomeric ERK in a passive manner across the nuclear pore is by Brownian motion, without ATP, and independent of soluble factors (Matsubayashi et al., 2001).

And just like nuclear import, the factors governing nuclear export of ERK are also not well understood. What is known is that the ERK1/2 protein does not contain nuclear export sequences (NES). However, a small 15 kDa protein named PEA-15 has been

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demonstrated to bind to ERK1/2 and blocks its translocation to the nucleus (Formstecher et al., 2001). PEA-15 contains a nuclear export sequence and upon binding to ERK anchors it to the cytoplasm (Formstecher et al., 2001). Genetically deleting this gene and thus its protein product resulted in an increased ERK localization to the nucleus (Formstecher et al., 2001). However, considerable work still needs to be done to understand the mechanisms that are involved in regulating subcellular localization of ERK.

The site of subcellular compartmentalization of activated/phosphorylated ERK may lead the cell down the path of apoptosis, survival, or proliferation. Apoptosis results from oxidative stress when chronically active ERK 1/2 is retained in the nucleus. A chronically active ERK1/2 is tolerated by the HT-22 cells if it is excluded from nuclei. Therefore, the specific compartmentalization of activated ERK1/2 is a major factor of neuronal cell death (Stanciu and DeFranco, 2002). Nilsen and Brinton (2003) observed in primary dissociated hippocampal neurons, ERK phosphorylation in a rapid and transient temporal manner does not necessarily commit ERK to translocation into the nucleus. In the hippocampus, E2 and P4 was neuroprotective whereas MPA was not. E2, P4 and MPA all elicited ERK phosphorylation in a similar temporal manner. However, only E2 and P4 resulted in the translocation of ERK into the nucleus. Interestingly, E2 induced translocation of ERK to the nucleus was blocked by the co-administration of MPA. Therefore, conclusions were made that MPA elicits differing downstream elements of MAPK promoting a divergence of events not leading to cellular survival

(Nilsen and Brinton, 2003). Given that the mechanisms of cytoprotection are not completely known, a better understanding of the crosstalk between the steroids, their receptors, and kinase pathways may be key to more effective HRT development (Hapgood et al., 2004).

The PI-3 Kinase / Akt Pathway

The other pathway of interest elicited by P4 is the phosphoinositide (PI)-3-kinase pathway. The PI-3 kinase pathway has been demonstrated to inhibit apoptosis and promote mechanisms leading to neuronal survival. PI-3K phosphorylates phosphoinositides, which in turn, can result in the activation of the downstream kinase Akt (also known as PKB). Akt then phosphorylates, among other targets, the pro-apoptotic protein BAD. Phosphorylated BAD is sequestered by the 14-3-3 class of proteins, and thus, rendering BAD unable to dimerize with the pro-survival Bcl-X$_L$ or Bcl-2. With Bad sequestered in the cytosol, the anti-apoptotic Bcl-2 / Bcl-X$_L$ proteins can inhibit the activity of Bax (another pro-apoptotic protein), thereby preventing the release of cytochrome c from the mitochondria and consequent activation of caspases. P4 alone or in combination with E2 increased Bcl-2 expression in primary hippocampal neurons whereas MPA blocked E2 induced Bcl-2 levels (Nilsen and Brinton, 2003). Singh has observed that P4 elicits the phosphorylation of Akt in explants (Singh, 2001). Because Akt phosphorylation results in a cascade of events leading to survival, P4's

ability to phosphorylate Akt leads us to hypothesize that P4 may be protective through this mechanism.

Pathologically, a decrease in PI-3 kinase activity and its downstream events are observed in AD patients when compared to age-matched controls (Zubenko et al., 1999; Singh, 2001; Rickle et al., 2004; Ryder et al., 2004). Postmortem analysis of brains from AD patients illustrated a significant decrease in the soluble form of PI-3 kinase relative to controls (Zubenko et al., 1999). Recently, the enzyme activities of the serine/threonine kinase Akt were compared with the mid-temporal and frontal cortices of Alzheimer's disease subjects and age-matched controls (Rickle et al., 2004). Glycogen synthase kinase - 3β (GSK-3β) is a direct substrate of Akt. The phosphorylation of N-terminal serine residues of GSK-3β is important for neuronal survival mediated by Akt. GSK-3β activity was significantly increased in these regions of Alzheimer's disease patients when compared with non-disease controls (Rickle et al., 2004). GSK-3β is also found to be co-localized with the tau protein in hippocampal pyramidal cells implicating a role for this protein in the phosphorylation of tau in familial Alzheimer's disease. Therefore, a significant reduction in the Akt protein results in the increased kinase activity of GSK-3β (Ryder et al., 2004).

Medroxyprogesterone Acetate

Medroxyprogesterone acetate (MPA) is a synthetic progestin derived from 17α-hydroxyprogesterone. Orally administered MPA does not undergo any first pass effects

and therefore the bioavailability is nearly 100% (Schindler et al., 2003) as compared to progesterone which is almost completely metabolized in one passage through the liver (Katzung, 2004). In addition to differences in bioavailability and half-life, MPA also displays many non-progestagenic effects (Schindler et al., 2003). For example, unlike P4, MPA binds to the AR and acts as a partial agonist (Winneker et al., 2003), with a Kd of 2.1 nM (Hackenberg et al., 1990), whereas progesterone does not bind to the AR (Schindler et al., 2003). Due to MPA's ability to bind the AR, recent studies in breast cancer cells expressing AR have illustrated that MPA has different effects than progesterone that are more similar to dihydrotestosterone (Ghatge et al., 2005). In fact, MPA in breast cancer cells is a strong androgen. This is of great relevance considering that the majority of grade 3 ductal carcinoma *in situ* are ER and PR negative but continue to be AR positive (Moinfar et al., 2003). In fact, androgen receptors, quantified by immunohistochemistry in 1026 metastatic tumors, were demonstrated to be present at double the frequency than PR and in one-fourth of the tumors AR was expressed as the only sex hormone receptor (Lea et al., 1989). With MPA treatment, the AR and ER is downregulated by 50 and 60% in MCF-7 cells (Hackenberg et al., 1990). MPA also binds to, and activates, glucocorticoid receptors (Koubovec et al., 2005, Schindler, 2003 #156), the consequence of which may be to increase the susceptibility to infection by immuno-suppression (Koubovec et al., 2004). MPA might increase the chances of contracting STDs in women using MPA as a contraceptive agent (Hapgood et al., 2004)

and could potentially explain the higher incidence of virally sexually transmitted diseases today.

From a historical perspective, MPA was added to the formulation of hormone therapy to prevent the hyperplasia of the endometrium that occurs with unopposed estrogen therapy, which may increase the risk of uterine cancer (Hirvonen, 1996). To note, MPA has about a two-fold greater affinity for the progesterone receptor in the human uterine endometrium than progesterone (Turgeon et al., 2004). In addition, the risk of breast cancer due to prolonged exposure to estrogen was also thought to be reduced with the inclusion of a progestin (Gambrell, 1986). However the recent 'Million Women Study' found that MPA substantially increased the risk of breast cancer in long-term users (Beral, 2003).

In addition to the Women's Health Initiative Memory Study (WHIMS), where a hazard ratio of 2.05 for dementia was reported in women receiving continuous combined hormone therapy (consisting of CEE and MPA)(Rapp et al., 2003), other studies have also been performed. At the Kyoto Prefectural University of Medicine in Japan, a trial of a combination of MPA, and CEE was performed. In group A, CEE was given to AD patients for seven weeks. At week 4, MPA was added to the regimen. Three dementia scales (revised version of Hasegawa's dementia scale (HDS-R), new screening test for dementia (NSD) (Japanese National Institute for Mental Health), mini-mental state examination (MMS) and clinical symptoms were used to assess patients at three weeks (before combination therapy), six weeks (during combination) and nine weeks. The

scores of patients improved significantly at three weeks using CEE however decreased non-significantly after MPA combination therapy at six weeks (Honjo et al., 2005).

Many of the clinical trials to date have used the formulation of conjugated equine estrogens with MPA; however the basic research does not support the use of this progestin in protection. In primary dissociated hippocampal neurons, MPA was not protective against glutamate toxicity, whereas P4 was effective at protecting these cells (Nilsen and Brinton, 2003). Furthermore, the protective effects of P4 appeared to be mediated, in part, by preventing the glutamate-induced increase in intracellular Ca^{2+} levels, whereas MPA had no effects on the glutamate-induced $[Ca^{2+}]_i$ rise. Of particular importance is that MPA blocked the beneficial effect of E2, whereas P4 did not (Nilsen and Brinton, 2002). With regards to the regulation of cell signaling, both P4 and MPA were shown to elicit ERK phosphorylation, although only P4 treatment resulted in nuclear translocation of ERK within the model of dissociated hippocampal cells (Nilsen and Brinton, 2003). The relevance of the nuclear translocation of ERK may be pertinent to the signaling pathways for the regulation of gene expression relevant to the promotion of cell survival. Supporting this assertion is the observation that P4, but not MPA, increased the expression of the anti-apoptotic Bcl-2 protein. In fact, not only did MPA fail to increase expression of Bcl-2, but actually inhibited that elicited by E2 (Nilsen and Brinton, 2002).

The disparity between the effects of P4 and MPA has also been observed *in vivo*. For example, a study using rhesus monkeys illustrated that E2 + P4 protects against

coronary vasospasm, whereas E2 + MPA did not (Miyagawa et al., 1997). P4 has also been illustrated in post-menopausal women to enhance the beneficial effects of estrogen on exercise-induced myocardial ischemia, whereas MPA has not (Rosano et al., 2000). After 22 hours of reperfusion, CEE and MPA diminished estrogen's ability to reduce stroke damage (120 min of reversible focal stroke using the intraluminal filament model) in the subcortical regions of the rodent stroke model. All of these studies imply that the choice of progestin used requires careful consideration.

Studies done with cynomolgus monkeys in 1990 with P4 did not attenuate estrogen's ability to reduce atherosclerosis. These studies were conducted with either a continuous estradiol or continuous estradiol plus cyclically administered P4 (28 days on; 28 days off) for 30 months. In 1997, studies by Adams demonstrated that monkeys treated with CEE showed a 72% reduction in coronary artery atherosclerosis whereas there were no benefits observed in CEE plus MPA group (Adams et al., 1997).

Therefore, while P4 does not interfere with the beneficial effects of estrogens, MPA appears to have the capacity to prevent estrogen's beneficial effects. This leads to the distinct possibility that the interactions of MPA with estrogen as demonstrated in the cardiovascular and nervous systems within the WHI trials may have contributed to the negative results observed.

As a consequence of reviewing the above literature, I pursued the cellular and molecular differences distinguishing progesterone and medroxyprogesterone acetate upon

cytoprotection in order to explain the negative observations of the WHI trials using an *in vitro* model.

Organotypic Explants of the Cerebral Cortex

Organotypic explants (slice cultures) derived from the mouse cerebral cortex were used to test the above hypotheses. The explants were prepared from C57Bl/6 mice and have been used in past and ongoing projects in the laboratory. The advantage of the explant model is that it preserves the native cytoarchitecture of the brain, at least within the confines of the slice. Thus, cell to cell interactions are retained and thus, offer a unique model to evaluate the effects of hormone action on the brain. Specifically, this experimental system provides our research with a model reflective of the complexity of the *in vivo* brain in terms of interactions between differing cell types, yet offers the ability to isolate only a particular region of the brain. Caveats to this model include the inability to distinguish the source of the signals (i.e. neurons vs. glial cells) being detected with the assays proposed herein (such as Western blot, LDH release, etc). That is, we may not be able to discriminate between those cells that are responding to P4 versus those that are not.

A Model of Cell Damage/Death - Glutamate Toxicity

Glutamate is the main excitatory neurotransmitter in the CNS. Excessive glutamate stimulation of the glutamate receptors results in a sustained increase in Ca^{2+}

influx and ROS (reactive oxygen species) production leading to oxidative stress and an excitotoxic death. Glutamate toxicity contributes to cellular damage associated with either acute insults such as ischemia and stroke or chronic neurodegenerative diseases such as Alzheimer's disease (Siesjo, 1981; Greenamyre et al., 1985). Cellular attrition in such diseases is resultant of both excitotoxicity and generation of oxidative species (Lazzarino et al., 1992; Coyle and Puttfarcken, 1993; Lei et al., 1994; Simonian and Coyle, 1996).

The excitotoxicity-associated rise in $[Ca^{2+}]_i$ that results from glutamate stimulation has been well established to play an important role in neuronal injury and death (Choi, 1987). This toxicity can be attenuated by E_2 and P4 (Goodman et al., 1996). However, MPA had no effect on the glutamate-induced $[Ca^{2+}]_i$ and blocked E_2's attenuation in primary hippocampal neurons (Nilsen and Brinton, 2003).

Glutamate-induced oxidative stress can occur by high concentrations of extracellular glutamate preventing cysteine uptake into the cells through the glutamate / cysteine antiporter (Murphy et al., 1989; Davis and Maher, 1994). The cellular insult occurs as a result of an imbalance between radical-generating and radical-scavenging systems. Cysteine is a precursor for glutathione synthesis and therefore in the presence of high extracellular concentrations of glutamate, glutathione levels are unable to be replenished. Glutathione eliminates reactive oxygen species (ROS). As the glutathione levels decrease, ROS accumulate under conditions of oxidative stress producing damage within the cell (Murphy et al., 1989). Thus, as a result of ROS accumulation there is

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damage as a result to proteins, lipids and nucleic acids. This further perpetuates the cell towards apoptosis (Mattson and Magnus, 2006).

Free radicals in the presence of oxygen may also induce the perioxidation of lipids within the membranes. This results in mitochondrial dysfunction and over time, ATP depletion. ATP depletion results in the activation of phospholipases and consequently the breakdown of phospholipids. These events then downstream lead to increased membrane permeability. It is also established that chronic elevations in Ca^{2+} results can also induce the membrane damage by increasing phospholipase activation leading to the an increase in the amount of phospholipid degradation. In addition, protease activation resulting from elevations in Ca^{2+}, may promote the enzymatic digestion of proteins within the membranes of cell organelles. Collectively, I believe that the model of insult/injury chosen is useful in mimicking certain pathogenic aspects of aging and age-associated diseases, including AD.

This preceding section, while outlining what we currently know about the neurobiology of progestins, points out the presence of a gap in our understanding of the cellular and molecular mechanisms that distinguish the neurobiological effects of progesterone and medroxyprogesterone acetate. Thus, to provide additional insight into the failure of the WHI studies to show beneficial effects of hormone therapy, I hypothesized that P4 and MPA have different mechanisms of action such that one is protective, while the other is not. The results from the studies I completed have helped confirm that MPA is not neuroprotective, and further, have provided a cellular

mechanism by which progesterone, in contrast, is. Such studies, along with those ongoing in the Singh lab will be instrumental in facilitating new drug development of hormone therapies without untoward effects.

MATERIALS AND METHODS

Preparation of Organotypic Explants

Procedure for Washing Instruments

Wearing powder-free gloves, the instruments were cleaned of any visible residue using gauze soaked in 80% ethanol. The instruments were placed in a stainless steel tray filled with 1% isoclean, making sure to sufficiently cover the instruments. The trays were placed on the gas burner and allowed to come to a 'slow boil' for 5 min. The trays were allowed to cool. Deionized water was then run over the tray in the sink to rinse away the soapy solution. The instruments were then rinsed with deionized water eight times. After the eighth rinse, the instruments were then rinsed with Milli-Q water another eight times. Following the final rinse with Mili-Q water, the tray was refilled with Mili-Q water and placed on a gas burner and allowed to come to a boil. If soapy bubbles were detected, the instruments were re-rinsed with Milli-Q water and then re-boiled. The tray was allowed to cool and the water was aspirated. The tray's base was then wiped with 80% alcohol and placed in the hood. The instruments were allowed to dry (approximately 1 hour). The instruments were then soaked in 95% alcohol for an additional hour. Then, the alcohol was poured out and the instruments were allowed to dry in the hood. Once dry, the instruments were placed into instrument containers that were subsequently wrapped in foil and baked for 2 hrs at 171 °C in a sterilizing oven. Once baked, they were stored until time of use.

Procedure for Washing Glassware

Glassware was placed into a large stainless steel stock pot containing 1% isoclean making sure to sufficiently cover the glassware. The pot was then placed onto the gas burner and allowed to come to a boil for 5 min. The pot was allowed to cool and the isoclean was decanted. The pot was placed in the sink and deionized water was run over the glassware (while still in the pot) to sufficiently rinse away the soapy solution. The glassware was then rinsed 4 times with deionized water, followed by 4 rinses with Milli-Q water. After the final rinse, the pot was re-filled with Mili-Q water and boiled for 5 min. If soapy bubbles were detected, the glassware was re-rinsed with Milli-Q water and then re-boiled. The pot was allowed to cool and the water was aspirated. Wearing a pair of powder-free gloves, the glassware was removed from the pot and placed into the drying oven (set at 60 °C). The glassware in the oven was covered and allowed to dry overnight. The glassware was then wrapped in foil and baked as described above. The sterilized glassware was then stored until used.

Procedure for Washing Brain Slicing Grids

Assuming that the grids were used previous to this, the threads and screws were removed from the grid and placed in a tray of 1% isoclean. The tray was placed on the gas burner and allowed to boil for 5 min. Upon cooling the tray was placed in the sink and deionized water was run over the grids to sufficiently rinse away the soapy solution.

The trays were allowed to cool. Deionized water was then run over the grids to sufficiently rinse away the soapy solution. The grids were then rinsed eight times with deionized water, followed by eight rinses with Milli-Q water. After the final rinse, the tray was re-filled with Milli-Q water and placed on the gas burner and allowed to come to a boil. If soapy bubbles were detected, the grids were re-rinsed with Milli-Q water and then re-boiled. The tray was allowed to cool and the water was aspirated. The tray's base was then wiped with 80% alcohol and placed in the hood. The grids were allowed to air dry in the tissue culture hood (approximately 1 hour). The grids were then wound with gold-plated tungsten wire using a custom made grid-winder. The wound grids were then again placed in a tray of 1% isoclean. The tray was placed on the gas burner and allowed to boil for 5 min. Once cooled, the deionized water was run over the grids to sufficiently rinse away the soapy solution. The grids were then rinsed with deionized water eight times, followed by eight rinses with Milli-Q water. After the final rinse, the tray was placed on the gas burner and allowed to come to boil. If soapy bubbles were detected, the grids were re-rinsed with Milli-Q water and then re-boiled. The tray was allowed to cool and the water was aspirated. The tray's base was then wiped with 80% alcohol and placed in the hood. The grids were allowed to dry (approximately 1 hour). Once the grids were dry, 95% alcohol was poured into the tray to sufficiently cover the grids. The grids were soaked as such for 1 hour. The alcohol was poured out and allowed to dry in the hood. The grids were then placed into a small Petri dish. The Petri

dish was then placed into an autoclave pouch, sealed and autoclaved (121 °C at 15 psi for 30 min).

Wrapping and Sterilization of Glassware

Wearing a pair of powder-free gloves, the glassware was removed from the drying oven. The glassware was wrapped initially in "light gauge" aluminum foil and finally in "heavy gauge" aluminum foil. The glassware was labeled using a Sharpie on the heavy-duty foil for identification purposes later.

Baking of the Glassware

Wrapped glassware was placed into the baking oven. The temperature was set at 171° C for 2 hours. The timer was started once the temperature reached the 171° C. The oven was not opened until contents reached room temperature (usually left undisturbed overnight). After baking, the items were stored until time of use.

Preparation of Coverslips

Acid Cleaning of Coverslips for use in Collagenization Protocol

Using clean flat ended forceps, 12 X 22 mm coverslips were placed into porcelain coverslip racks. Within the fume hood and using appropriate safety measures (i.e. safety glasses, lab coat and gloves), the porcelain racks containing coverslips were placed into a glass container containing 500 mL of 15.7 M nitric acid. The glass container was covered

and allowed to soak overnight in the fume hood. The next day, the nitric acid was removed. The porcelain rack containing the coverslips were then transferred into a fresh glass container in which the coverslips were rinsed with deionized water, and then with Milli-Q water. After the fourth rinse with Milli-Q water, the container was filled again with Milli-Q water and allowed to sit for an hour. This was repeated two more times. After the third rinse and aspiration, the container was filled again with Milli–Q water, covered and allowed to sit overnight. The next day, the water was drained and rinsed twice more, allowing to sit an hour between rinses and aspirated. The porcelain holder containing the clean coverslips was then placed in the drying oven. Once dry, the coverslips were placed in a pre-cleaned medium Petri dish (see Glassware cleaning method above). The Petri dish was then wrapped in 2 layers of aluminum foil and baked for 171°C for 2 hours. Upon cooling, the wrapped Petri dish was labeled as baked coverslips and stored until time of use during the collagenization step.

Poly-L-Lysine Pre-coating of Coverslips

The protocol herein was for the preparation of 28 coverslips. Seven clean coverslips were placed into each of the four washing dishes. Poly-L-Lysine (molecular weight > 300,000, Sigma, Cat. P-5899 5mg) was reconstituted in sterile filtered Milli-Q water for a final concentration of 0.2 mg/mL. Poly-L-Lysine was made in advance (at least 1.5 hours) and refrigerated. The solution was discarded after 4 uses. To each of the 4 washing dishes, 5 mL of poly-L-lysine was added. The dishes were allowed to stand

for 1 hour. After 1 hour, the poly-L-lysine was pipetted back into the original bottle, sealed and then refrigerated. Four new washing dishes were labeled with the date and to also mark the front of the dishes. To the washing dishes, 7 mL of sterile filtered Milli Q was added. Using flat forceps, the coverslips were transferred from the poly-L-lysine washing dishes into the Milli-Q water containing dishes. The coverslips were then washed 6 times for 10 min with 7 mL with sterile filtered Milli-Q water. Aspiration was accomplished using a sterilized fine tip Pasteur pipet. After the final wash, the water was aspirated. Two Maximow slides (depression side down, side by side) were placed into each of two large (150 mm) Petri dishes containing a single sheet of black filter paper (black Petri). The coverslips were set onto the maximow slides (14 per Petri dish). The Petri dishes were then cracked open to allow air drying of the coverslips.

Collagen Coating of Coverslips

With a bent-tip Pasteur pipette, one drop of collagen (Rat tail, Type I, UBI, Cat. No. 08-115) was added to each of the Poly-L-lysine coated coverslips. The drop was then spread with the "knee" of the bent tip pipette to entirely cover it. Immediately after the collagen was spread, the remaining collagen stock was stored into the refrigerator. A piece of foil was then cut to sufficiently wrap and cover the large Petri dishes. Two Petri dishes were retrieved that contained white filter paper and placed into the hood. About 20 drops of ammonium hydroxide was placed onto the white filter paper. The covers of the black Petri dishes were removed and replaced with the Petri cover with white filter

paper soaked in ammonium hydroxide. As quickly as possible, the Petri dishes were wrapped in aluminum foil for 3 minutes. The ammonia fumes caused the collagen to congeal. After the 3 minutes, the coverslips were then transferred into a fresh set of washing dishes containing 7 mL of sterile filtered "ammoniated" Milli-Q water (Milli-Q water whose pH had been made slightly alkaline using 70 µL of ammonium hydroxide per liter of Milli-Q water) and left in the hood for at least 24 hours.

The following day, the coverslips were rinsed with ammoniated water 4 times for 15 min each. After the last ammoniated water wash, the washing dish was rinsed with 7 mL Gey's BSS and aspirated. A final 6 mL of Gey's BSS was added to these washing dishes.

The coverslips were then transferred to fresh washing dishes containing 7 mL of Gey's BSS was added and 4 hanging drops (~ 200 µL) of heat inactivated gelding horse serum with a bent tip glass pipette. The washing dishes containing the coverslips were then placed into a tray and placed into the refrigerator until needed.

Preparation of 2% Noble Agar

1.2 g of Noble Agar was weighed out and transferred into a clean Erlenmeyer (250mL) flask containing 60 mL of Milli-Q water. This was minimally disturbed and not mixed. The cap was placed onto the flask but not tightened. The cap was then covered with autoclave paper and secured using autoclave tape. The flask was then autoclaved (liquid cycle). Once the Erlenmeyer flask could be handled, but ensuring that the agar

was in liquid form, the agar was poured into a sterile medium Petri dish in the hood. The agar was allowed to cool and solidify with the Petri dish cracked open. Once solidified, the agar was allowed to sit in the hood for an additional hour, after which the Petri containing the agar was placed into a sterile can and placed in the refrigerator at 4°C.

Derivation of Cultures

Organotypic explants were derived from ~360- μm-thick hemicoronal slices of postnatal day 3 (P3) frontal and cingulate cerebral cortex (day of birth = P1), obtained from pups born of pregnant C57B1/6J mice. As described by Singh et al. 1999, cultures were maintained as roller tube cultures (Gahwiler, 1981). Three hemicoronal slices were placed on each coverslip per Leighton tube. Antibiotics were never used during the procedure.

Craniotomy, removal of the brain and dissection

The sex of the animal was determined by visualizing the ano-genital distance (AGD) (Greenham and Greenham, 1977). Males displayed greater anogenital distances than females (Greenham and Greenham, 1977). In addition to AGD, the male genitalia are more prominent and dark pigmentation is often observable below the pre-scrotal region. The pup (postnatal day 2) was anesthetized, via hypothermia, by placing the animal in ice for 2 min. The procedure, hereafter, was done in the horizontal flow hood. The animal, after being anesthetized, was euthanized using cardiac puncture and

subsequently stabilized onto a gauze dissection pad, using 24 Ga needles. Tincture of iodine was applied onto the skin of the pup and allowed to dry to disinfect the skin before craniotomy. A small wind shield was placed behind the tripod stand to prevent drying out of the brain following craniotomy. The skin was excised from the head while being careful not to damage the underlying skull, which at this postnatal age is quite thin. Then, iris scissors and another curved dull forceps were used to cut horizontally through the orbits of the eyes. Subsequently, another incision was made vertically from in between the orbits to the back of the head. This incision was made carefully so as not to damage the underlying brain. Once the midline cut was made, the point of the iris scissors was used to gently lift the one side of the cranium. Lifting it gently with the scissors, the forceps were used to gently grab it. The forceps were pulled laterally, enabling an area available for the iris scissors to cut away the cranium. As the cranium was separated from the brain, the forceps were repositioned to enable the iris scissors to cut completely around the skull. Once the brain was exposed, a sterile blade was used to cut horizontally in front and behind the brain. A large sterile depression slide (Maximow) was placed upon the Radnotti cooling device allowing the slide to stay at 4°C. Ice-cold dissection feed (93% Gey's Balanced Salt Solution (Sigma), 30 mM D-(+) - glucose (Sigma), 10 mM magnesium chloride (EM Science), 270 µg/mL ketamine-HCl (VedCo), 2 mM L-glutamine (MediaTech, Inc., Herndon, VA), 50 µg/mL of L-ascorbic acid (Sigma)) was placed into the depression. Quickly, curved dull forceps were dipped in dissection feed (to prevent the brain from actually sticking to the forceps themselves),

and placed on either side of the brain and very gently lifted out. The brain was placed in the Maximow slide sufficiently covered with dissection feed. Another brain, from the same sex, was removed. The meninges from both of the brains were removed with 45° fine forceps under the aid of the dissection microscope (Zeiss). After placing the grid onto the chopper with sterile surgical gloves, a piece of 2% noble agar was cut to the size of the grid and placed onto the base plate. The brains were positioned such that they were placed perfectly perpendicular to the orientation of the wired grid. The brains were chopped and the grid loosened from the chopper. The grid and the brains sitting upon the agar were placed into a small sterile Petri containing 19 mL of cold dissection feed. This Petri was then placed under the microscope. With the aid of the microscope, the dull end of the Graefe knife was used to separate the slices. The separated slices were then transferred to two 3-well depression slides that had been placed on a Petri dish set on a block of ice. Hemi-coronal slices were then micro-dissected for frontal and cingulate cerebral cortex using the Graefe knife. Three hemicoronal slices were then transferred onto a collagenized cover slip using a bent tip glass pipet. The slices placed on each coverslip were chosen so that the rostro-caudal extent of the brain was represented. That is, a slice from the rostral, mid and caudal part of the brain was placed onto a single coverslip. This method helped eliminate rostro-caudal bias. Excess amount of fluid was absorbed using a surgical absorbent spear (to ensure that the slices did not slide off the coverslip). At the edge of the coverslip, a minimal amount of steroid-deficient and phenol red-free maintenance medium (25% horse serum heat inactivated (Sigma), 22.5%

Hank's Balanced Salt Solution (Mediatech, Inc., Herndon, VA), 50% minimum essential medium Eagle MEM (Sigma), 30 mM of D-(+)-glucose (Sigma), 2 mM L-glutamine (MediaTech, Inc., Herndon, VA), 50 µg/mL of L-ascorbic acid (Sigma) supplemented with 2 nM 17-β estradiol (Sigma)) was placed. The slide was then handled with long forceps and placed into a Leighton tube containing 4 drops (~200 µL) of Final Feed supplemented with 2 nM estradiol. The Leighton tubes were placed into a roller drum. The roller drum was placed inside a 5% CO_2 incubator kept at 34.5°C and humidified with Milli-Q water.

Maintenance of Cultures

The cultures were maintained with the previously mentioned Basic Final Feed media supplemented with 2 nM 17-β estradiol (Sigma). The addition of this low concentration of estradiol helps ensure healthy cultures (Toran-Allerand, personal communication). Day one, day of dissection, the cultures were maintained with only 4 hanging drops of Basic Final Feed media supplemented with 2 nM 17-β estradiol (Sigma). The minimal fluid allows the slices to adhere to the collagenized coverslip over the initial 24 hr period. On the second day *in vitro*, 500 µL of the Basic Final Feed media supplemented with 2 nM 17-β estradiol (Sigma) media was added to the tubes containing the cultures. After replacing the Leighton tubes into the roller drum, the roller drum was

turned on and set at a rotation speed of 6 revolutions/ hour. Media was replenished every 2-3 days.

Treatment of Cultures

The cultures were maintained *in vitro* for 6 days. On the 6th day *in vitro* a 24 hour "hormone washout" was performed, consisting of replacing the maintenance medium with media that did not contain exogenously added 17-β estradiol. On the 7th day *in vitro*, cultures were spiked with the appropriate treatment (see below) for the specified amount of time. Controls were also hormone deprived and sham (vehicle)-treated to account for any consequences of procedural manipulation of the explants.

Progesterone (4-pregnen-3, 20-dione, Sigma) was dissolved in DMSO to a stock concentration of 100 μM; and applied to the culture at a dilution of 1:1000, resulting in a final concentration of 100 nM. This dilution also ensured that the concentration of vehicle (DMSO) never exceeded 0.1%. Due to the labor intensiveness of the explant procedure, controls for sham (vehicle) treatment were only performed in replicate experiments to which the resulting LDH values were normalized. Sham (vehicle)-treatments, progesterone treatment and untreated controls were not statistically significant from each other (data not shown).

To evaluate the involvement of the ERK/MAPK or PI3-K pathways in progesterone's ability to protect cultures from L-glutamate, pharmacological inhibitors were used. UO126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylalthio] butadiene,

(Calbiochem Cat. No. 662005, San Diego, CA), a MEK1 and MEK2 inhibitor, was applied to the explants at a concentration of 10 μM for 30 min. prior to P4 pre-treatment. The IC50 for MEK1 is 72 nM and for MEK2 is 58 nM. UO126 has very little effect on other kinases such as Abl, Cdk2, ERK, JNK, MEKK, MKK-3, MKK-4/SEK, MKK-6 PKC and Raf (Calbiochem, San Diego, CA, Technical Resources). At concentrations similar to that inhibiting MEK1/2, ERK5/MKK5 is also inhibited (Mody et al., 2001).

LY294002, (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one, Calbiochem, San Diego, CA), a highly selective inhibitor of PI3-Kinase was applied at a concentration of 15 μM concentration for 30 min. prior to P4 pre-treatment. The IC50 for PI-3K is 1.4 μM. This compound acts directly by binding to the ATP-binding site. It does not affect the activities of EGF receptor kinase, MAP kinase, PKC, PI-4 kinase, S6 kinase, and c-Src up to 50 μM (Calbiochem, San Diego, CA, Technical Resources).

Twenty-four hours after the treatment with progesterone (with or without pharmacological inhibitor), L-glutamic acid (Sigma) diluted in Gey's BSS was administered to the cultures for a duration of 6 hrs. This duration of glutamate treatment was chosen to ensure minimal degradation of the LDH in the conditioned media [$t_{1/2}$ of LDH in solution has been reported to be ~9 hrs (CytoTox-ONE Homogeneous Membrane Integrity Assay Technical Bulletin #TB306, Promega, Madison, WI].

Lactate Dehydrogenase Assay

A fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase (LDH) released from damaged or dying cells into the media. This assay for cytotoxicity was carried out according to the method provided and is based on the ability of LDH to promote the formation of a fluorescent product, resorufin, from the substrate, resazurin. Briefly, 100 μL of conditioned media was aliquoted into a black 96-well plate. The media was allowed to equilibrate to ambient temperature (~15 min), after which the CytoTox-One reagent was added to each well and incubated for 10 min at room temperature. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA). Relative fluorescent units (RFU) were normalized to μg of protein associated with the explant culture from which the conditioned media was derived. The values were expressed as a percentage of vehicle control.

BDNF Enzyme-linked Immunosorbant Assay

An enzyme-linked immunosorbant assay was used to detect and quantify total cellular BDNF levels (Promega). A 96-well Nunc MaxiSorp surface polystyrene flat bottom immuno-plate plate was pre-coated with an anti-BDNF monoclonal antibody [diluted 1:1000 in coating buffer (25 mM sodium bicarbonate and 25 mM sodium carbonate, pH 9.7)]. After rinsing off unbound antibody with TBS-T buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween-20), and blocking the plate non-

specific binding, 100 μg of sample lysate (in a final volume of 100 μL) or volume of appropriate BDNF standard, ranging in concentration from 7.8 – 500 pg/mL, was added. After 5 washes with TBS-T, the captured BDNF was then incubated with the polyclonal anti-human BDNF antibody. The amount of specifically bound polyclonal antibody was then detected through the use of the anti-IgY-horseradish peroxidase (HRP) tertiary antibody, which when treated with the chromogenic substrate (TMP reagent, Promega), changes color in proportion to the amount of BDNF present in the sample. The color intensity was quantified by measuring the absorbance at 450 nm using a Viktor3 ELISA plate reader (Perkin Elmer). Only values that were within the linear range of the standard curve were considered valid. BDNF levels were reported as % of vehicle control.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Experiments involving this methodology were carried out by Dr. Parmeet Jodhka, a post-doctoral fellow in the laboratory.

RNA isolation and cDNA synthesis

Total RNA was extracted from explant cultures and DNase-treated using the RNeasy Lipid Kit (Qiagen) according to manufacture's instructions. RNA concentrations of extracted RNA were calculated from the absorbance at 260 nm. The quality of RNA was assessed by absorption at 260 nm and 280 nm (A_{260}/A_{280} ratios of 1.9 to 2.10 were considered acceptable) and by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S rRNA bands were visualized under UV light. Total

RNA (1.5 μg) was reverse transcribed into cDNA in a total volume of 50 μL using the High Capacity DNA Archive Kit (Applied Biosystems) according to manufacture's instructions.

Primers and Probes for Quantitative Real-Time Reverse Transcription PCR

PCR primers and probes for the target gene, BDNF, and the endogenous control, 18S rRNA, were purchased as Assays-On-Demand (Applied Biosystems). The assays were supplied as 20X mix of PCR primers (900 nM) and TaqMan probes (200 nM). The BDNF assay (Mm00432069_m1) contained FAM (6-carboxy-fluorescein phosphoramidite) dye label at the 5' end of the probes and minor groove binder and nonfluorescent quencher at the 3'end of the probes. The 18S rRNA assays (4319413E) contained VIC-labeled probes. The assays are optimized for use on ABI prism Sequence Detection System using the default machine settings.

Quantitative Real-Time Reverse Transcription-PCR

The reaction mixture containing water, 2X qPCR™ Master Mix (Eurogentec), and 20X Assay-On-Demand for BDNF was prepared. A separate reaction mixture was prepared for the endogenous control, 18S rRNA. The reaction mixture was aliquoted in a 96-well plate and cDNA (11 ng of RNA converted to cDNA) was added to give a final volume of 30 μl. Each sample was analyzed in triplicate. Two nontemplate controls (RNase-free water) were included on each plate for reaction mixture containing BDNF

and 18S rRNA assays. Amplification and detection were performed using the ABI 7300 Sequence Detection System (Applied Biosystems) with the following profile: 2 min hold at 50°C (UNG activation), 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). Sequence Detection Software 1.3 (Applied Biosystems) was used for data analysis. A threshold was determined for a gene by the software, and the point at which the amplification plot crossed the threshold was defined as the critical threshold cycle, C_T. The cycle number at which fluorescence of the reporter exceeds a fixed threshold above baseline and is inversely proportional to the logarithm of the initial number of template molecules is referred to as the C_T (Livak, 1999).

Relative Quantitation of target mRNAs

The comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to calculate the relative changes in target gene expression. In the Comparative C_T Method, the amount of target, normalized to an endogenous control (18S) and relative to a calibrator (untreated control), was given by the $2^{-\Delta\Delta C_T}$ equation. Quantity was expressed relative to a calibrator sample that was used as the basis for comparative results. Therefore, the calibrator was the baseline (control) sample and all other treatment groups were expressed as an n-fold (or %) difference relative to the calibrator. Calculations for relative quantitation were performed as outlined in User Bulletin #2: ABI Prism 7300 Sequence Detection System (Applied Biosystems). The average and standard deviation of $2^{-\Delta\Delta CT}$ was calculated for the values

from three independent experiments, and the relative amount of target gene expression for each sample was plotted in bar graphs using GraphPad Prism 4 software.

Statistical Analysis

Data from at least three independent experiments were subjected to ANOVA, followed by Tukey's post hoc analysis for the evaluation of group differences, and are presented as bar graphs depicting the means ± SEM, using GraphPad Prism 4 software (San Diego, CA).

CHAPTER II

PROGESTERONE INCREASES BDNF EXPRESSION AND PROTECTS AGAINST GLUTAMATE TOXICITY IN A MAPK- AND PI3-K - DEPENDENT MANNER IN CEREBRAL CORTICAL EXPLANTS.

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Abbreviations: BDNF: Brain-derived neurotrophic factor; ERK: extracellular-signal regulated kinase; MAPK: mitogen-activated protein kinase; MEK: MAPK/ERK kinase; P4: progesterone; PI3-K: phosphatidylinositol-3 kinase; RT-PCR: reverse transcriptase-polymerase chain reaction.

ABSTRACT

Women have a higher prevalence for Alzheimer's disease (AD) than men, suggesting that the precipitous decline in gonadal hormone levels following the menopause may contribute to the risk of developing AD. While considerable attention has focused on the consequence of estrogen loss, or alternatively, estrogen's neuroprotective potential, it is important to recognize that the menopause results in a precipitous decline in progesterone (P4) levels as well. In fact, progesterone has been shown to be neuroprotective, although the precise mechanisms involved remain unclear. We have previously shown that P4 elicits the phosphorylation of ERK and Akt, key effectors of the neuroprotective MAPK and PI3-K pathways, respectively. Using organotypic explants of the cerebral cortex, we determined if the ability of P4 to elicit the phosphorylation of these pathways was predictive of its neuroprotective potential, and evaluated if activation of either of these pathways was necessary for P4-induced protection. We found that P4 (100nM) protected against glutamate- induced toxicity, and further, that the protective effects of P4 were inhibited by either the MEK1/2 inhibitor (UO126, 10 μM) or the PI-3K inhibitor (LY294002, 15μM), supporting the requirement of both the MAPK and PI-3K pathways in P4-mediated protection. In addition, P4, at a concentration and duration of treatment consistent with our neuroprotection data, also increased the levels of BDNF (both at the level of protein and mRNA). Collectively, these data suggest that P4 is protective via multiple, and potentially related mechanisms.

INTRODUCTION

The menopause is characterized by a precipitous decline in the levels of circulating estrogen and progesterone. As the average lifespan of women has increased to approximately 80 years of age (Arias and Smith, 2003), a more substantial portion of a woman's life is spent in a hormone-deprived state. Since post-menopausal women have a two to three fold higher prevalence of Alzheimer's disease (AD) than men (Henderson, 1997; Gao et al., 1998; Andersen et al., 1999; Sherwin, 1999), it has been suggested that these hormone deficits may play a significant role in enhancing the risk for the disease.

Numerous studies have indeed reported behavioral, neurochemical and molecular deficits following ovariectomy, and found that estrogen can at least partially normalize these deficits (Luine et al., 1975; Singh et al., 1994; Bishop et al., 1995; Singh et al., 1995; Panickar et al., 1997; Dubal et al., 1998; Gibbs, 1998; Shi et al., 1998; Jezierski and Sohrabji, 2000; Shi et al., 2001; Wise, 2002; Fan et al., 2003; Hoffman et al., 2003; Nordell et al., 2003; Simpkins et al., 2004; Wen et al., 2004; Simpkins et al., 2005). For example, ovariectomy results in a decrease in BDNF levels (Singh et al., 1995) while estrogen has been shown in multiple models to elicit an increase in the expression of BDNF (Singh et al., 1995; Sohrabji et al., 1995; Gibbs, 1999; Jezierski and Sohrabji, 2000; Solum and Handa, 2002; Gonzalez et al., 2004). Interestingly, our previous data suggested that estrogen only partially normalized the ovariectomy-induced deficit in BDNF expression in the cerebral cortex (Singh et al., 1995), leading us to suggest that the

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incomplete restoration of BDNF levels could have been due to the lack of added progesterone, the other major ovarian hormone that also diminished following ovariectomy.

Progesterone has also been shown to exert neuroprotective effects. For example, progesterone pre-treatment protects hippocampal neurons from $FeSO_4$-, amyloid β- (Goodman et al., 1996) as well as glutamate-induced cell death (Nilsen and Brinton, 2002; Nilsen and Diaz Brinton, 2003). In addition, progesterone reduces MPTP-induced toxicity (Callier et al., 2001), a neurotoxin used in models of Parkinson's disease. Further, secondary neuronal loss following cortical contusion injury and resulting cognitive impairment was significantly reduced in mice that received progesterone treatment relative to untreated controls (Roof et al., 1994; Asbury et al., 1998). And though evidence continues to increase with respect to the ability of progesterone to protect against a variety of insults, our understanding of the precise mechanisms underlying the neuroprotective effects of progesterone remain incomplete.

The purpose of the present study was to determine whether progesterone is protective in slice cultures (organotypic explants) of the cerebral cortex. Further, given our previously described progesterone-induced phosphorylation of ERK and Akt (Singh, 2001), two key effectors of the ERK/MAPK and PI3-K pathways, we sought to determine if the activation of either of these pathways was required for progesterone's protective effects. Additionally, since our earlier work suggested that estrogen only partially normalized the ovariectomy-induced deficit in BDNF mRNA, we hypothesized

that the lack of progesterone contributed to the incomplete restoration of BDNF signal, and as such, progesterone may also increase the expression of BDNF. Our results demonstrate that progesterone is protective against glutamate-induced cytotoxicity in the organotypic explants of the cerebral cortex and that this protection was mediated by the ERK/MAPK and PI-3K/Akt pathways. Further, our data also support the ability of progesterone to increase the expression of BDNF, implicating this "genomic" mechanism as another, potentially related, mechanism by which progesterone is protective.

MATERIALS AND METHODS

Tissue Culture

Tissue Culture.

Organotypic explants were derived from ~360- μm thick hemicoronal slices of postnatal day 2 (P2) frontal and cingulate cerebral cortex (day of birth = P1), obtained from pups born of C57Bl/6J mice and maintained as roller tube cultures(Gahwiler, 1981) on rat tail collagen-coated/poly-D-lysine pre-coated glass cover slips, as previously described (Singh et al., 2000). The cultures were maintained in steroid-deficient and phenol red-free maintenance medium (25% heat inactivated horse serum (Sigma, St. Louis, MO), 22.5% Hank's Balanced Salt Solution (Mediatech, Inc., Herndon, VA), 50% minimum essential medium Eagle MEM (Sigma), 5.25 mg/ml of D(+)-glucose (Sigma), 2 mM L-glutamine (MediaTech, Inc.), 50 μg/mL of L-ascorbic acid (Sigma) supplemented with 2 nM 17-β estradiol (Steraloids, Newport, RI). Three hemicoronal slices were placed on each coverslip per Leighton tube. Antibiotics were not used.

Treatment of Cultures

The cultures were maintained *in vitro* for 6 d. On the 6th d *in vitro* a 24 h "hormone washout" was performed, consisting of replacing the maintenance medium with media that did not contain exogenously added 17-β estradiol. On the 7th day *in vitro*, cultures were spiked with the appropriate treatment (see below) for the specified

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amount of time. Controls were also hormone deprived and sham (vehicle)-treated to account for any consequences of procedural manipulation of the explants.

Progesterone (4-pregnen-3, 20-dione, Sigma) was dissolved in DMSO to a stock concentration of 100 µM; and applied to the culture at a dilution of 1:1000, resulting in a final concentration of 100 nM. This dilution also ensured that the concentration of vehicle (DMSO) never exceeded 0.1%.

To evaluate the involvement of the ERK/MAPK or PI3-K pathways in progesterone's ability to protect cultures from L-glutamate, pharmacological inhibitors were used. UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyalthio] butadiene, Cell Signaling, Danvers, MA), a MEK1 and MEK2 inhibitor, was applied to the explants at a concentration of 10 µM for 30 min. prior to P4 pre-treatment. LY294002, (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one, Cell Signaling), a highly selective inhibitor of PI3- Kinase was applied at a concentration of 15 µM concentration for 30 min. prior to P4 pre-treatment.

Twenty-four hours after the treatment with progesterone (with or without pharmacological inhibitor), L-glutamic acid (Sigma) diluted in Gey's BSS was administered to the cultures for a duration of 6 hrs. This duration of glutamate treatment was chosen to ensure minimal degradation of the LDH in the conditioned media [$t_{1/2}$ of LDH in solution has been reported to be ~9hrs (CytoTox-ONE Homogeneous Membrane Integrity Assay Technical Bulletin #TB306, Promega, Madison, WI)].

Lactate Dehydrogenase Assay

A fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase (LDH) released from damaged or dying cells into the media. This assay for cytotoxicity was carried out according to the method provided and is based on the ability of LDH to promote the formation of a fluorescent product, resorufin, from the substrate, resazurin. Briefly, 100 μL of conditioned media was aliquoted into a black 96-well plate. The media was allowed to equilibrate to ambient temperature (~15 min), after which the CytoTox-One reagent was added to each well and incubated for 10 min at room temperature. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA). Relative fluorescent units (RFU) were normalized to μg of protein associated with the explant culture from which the conditioned media was derived. The values were expressed as a percentage of vehicle control.

BDNF Enzyme-linked Immunosorbant Assay

An enzyme-linked immunosorbant assay was used to detect and quantify total cellular BDNF levels (Promega). A 96-well Nunc MaxiSorp surface polystyrene flat bottom immuno-plate plate was pre-coated with an anti-BDNF monoclonal antibody [diluted 1:1000 in coating buffer (25 mM sodium bicarbonate and 25 mM sodium carbonate, pH 9.7)]. After rinsing off unbound antibody with TBS-T buffer (20 mM

Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween-20), and blocking the plate non-specific binding, 100 µg of sample lysate (in a final volume of 100 µL) or volume of appropriate BDNF standard, ranging in concentration from 7.8 – 500 pg/ml, was added. After 5 washes with TBS-T, the captured BDNF was then incubated with the polyclonal anti-human BDNF antibody. The amount of specifically bound polyclonal antibody was then detected through the use of the anti-IgY-horseradish peroxidase (HRP) tertiary antibody, which when treated with the chromogenic substrate (TMP reagent, Promega), changes color in proportion to the amount of BDNF present in the sample. The color intensity was quantified by measuring the absorbance at 450 nm using a Viktor3 ELISA plate reader (Perkin Elmer). Only values that were within the linear range of the standard curve were considered valid. BDNF levels were reported as % of vehicle control.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA isolation and cDNA synthesis.

Total RNA was extracted from explant cultures and DNase-treated using the RNeasy Lipid Kit (Qiagen) according to manufacture's instructions. RNA concentrations of extracted RNA were calculated from the absorbance at 260 nm. The quality of RNA was assessed by absorption at 260 nm and 280 nm (A_{260}/A_{280} ratios of 1.9 to 2.10 were considered acceptable) and by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S rRNA bands were visualized under UV light. Total RNA (1.5 µg) was reverse transcribed into cDNA in a total volume of 50 µL using the

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High Capacity DNA Archive Kit (Applied Biosystems) according to manufacture's instructions.

Primers and Probes for Quantitative Real-Time Reverse Transcription PCR.

PCR primers and probes for the target gene, BDNF, and the endogenous control, 18S rRNA, were purchased as Assays-On-Demand (Applied Biosystems). The assays were supplied as 20X mix of PCR primers (900 nM) and TaqMan probes (200 nM). The BDNF assay (Mm00432069_m1) contained FAM (6-carboxy-fluorescein phosphoramidite) dye label at the 5' end of the probes and minor groove binder and nonfluorescent quencher at the 3'end of the probes. The 18S rRNA assays (4319413E) contained VIC-labeled probes. The assays are optimized for use on ABI prism Sequence Detection System using the default machine settings.

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The reaction mixture containing water, 2X qPCR™ Master Mix (Eurogentec), and 20X Assay-On-Demand for BDNF was prepared. A separate reaction mixture was prepared for the endogenous control, 18S rRNA. The reaction mixture was aliquoted in a 96-well plate and cDNA (11 ng of RNA converted to cDNA) was added to give a final volume of 30 μL. Each sample was analyzed in triplicate. Two nontemplate controls (RNase-free water) were included on each plate for reaction mixture containing BDNF and 18S rRNA assays. Amplification and detection were performed using the ABI 7300

Sequence Detection System (Applied Biosystems) with the following profile: 2 min hold at 50°C (UNG activation), 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). Sequence Detection Software 1.3 (Applied Biosystems) was used for data analysis. A threshold was determined for a gene by the software, and the point at which the amplification plot crossed the threshold was defined as the critical threshold cycle, C_T. The cycle number at which fluorescence of the reporter exceeds a fixed threshold above baseline and is inversely proportional to the logarithm of the initial number of template molecules is referred to as the C_T (Livak and Schmittgen, 2001).

Relative Quantitation of Target mRNAs

The comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to calculate the relative changes in target gene expression. In the Comparative C_T Method, the amount of target, normalized to an endogenous control (18S) and relative to a calibrator (untreated control), is given by the $2^{-\Delta\Delta C_T}$ equation. Quantity is expressed relative to a calibrator sample that is used as the basis for comparative results. Therefore, the calibrator was the baseline (control) sample and all other treatment groups were expressed as an n-fold (or %) difference relative to the calibrator (Livak and Schmittgen, 2001). The average and standard deviation of $2^{-\Delta\Delta C_T}$ was calculated for the values from three independent experiments, and the relative amount of target gene expression for each sample was plotted in bar graphs using GraphPad Prism 4 software.

Statistical Analysis.

Data from at least three independent experiments were subjected to ANOVA, followed by Tukey's post hoc analysis for the evaluation of group differences, and are presented as bar graphs depicting the means $\pm$ SEM, using GraphPad Prism 4 software (San Diego, CA).

RESULTS

Progesterone protects against glutamate – induced cytotoxicity.

In order to determine if P4 protected against an excitotoxic/oxidative insult, we pre-treated organotypic explants of the cerebral cortex with progesterone for 24 hours prior to treatment with L-glutamate (15 mM, 6 hr). P4 (100nM) significantly reduced the amount of glutamate-induced LDH release (Figure 1) while the effect of a lower concentration of P4 (10nM) did not result in a statistically significant reduction in glutamate-induced LDH release (Figure 2). The concentration of glutamate chosen for these experiments was based on concentration-response curves in which we found that concentrations between 5 and 15 mM resulted in significant (but not maximal) increases in LDH release (data not shown). Further, our data suggest that glutamate-induced cytotoxicity appears to be mediated primarily by an NMDA-receptor sensitive mechanism, as MK801 completely prevented the glutamate-induced LDH release (Figure 3).

Progesterone-induced cytoprotection is inhibited by inhibitors of MEK and PI3-kinase.

Our laboratory has previously shown that progesterone elicits the phosphorylation of ERK and Akt, key effectors of the neuroprotection-associated ERK/MAPK and PI3-K pathways, respectively, in explants of the cerebral cortex (Singh, 2001). In order to determine if the activation of such signaling pathways was relevant to the protection

afforded by progesterone treatment, we determined if inhibitors of these pathways would alter progesterone's ability to exert its protective effects. Pre-treatment of the cortical cultures with the MEK inhibitor (UO126, 10μM) significantly attenuated progesterone's ability to protect against glutamate-induced LDH release (Figure 4). Similarly, pre-treatment of the cortical cultures with the PI3-K inhibitor also attenuated progesterone's ability to protect against glutamate-induced LDH release (Figure 5). Neither the vehicle (0.1% dimethylsulfoxide), UO126 or LY294002 alone were cytotoxic after 30 hrs of treatment (from time of initial application to the time of insult).

Progesterone elicits an increase in both BDNF protein and mRNA.

We have previously shown that ovariectomy results in a substantial decrease in the expression of BDNF mRNA in both the hippocampus and cerebral cortex (Singh et al., 1995). Interestingly, however, estrogen replacement was able to only partially restore BDNF levels in the cerebral cortex (Singh et al., 1995), leading us to hypothesize that this incomplete normalization of BDNF mRNA may have been due to the omission of progesterone in the replacement paradigm. As such, we evaluated the effect of progesterone treatment, at a concentration and duration of treatment consistent with the cytoprotection studies above, on the expression of BDNF mRNA and protein in explants of the cerebral cortex. Using real time RT-PCR, our results showed that progesterone induces an approximately 75% increase in BDNF mRNA expression (Figure 6). This effect size that was nearly identical to that seen for BDNF protein levels (Figure 7).

DISCUSSION

Numerous studies have been published that support the neuroprotective effects of estrogen. However, only recently has the role of the other major ovarian hormone, progesterone, received attention with respect to its neuroprotective effects. An important driving force behind the need to study the neurobiology of progesterone more completely is the fact that the molecular, biochemical and behavioral deficits that have been reported to result following ovariectomy, and additionally, the increased risk for AD following the menopause, may not only be a consequence of estrogen loss, but could also be due to a loss in circulating progesterone. This is because ovariectomy, like the menopause, results in a precipitous decline in not only circulating estrogen, but also in levels of progesterone. As such, progesterone may have beneficial effects on its own.

We and others have previously reported that progesterone elicits the phosphorylation of extracellular-signal regulated kinase (ERK), a signaling protein within the Ras/Raf/MAP Kinase (MAPK) pathway (Singh, 2001; Nilsen and Brinton, 2002). In addition, we have also shown that progesterone elicits the phosphorylaiton of Akt, a key effector of the phosphoinositide (PI)-3 kinase pathway in cerebral cortical explants (Singh, 2001). Both these pathways have been implicated in mechanisms of neuroprotection (Singer et al., 1996; D'Mello et al., 1997; Datta et al., 1997; Dudek et al., 1997; Mills et al., 1997; Desdouits-Magnen et al., 1998; Yan and Greene, 1998; Klesse et

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al., 1999; Singer et al., 1999). In the present study, we demonstrated that progesterone protected organotypic explants of the cerebral cortex against glutamate-induced cytotoxicity. Moreover, we found that this protection was mediated by the ERK/MAPK and PI3-K signaling pathways since pharmacological inhibitors of either of these pathways effectively inhibited progesterone's protective effects.

The model in which we chose to assess the protective effects of progesterone (organotypic explants) has the advantage of retaining the cytoarchitecture of the brain, at least within the confines of the slice, and as such, complements previous published work that demonstrates the ability of progesterone to protect in primary dissociated cultures of the brain (Nilsen and Brinton, 2002). The co-existence of both neurons and glia in this model, however, did require a higher concentration of glutamate to elicit cell damage/death. While primary dissociated cells typically require mid-micromolar concentrations of glutamate to promote cytotoxicity, concentration response curves for glutamate-induced LDH release in cerebral cortical explants revealed that only low millimolar concentrations of glutamate were effective (data not shown). With regards to the mechanism of glutamate induced toxicity in this tissue culture system, our data strongly implicate the NMDA receptor as the primary mediator (Figure 3). As such, progesterone's protective effects may target the NMDA receptor.

To further explore the mechanism by which progesterone protects against glutamate induced LDH release, we addressed the ability of progesterone to regulate BDNF levels. Estrogen has been demonstrated to regulate the levels of neurotrophins in a

variety of experimental models, including the ovariectomized rodent. However, there is a paucity of information on the effect of progesterone alone in this regard. Further, given our previous data that showed an incomplete restoration of BDNF mRNA in the cerebral cortex of estrogen-treated, ovariectomized animals, we proposed that progesterone may play an important role in upregulating BDNF levels. As such, we evaluated if a correlation existed between progesterone's protective effects and its ability to increase the expression of the neurotrophin, BDNF. We found, for the first time, that progesterone elicits an increase in both BDNF protein and mRNA levels. These data expand our understanding of progesterone neurobiology and offer another plausible mechanism through which progesterone may have protective effects. Knowledge of these novel mechanisms of action will be instrumental towards developing novel neuroprotective strategies for treating brain injury or age-associated disease pathologies of the brain, including Alzheimer's disease.

ACKNOWLEDGEMENTS

FIGURES

Figure 1. Progesterone protects against glutamate-induced LDH release.

Cerebral cortical explants were pre-treated with P4 (100nM) for 24 hr prior to the administration of L-glutamate, 10 mM or 15 mM for 6 hr. P4 prevented glutamate-induced LDH release. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). LDH release, the marker of cytotoxicity, is expressed as a percentage of vehicle control. The graph shown represents data from three independent experiments. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.05$ and **, $p < 0.001$).

Figure 2. 100 nM progesterone protects against glutamate-induced LDH release.

Cerebral cortical explants were pre-treated with either 100 nM or 10 nM P4 for 24 hr prior to the administration of L-glutamate, 5 mM for 6 hr. 100 nM P4 prevented glutamate-induced LDH release whereas 10 nM P4 did not. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). LDH release, the marker of cytotoxicity, is expressed as a percentage of vehicle control. The graph shown represents data from two independent experiments. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (**, $p < 0.001$).

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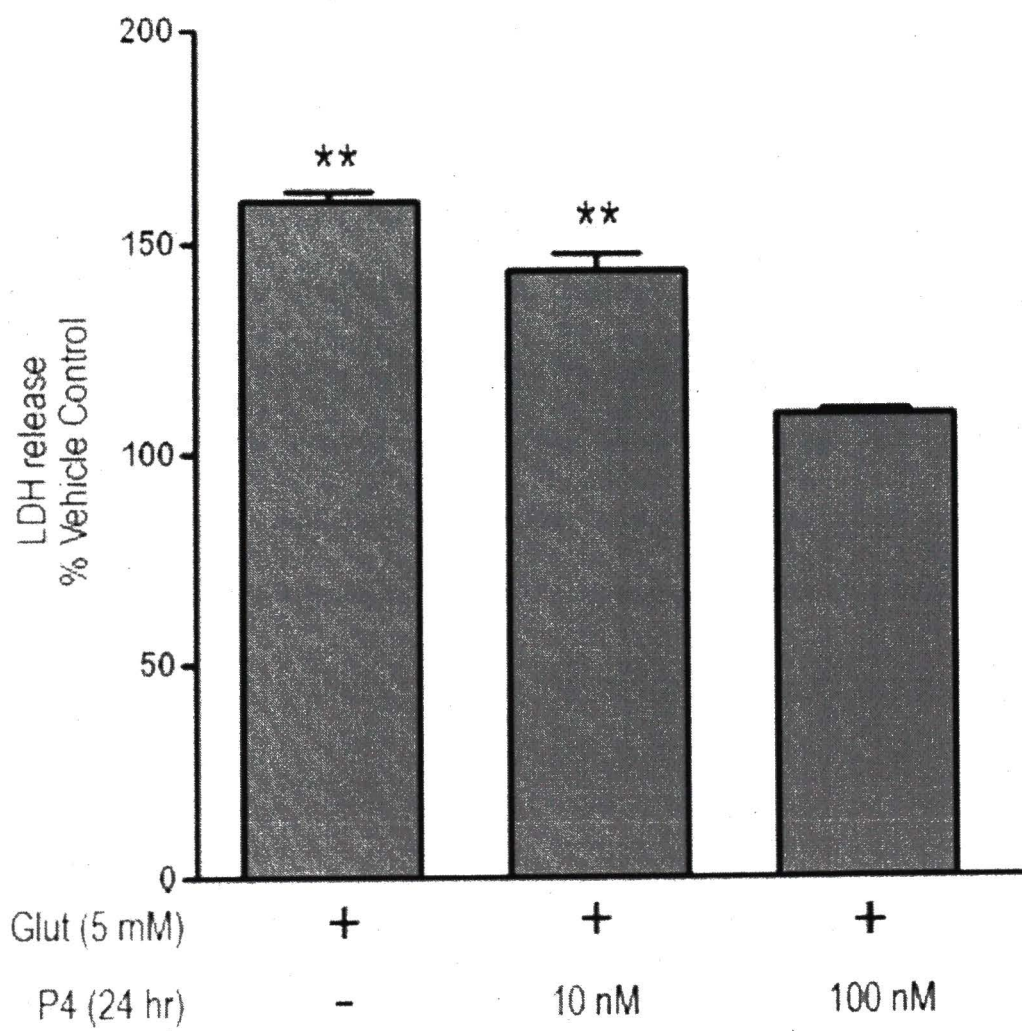
Figure 3. L-glutamate induces cellular death mediated by the NMDA receptor.

Cerebral cortical explants were pre-treated with the NMDA receptor antagonist, MK801 (10 or 20 μM), for 10 min. prior to the administration of L-glutamate (10 mM or 15 mM for 6 hr). MK801 prevented glutamate – induced LDH release. MK801 binds to a site located within the NMDA-associated ion channel reducing the ability of glutamate to induce LDH release. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). LDH release, the marker of cytotoxicity, is expressed as a percentage of vehicle control. The graph shown represents data from two independent experiments. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.001$).

Glut (15 mM)	+	+	+	+	−	−	−
P4 (100 nM)	−	+	−	−	−	−	+
MK801	−	−	10 μM	20 μM	10 μM	20 μM	−
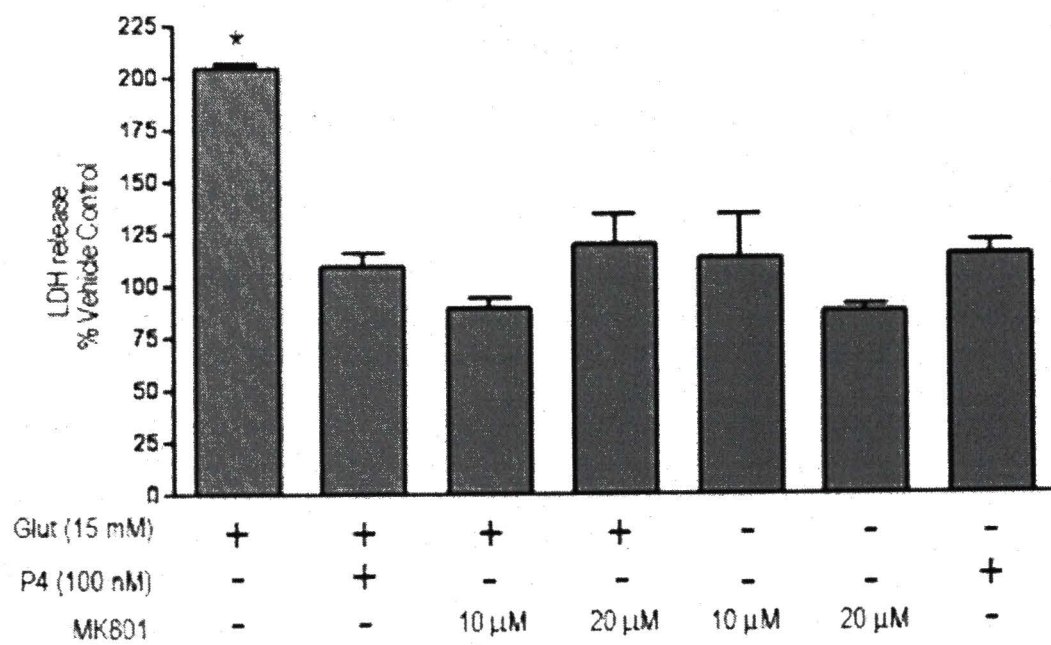
Figure 4. Progesterone- induced protection is dependent on MEK 1/2 kinase activity. Cerebral cortical explants were pre-treated with the MEK1/2 inhibitor, UO126 (10 μM, 30min), prior to the administration of 100 nM progesterone (P4) for 24 h prior to insult with glutamate (Glu, 10 mM, 6 hr). UO126 reduced the ability of P4 to protect against glutamate-induced LDH release. LDH release, the marker of cytotoxicity, is expressed as a percentage of vehicle control. The graph shown represents data averaged from two or three independent experiments. Data involving concentrations of 10 mM glutamate were performed in triplicate whereas data accrued for concentrations of 15 mM glutamate were performed in duplicate. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.05$ and **, $P < 0.001$).
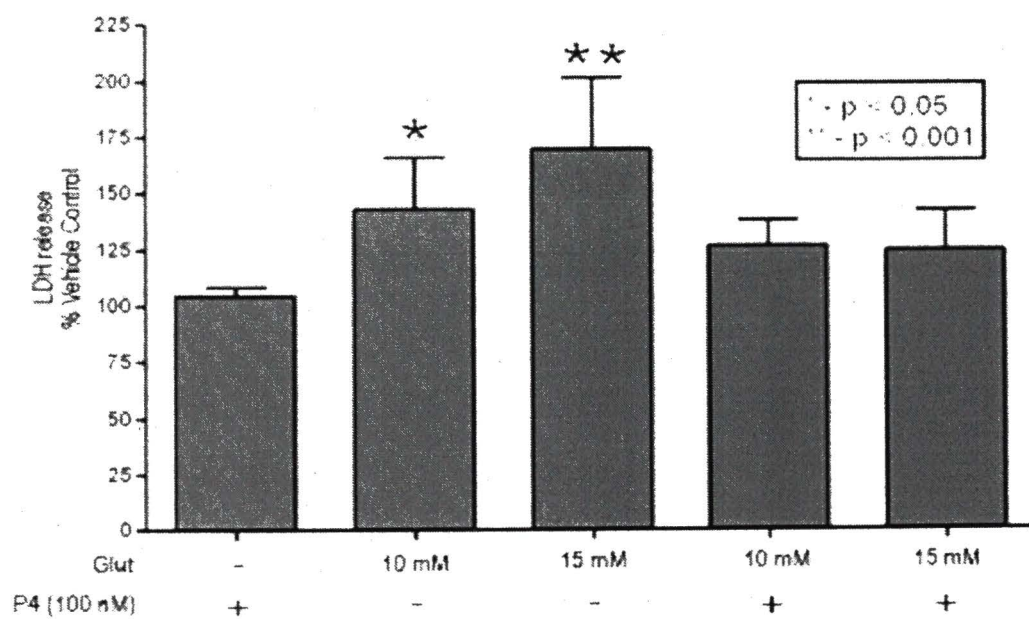
Glut	10 mM	15 mM	10 mM	15 mM	10 mM	15 mM	10 mM	15 mM	–
P4 (100 nM)	–	–	+	+	+	+	–	–	–
UO126 (10 μM)	–	–	–	–	+	+	+	+	+
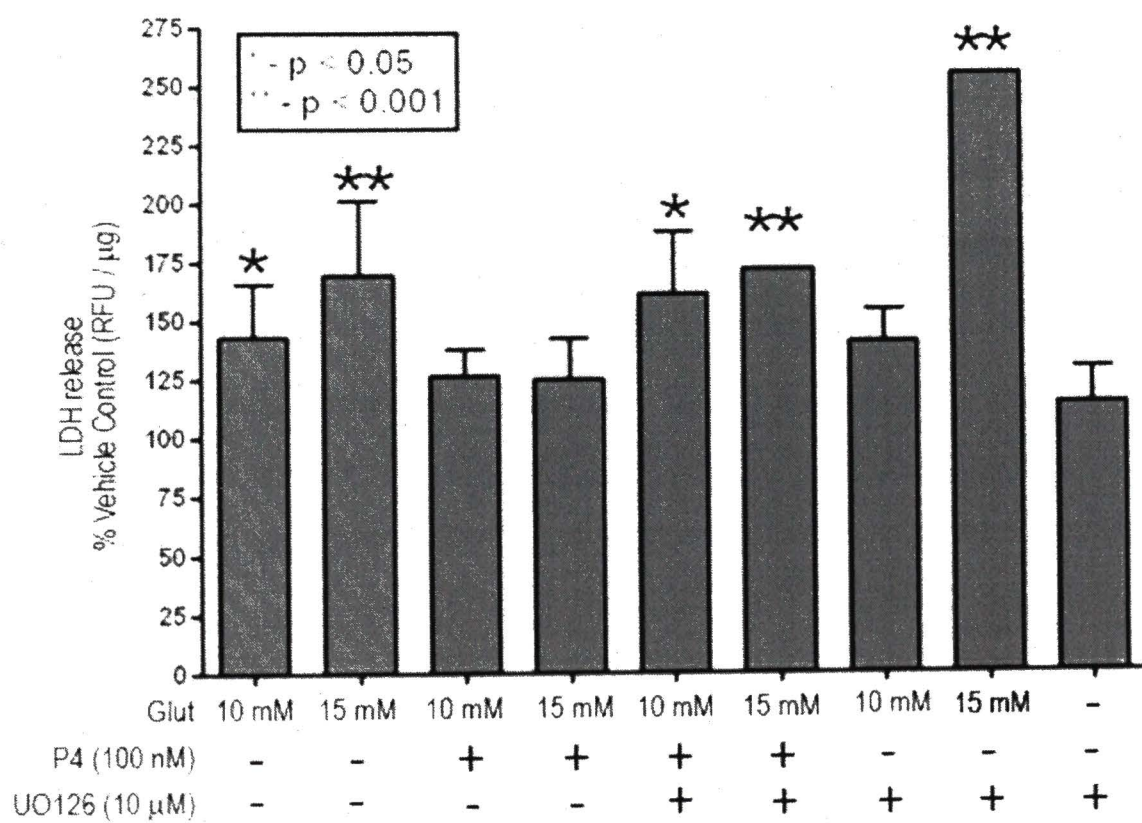
Figure 5. Progesterone-induced protection is dependent on the PI-3K activity.

Cerebral cortical explants were pre-treated with the PI3-kinase inhibitor, LY294002 (15 µM, 30min), prior to the administration of 100 nM progesterone (P4) for 24 h prior to insult with glutamate (Glu, 10 mM, 6 hr). LY294002 reduced the ability of P4 to protect against glutamate-induced LDH release. LDH release, the marker of cytotoxicity, is expressed as a percentage of vehicle control. The graph shown represents data averaged from either two or three independent experiments. Data involving concentrations of 10 mM glutamate were performed in triplicate whereas data accrued for concentrations of 15 mM glutamate were performed in duplicate. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.05$ and **, $p < 0.001$).
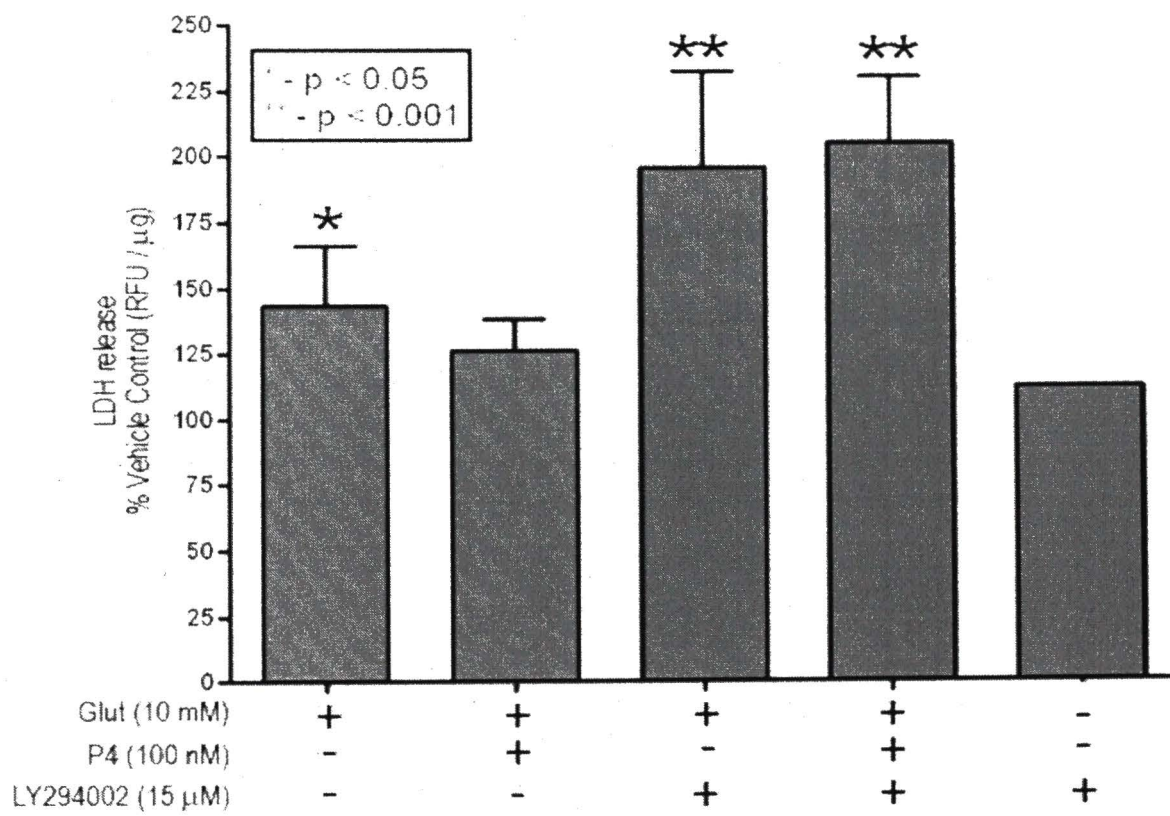
Figure 6. Progesterone elicits an increase in total cellular BDNF content.

BDNF protein levels were assessed in cerebral cortical explants treated with progesterone (P4, 100 nM) for 18 hr. Using an enzyme-linked immunosorbent assay (ELISA), we found that P4 induced an increase in BDNF expression. Statistical significance was determined using a single sample t-test (*: $p \leq 0.05$). Data are presented as mean $\pm$ SEM of three different experiments, performed independently.
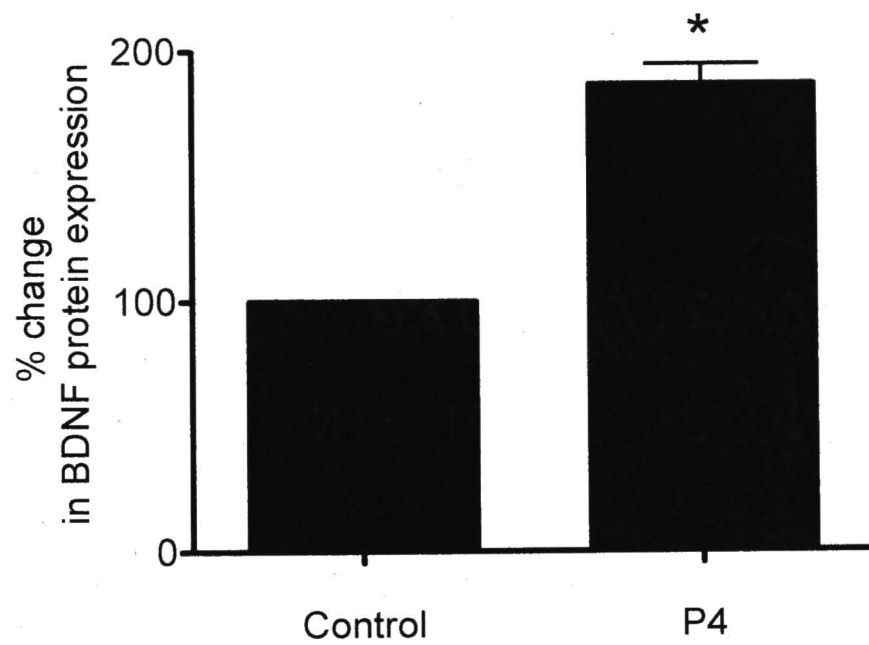
Figure 7. Progesterone elicits an increase in BDNF mRNA.

BDNF mRNA was assessed in cerebral cortical explants treated with progesterone (P4, 100nM) for 18 hr. Using real time RT-PCR, we found that both P4 induced an increase in BDNF mRNA. Statistical significance was determined using a single sample t-test (*: $p \leq 0.05$). Data are presented as mean ± SEM of four different experiments, performed independently.

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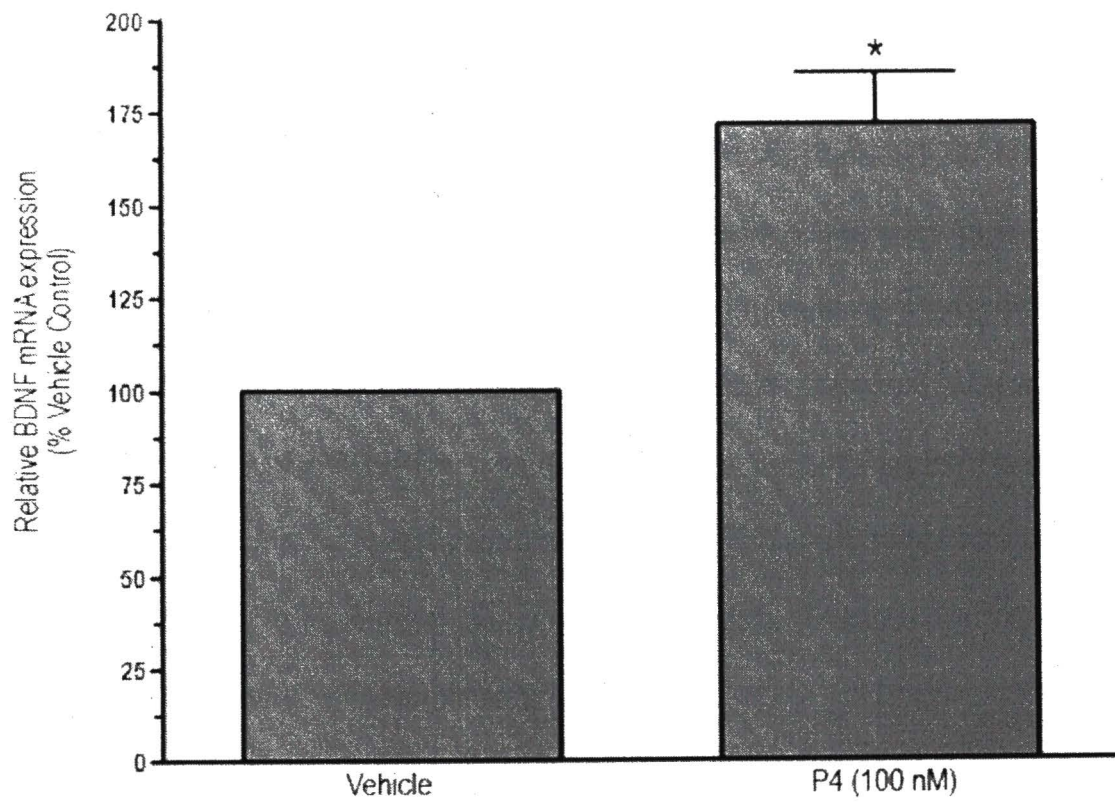
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CHAPTER III

PROGESTERONE AND MEDROXYPROGESTERONE ACETATE DIFFER IN THEIR ABILITY TO PROTECT AGAINST GLUTAMATE TOXICITY IN CEREBRAL CORTICAL EXPLANTS.

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Number of Tables: n/a

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Abbreviations: BDNF: Brain-derived neurotrophic factor; ERK: extracellular-signal regulated kinase; MAPK: mitogen-activated protein kinase; MEK: MAPK/ERK kinase; MPA: medroxyprogesterone acetate; P4: progesterone; PI3-K: phosphatidylinositol-3 kinase; RT-PCR: reverse transcriptase-polymerase chain reaction.

ABSTRACT

Women have a higher prevalence for Alzheimer's disease (AD) than men, suggesting that the precipitous decline in gonadal hormone levels following the menopause may contribute to the risk of developing AD. However, principal results from the Women's Health Initiative concluded that women taking conjugated equine estrogens combined with medroxyprogesterone acetate (MPA, tradename: Prempro) incurred more harmful than beneficial outcomes versus the placebo group (Rossouw et al., 2002). To determine if the discrepancy between basic science reports and these clinical studies could have been due to the synthetic progestin, MPA, we sought to distinguish the protective effects of the natural, P4 from MPA, and explore potential mechanistic differences in its actions in the brain. Using organotypic explants of the cerebral cortex, we found that both P4 and MPA elicit the phosphorylation of ERK and Akt, two signaling pathways implicated in neuroprotection. Interestingly, P4 (100 nM) protected against glutamate- induced toxicity however, while MPA (100 nM) did not. Further, P4 seemed to result in an increase in BDNF, while MPA did not. Collectively, these data suggest that P4 is protective via multiple, and potentially related mechanisms, and importantly, its neurobiology is different from the clinically used progestin, MPA.

Section: Cellular and Molecular Biology of Nervous Systems

Keywords: Progesterone, MPA, ERK, PI3-K, BDNF, organotypic explants.

INTRODUCTION

In order to alleviate the symptoms of the menopause, clinicians have widely prescribed the use of hormone therapy such that an estimated 37.6% of all post-menopausal women in the United States used hormone therapy in 1999 (Keating et al., 1999). Basic research in this area also supports the beneficial effects of estrogen against a wide variety of injuries such as oxidative stress, excitotoxic insults and β – amyloid in the *in vitro* models of the brain (Wise, 2002; Simpkins et al., 2004; Simpkins et al., 2005). In contrast, principal results from the Women's Health Initiative (WHI) concluded that women taking conjugated equine estrogens combined with MPA (Prempro) had an increased risk of invasive breast cancer, coronary heart disease (CHD) including myocardial infarction, stroke, pulmonary emboli, and deep vein thrombosis than placebo (Rossouw et al., 2002). Additional studies published from the WHI investigating the effect of treatment with conjugated equine estrogen and MPA on central nervous system (CNS) outcomes (Shumaker et al., 2003; Espeland et al., 2004; Shumaker et al., 2004) concluded that women who took hormones for an average of more than four yearsdoubled their risk of developing Alzheimer's disease (AD) or other forms of dementia, compared with those on placebo.

The discrepancies between the basic research and earlier clinical trials that supported the potential benefit of hormone therapy and the published results of the WHI may be attributed to many aspects of the trial. We hypothesize that the choice of

progestin included in this study may have contributed to the negative outcomes of the trial.

The purpose of the present study was to determine whether progesterone and/or medroxyprogesterone acetate is protective in slice cultures (organotypic explants) of the cerebral cortex. Further, we sought to determine if differences exist in the ability of P4 and MPA to elicit the ERK and/or Akt signaling pathways. Additionally, since our earlier work suggested that estrogen replacement only partially normalized the ovariectomy-induced deficit in BDNF mRNA seen in rodents (Singh et al., 1995). We hypothesized that the incomplete restoration of BDNF signal was a result of not replacing the other major ovarian hormone that declines following ovariectomy, progesterone. Our results demonstrate that progesterone is protective against glutamate-induced cytotoxicity in the organotypic explants of the cerebral cortex whereas MPA is not. Further, our data also supports the ability of progesterone to increase the expression of BDNF, implicating this "genomic" mechanism as another, potentially related, mechanism by which progesterone is protective. However, the ability to promote the expression of BDNF mRNA did not qualify a progestin to be protective as demonstrated by MPA's ability to increase BDNF mRNA expression, and yet not have the ability to protect against glutamate induced cytotoxicity.

MATERIALS AND METHODS

Tissue Culture

Organotypic explants were derived from ~360- μm thick hemicoronal slices of postnatal day 2 (P2) frontal and cingulate cerebral cortex (day of birth = P1), obtained from pups born of C57B1/6J mice and maintained as roller tube cultures(Gahwiler, 1981) on rat tail collagen-coated/poly-D-lysine pre-coated glass cover slips, as previously described (Singh et al., 2000). The cultures were maintained in steroid-deficient and phenol red-free maintenance medium (25% heat inactivated horse serum (Sigma, St. Louis, MO), 22.5% Hank's Balanced Salt Solution (Mediatech, Inc., Herndon, VA), 50% minimum essential medium Eagle MEM (Sigma), 5.25 mg/mL of D(+)-glucose (Sigma), 2 mM L-glutamine (MediaTech, Inc.), 50 μg/mL of L-ascorbic acid (Sigma) supplemented with 2 nM 17-β estradiol (Steraloids, Newport, RI). Three hemicoronal slices were placed on each coverslip per Leighton tube. Antibiotics were not used.

Treatment of Cultures.

The cultures were maintained *in vitro* for 6 d. On the 6th d *in vitro* a 24 h "hormone washout" was performed, consisting of replacing the maintenance medium with media that did not contain exogenously added 17-β estradiol. On the 7th day *in vitro*, cultures were spiked with the appropriate treatment (see below) for the specified

amount of time. Controls were also hormone deprived and sham (vehicle)-treated to account for any consequences of procedural manipulation of the explants.

Progesterone (4-pregnen-3, 20-dione, Sigma) and medroxyprogesterone acetate (6α-methyl - 17α –hydroxy-progesterone acetate, Depo-provera, Sigma) were dissolved in DMSO to a stock concentration of 100 μM; and applied to the culture at a dilution of 1:1000, resulting in a final concentration of 100nM. This dilution also ensured that the concentration of vehicle (DMSO) never exceeded 0.1%.

Twenty-four hours after the treatment with P4 or MPA, L-glutamic acid (Sigma) diluted in Gey's BSS was administered to the cultures for the duration of 6 hrs. This duration of glutamate treatment was chosen to ensure minimal degradation of the LDH in the conditioned media [$t_{1/2}$ of LDH in solution has been reported to be ~9hrs (CytoTox-ONE Homogeneous Membrane Integrity Assay Technical Bulletin #TB306, Promega, Madison, WI)].

Lactate Dehydrogenase Assay

A fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase (LDH) released from damaged or dying cells into the media. This assay for cytotoxicity was carried out according to the method provided and is based on the ability of LDH to promote the formation of a fluorescent product, resorufin, from the substrate, resazurin. Briefly, 100 μL of conditioned media was aliquoted into a black 96-well plate. The media was

allowed to equilibrate to ambient temperature (~15 min), after which the CytoTox-One reagent was added to each well and incubated for 10 min at room temperature. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor^3 ELISA plate reader (Perkin Elmer, Boston, MA). Relative fluorescent units (RFU) were normalized to µg of protein associated with the explant culture from which the conditioned media was derived. The values were expressed as a percentage of vehicle control.

BDNF Enzyme-linked Immunosorbant Assay.

An enzyme-linked immunosorbant assay was used to detect and quantify total cellular BDNF levels (Promega). A 96-well Nunc MaxiSorp surface polystyrene flat bottom immuno-plate plate was pre-coated with an anti-BDNF monoclonal antibody [diluted 1:1000 in coating buffer (25 mM sodium bicarbonate and 25 mM sodium carbonate, pH 9.7)]. After rinsing off unbound antibody with TBS-T buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween-20), and blocking the plate for non-specific binding, 100 µg of sample lysate (in a final volume of 100 µL) or volume of appropriate BDNF standard, ranging in concentration from 7.8 – 500 pg/mL, was added. After 5 washes with TBS-T, the captured BDNF was then incubated with the polyclonal anti-human BDNF antibody. The amount of specifically bound polyclonal antibody was then detected through the use of the anti-IgY-horseradish peroxidase (HRP) tertiary antibody, which when treated with the chromogenic substrate (TMP reagent, Promega),

changes color in proportion to the amount of BDNF present in the sample. The color intensity was quantified by measuring the absorbance at 450 nm using a Viktor[3] ELISA plate reader (Perkin Elmer). Only values that were within the linear range of the standard curve were considered valid. BDNF levels were reported as % of vehicle control.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA isolation and cDNA synthesis.

Total RNA was extracted from explant cultures and DNase-treated using the RNeasy Lipid Kit (Qiagen) according to manufacture's instructions. RNA concentrations of extracted RNA were calculated from the absorbance at 260 nm. The quality of RNA was assessed by absorption at 260 nm and 280 nm (A_{260}/A_{280} ratios of 1.9 to 2.10 were considered acceptable) and by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S rRNA bands were visualized under UV light. Total RNA (1.5 μg) was reverse transcribed into cDNA in a total volume of 50 μL using the High Capacity DNA Archive Kit (Applied Biosystems) according to manufacture's instructions.

Primers and Probes for Quantitative Real-Time Reverse Transcription PCR.

PCR primers and probes for the target gene, BDNF, and the endogenous control, 18S rRNA, were purchased as Assays-On-Demand (Applied Biosystems). The assays were supplied as 20X mix of PCR primers (900 nM) and TaqMan probes (200 nM). The

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BDNF assay (Mm00432069_m1) contained FAM (6-carboxy-fluorescein phosphoramidite) dye label at the 5' end of the probes and minor groove binder and nonfluorescent quencher at the 3'end of the probes. The 18S rRNA assays (4319413E) contained VIC-labeled probes. The assays are optimized for use on ABI prism Sequence Detection System using the default machine settings.

Quantitative Real-Time Reverse Transcription-PCR.

The reaction mixture containing water, 2X qPCR^{TM} Master Mix (Eurogentec), and 20X Assay-On-Demand for BDNF was prepared. A separate reaction mixture was prepared for the endogenous control, 18S rRNA. The reaction mixture was aliquoted in a 96-well plate and cDNA (11 ng of RNA converted to cDNA) was added to give a final volume of 30 µL. Each sample was analyzed in triplicate. Two nontemplate controls (RNase-free water) were included on each plate for reaction mixture containing BDNF and 18S rRNA assays. Amplification and detection were performed using the ABI 7300 Sequence Detection System (Applied Biosystems) with the following profile: 2 min hold at 50°C (UNG activation), 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). Sequence Detection Software 1.3 (Applied Biosystems) was used for data analysis. A threshold was determined for a gene by the software, and the point at which the amplification plot crossed the threshold was defined as the critical threshold cycle, C_T. The cycle number at which fluorescence of the reporter exceeds a fixed threshold above baseline and is

inversely proportional to the logarithm of the initial number of template molecules is referred to as the C_T (Livak and Schmittgen, 2001).

Relative Quantitation of Target mRNAs

The comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to calculate the relative changes in target gene expression. In the Comparative C_T Method, the amount of target, normalized to an endogenous control (18S) and relative to a calibrator (untreated control), is given by the $2^{-\Delta\Delta C_T}$ equation. Quantity is expressed relative to a calibrator sample that is used as the basis for comparative results. Therefore, the calibrator was the baseline (control) sample and all other treatment groups were expressed as an n-fold (or %) difference relative to the calibrator (Livak and Schmittgen, 2001). The average and standard deviation of $2^{-\Delta\Delta CT}$ was calculated for the values from three independent experiments, and the relative amount of target gene expression for each sample was plotted in bar graphs using GraphPad Prism 4 software.

Statistical Analysis

Data from at least three independent experiments were subjected to ANOVA, followed by Tukey's post hoc analysis for the evaluation of group differences, and are presented as bar graphs depicting the means ± SEM, using GraphPad Prism 4 software (San Diego, CA).

RESULTS

Progesterone and medroxyprogesterone acetate result in a time dependent increase in the phosphorylation of ERK and Akt.

A rapid onset but limited duration of ERK phosphorylation may be indicative of events leading to neuroprotection (Marshall, 1995). The concentration of P4 used in our signal transduction studies (100 nM) was chosen on the basis of previously published work that described P4's ability to protect neuronal cultures from glutamate, $FeSO_4$ and β-amyloid toxicity (Kaur et al., SFN abstract) (Goodman et al., 1996) Our laboratory has also shown that progesterone elicits the phosphorylation of ERK and Akt, key effectors of the cytoprotection-associated ERK/MAPK and PI3-K pathways, respectively, in explants of the cerebral cortex (Singh, 2001). Figure 1 demonstrates the ability of P4 to elicit a time-dependent increase in the phosphorylation of ERK, a pattern consistent with our laboratory's previously published observations (Singh, 2001) and extends this earlier findingfinding by demonstratinging that MPA also elicits ERK in a similar time-dependent manner. An increase in ERK phosphorylation was evident within 5 min., became maximal between 15 and 30 min and declined (though still above baseline) thereafter.

100 nM progesterone and medroxyprogesterone acetate maximally phosphorylated ERK.

In order to determine if P4 and MPA exhibited different concentration-response profiles with respect to ERK phosphorylation, a concentration-response analysis was performed. The previous figure (Figure 1) demonstrated that P4 and MPA resulted in maximal phosphorylation at 15 min. A concentration-response for ERK phosphorylation therefore at the 15 min. time point was executed. Cerebral cortical explants were treated with 1 nM, 10 nM, 100 nM, and 1 μM medroxyprogesterone acetate for 15 min., resulting in a phosphorylation of ERK1, ERK2 and Akt. MPA elicited a concentration-dependent increase in ERK and Akt phosphorylation, although at micromolar concentrations of MPA, an inhibition of ERK and Akt phosphorylation was also observed. The lower panel represents the reprobing of the ERK1/2 blot for actin to verify equal loading of protein across the lanes. Densitometric representation of the relative intensities of phosphorylated ERK2 is also included in panel B. The results revealed that at the concentration of 100 nM both progesterone (Figure 2) and medroxyprogesterone acetate (Figure 3) maximally phosphorylated ERK.

Progesterone protects against glutamate-induced cytotoxicity whereas medroxyprogesterone acetate does not.

Based on the observation that P4 and MPA had a relatively similar ability to elicit the phosphorylation of ERK and Akt, two signaling effectors of the neuroprotective ERK/MAPK and PI3-K pathways, respectively, we predicted that both P4 and MPA would be equally protective against glutamate-induced cytotoxicity. We examined if, at

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the concentrations of steroids that resulted in the maximal phosphorylation of ERK and Akt, these hormones resulted in protection against glutamate-induced cytotoxicity. Pre-treatment of the cortical cultures with progesterone (100 nM) significantly attenuated glutamate-induced LDH release (Figure 4) while an equimolar concentration of did not (Figure 5).

Progesterone elicits BDNF mRNA whereas medroxyprogesterone acetate cannot.

We have previously shown that ovariectomy results in a substantial decrease in the expression of BDNF mRNA in both the hippocampus and cerebral cortex (Singh et al., 1995). Interestingly, however, estrogen replacement was able to only partially restore BDNF levels in the cerebral cortex (Singh et al., 1995), leading us to hypothesize that this incomplete normalization of BDNF mRNA may have been due to the omission of progesterone in the replacement paradigm. As such, we evaluated the effect of progesterone treatment, at a concentration and duration of treatment consistent with the cytoprotection studies above, on the expression of BDNF mRNA and protein in explants of the cerebral cortex. Using real time RT-PCR, our results showed that progesterone induces an approximately 75% increase in BDNF mRNA expression (Figure 6). Interestingly, despite the inability of MPA to protect cortical explants from glutamate-induced cytotoxicity, we found that MPA (100 nM for 18 hours) resulted in approximately 78% increase in BDNF mRNA expression (Figure 6).

DISCUSSION

Within this paper, we effectively demonstrate that the progestins, MPA and P4, are not equivalent when considering their ability to protect against glutamate induced cytotoxicity. This is despite the fact that both MPA and P4 induce similar patterns of ERK and Akt phosphorylation. Our results are similar to those reported in a study using primary dissociated hippocampal neurons. In this study, Nilsen and Brinton revealed that both P4 and MPA elicited ERK phosphorylation, yet only P4 was protective (Nilsen and Brinton, 2003). Further exploration revealed that though the temporal pattern of ERK phosphorylation was similar between P4 and MPA, only P4 promoted the nuclear translocation of ERK (Nilsen and Brinton, 2003). Thus, one possible reason for the difference in the ability of P4 and MPA to protect against cytotoxicity in our cortical explant model may have been related to the subcellular localization of the phosphorylated ERK. Therefore, the phosphorylation of ERK is necessary yet not sufficient for the cytoprotection.

P4 has the ability to be converted to the potentially neuroprotective neurosteroid, allopregnanolone (3α, 5α-THP). P4's 5α reduced metabolite, is a potent allosteric enhancer of GABA-gated currents. Therefore such a mechanism would counter glutamate induced cytotoxicity. MPA does not get metabolized to allopregnanolone and therefore would be able to impose neuroprotection via this mechanism.

The explant model system is a complex model system that retains the cell- to- cell interactions between both the neurons and glia. Therefore to demonstrate that P4 and not MPA can protect cultures within this model system recognizes that the hormone is protective against glutamate within the confines of the slice of cortex, which includes differing cell types. Although we did not ascertain which cells were protected against glutamate induced cytotoxicity, Singh has determined that progesterone has the ability to phosphorylate ERK in neuronal populations within cerebral cortical explants (Singh, 2001). As such, we can be assured that at least neurons are responsive to progesterone treatment.

To further explore the mechanism by which progesterone protects against glutamate induced LDH release, we previously addressed the ability of progesterone to regulate BDNF levels. Estrogen has been demonstrated to regulate the levels of neurotrophins in a variety of experimental models, including the ovariectomized rodent. Based on our previous data that showed an incomplete restoration of BDNF mRNA in the cerebral cortex of estrogen-treated, ovariectomized animals, we proposed that progesterone may play an important role in upregulating BDNF levels, and that the incomplete restoration of BDNF in estrogen-alone treated animals was due to the lack of added P4. We found, for the first time, that progesterone elicits an increase in both BDNF protein and mRNA levels. This data set was expanded to explore the effects of MPA upon BDNF levels. We were able to demonstrate that MPA did not have the ability to up- regulate BDNF mRNA levels in slice cultures of the cerebral cortex as P4 did.

Mechanisms to explore the divergent mechanisms of protection by these progestins to regulate BDNF mRNA observed after incubation with the progestins is underway in the laboratory. It is plausible that MPA and P4 differ in the levels of protein expression of BDNF, the expression of pro- and mature proteins in different levels, and/ or finally the receptor expression levels of TrkB and p75. All of these scenerios are possible in addition to having differences in the regulation of the mRNA protein.

A caveat to our observation that total cellular protein is increased in response to progesterone is derived from the knowledge that neurotrophins, such as BDNF, are synthesized as precursors and cleaved to produce a mature and pro-hormone. The pro-neurotrophin is proteolytically cleaved to produce the mature protein by extracellular proteases. Pro-BDNF is thought to elicit pro-apoptotic pathways whereas the mature peptide may be involved in more survival promoting signaling. There is also evidence that BDNF has the ability to be secreted and thus may act as a signaling molecule. The pro-BDNF protein has a relatively higher binding affinity towards $p75^{NTR}$ whereas the mature cleavage product binds preferentially to the TrkB receptor (Dechant, 2001). Preferential activation of the $p75^{NTR}$ receptor has been postulated to favor the induction of apoptotic cascades, via activation of $p75^{NTR}$ and sortilin (Teng et al., 2005), whereas activation of the TrkB receptor mediates survival pathways (Lu et al., 2005). Ongoing studies in the Singh laboratory are aimed at addressing whether the effects of progesterone on total cellular BDNF reflects a change in a particular species of BDNF (i.e., pro- versus mature-BDNF).

AD patients have been observed to have decreased amounts of BDNF mRNA levels in the cortex and hippocampus (Holsinger et al., 2000). Particularly in the nucleus basalis of AD patients there is a 50% decrease in the amount of BDNF mRNA when compared to controls (Fahnestock et al., 2002). In the AD patient, an up to 40% reduction has been observed in the pro-BDNF levels of the parietal cortex as observed using western blot techniques (Fahnestock et al., 2002; Michalski and Fahnestock, 2003). Thus, the ability of progesterone to induce BDNF may have therapeutic value for treating/preventing such neurodegenerative diseases as Alzheimer's disease.

We also determined whether MPA altered BDNF levels. Preliminarily, it would appear that MPA may inhibit BDNF mRNA levels in slice cultures of the cerebral cortex (Figure 6). Such a dichotomy in the regulation of BDNF could provide an additional mechanism that explains the ability of P4 to protect and MPA's inability to do so. Additional studies are underway to explore this effect further.

Since the findings of the clinical trials, the prescribing patterns of hormone therapy have drastically changed. In 2004, there was a 46% and 28% decline in the initiation of therapies of estrogen therapy and hormone therapy (Buist et al., 2004). We hope that by analyzing the molecular differences between the progestins, MPA and progesterone, we may contribute to the understanding of the beneficial effects observed in basic research and its discordance between the clinical body of knowledge in order to change and implement the existing hormone therapies.

ACKNOWLEDGEMENTS

FIGURES

Figure 1. MPA elicits the phosphorylation of ERK and Akt.

Cerebral cortical explants were treated with 100 nM medroxyprogesterone acetate (MPA) for various lengths of time. This resulted in a time dependent increase in the phosphorylation of ERK1/2 and Akt as determined by western blot analysis. The lower blot represents the re-probe of the p-ERK blot with antibodies towards total ERK2, verifying equal loading across lanes. This blot is representative of two independent experiments.

| Vehicle | 5 | 15 | 30 | 60 | 120 | MPA (100 nM) |

— P-Akt

— P-ERK1
— P-ERK2

— ERK2

Figure 2. Concentration-response analysis for the effect of progesterone on ERK and Akt phosphorylation.

Cerebral cortical explants were treated with 1 nM, 10 nM, 100 nM, and 1 μM progesterone for 15 min., resulting in a phosphorylation of ERK1, ERK2 and Akt. P4 elicited a concentration-dependent increase in ERK and Akt phosphorylation, although at micromolar concentrations of P4, an inhibition of ERK and Akt phosphorylation was observed. The lower panel represents the reprobing of the phosphorylated ERK1/2 blot for total ERK1 protein to verify equal loading of protein across the lanes. The data shown are representative of two independent experiments.
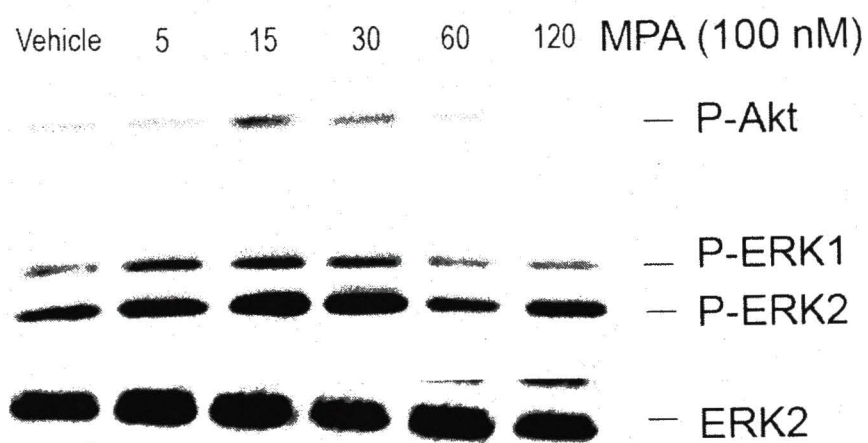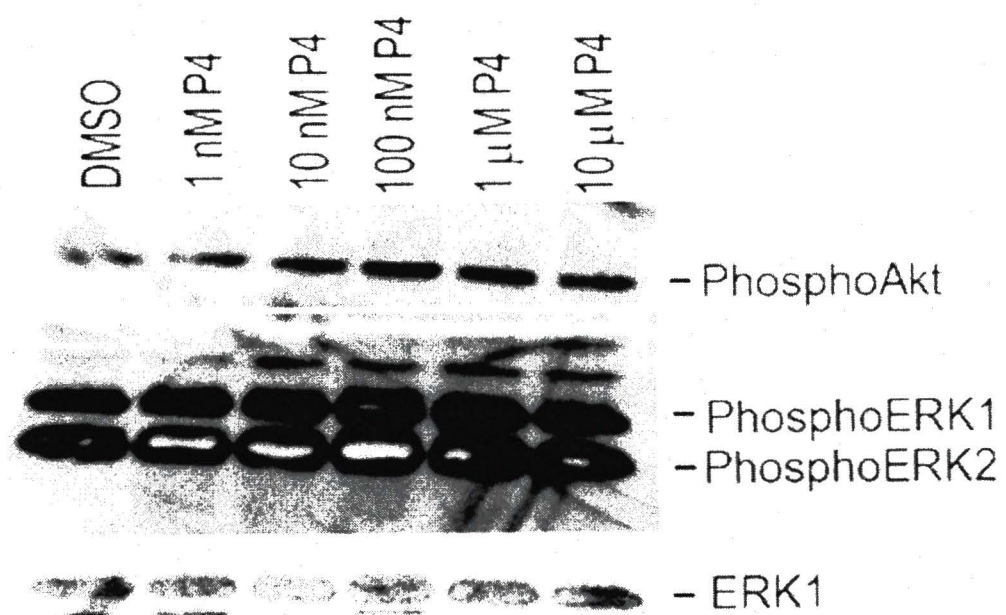
Figure 3. Concentration-response analysis for the effect of MPA on ERK and Akt phosphorylation.

Cerebral cortical explants were treated with 1 nM, 10 nM, 100 nM, and 1 μM medroxyprogesterone acetate for 15 min., resulting in a phosphorylation of ERK1, ERK2 and Akt. MPA elicited a concentration-dependent increase in ERK and Akt phosphorylation, although at micromolar concentrations of MPA, an inhibition of ERK and Akt phosphorylation was observed. The lower panel represents the reprobing of the phosphorylated ERK1/2 blot for total ERK1 protein to verify equal loading of protein across the lanes. The data shown are representative of two independent experiments.

Figure 4. Progesterone protects against glutamate-induced LDH release.

Cerebral cortical explants were pre-treated with P4 (100 nM) for 24 hr prior to the administration of L-glutamate (10 mM or 15 mM for 6 hr). P4 prevented glutamate-induced LDH release. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). The numerical values in the bar graphs represent the LDH values normalized to protein and vehicle treated controls. LDH release was expressed as a percentage of the vehicle control. The graph shown represents data from three independent experiments. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences differences (*, $p < 0.05$ and **, $p < 0.001$).

Glut   –   10 mM   15 mM   10 mM   15 mM

P4 (100 nM)   +   –   –   +   +

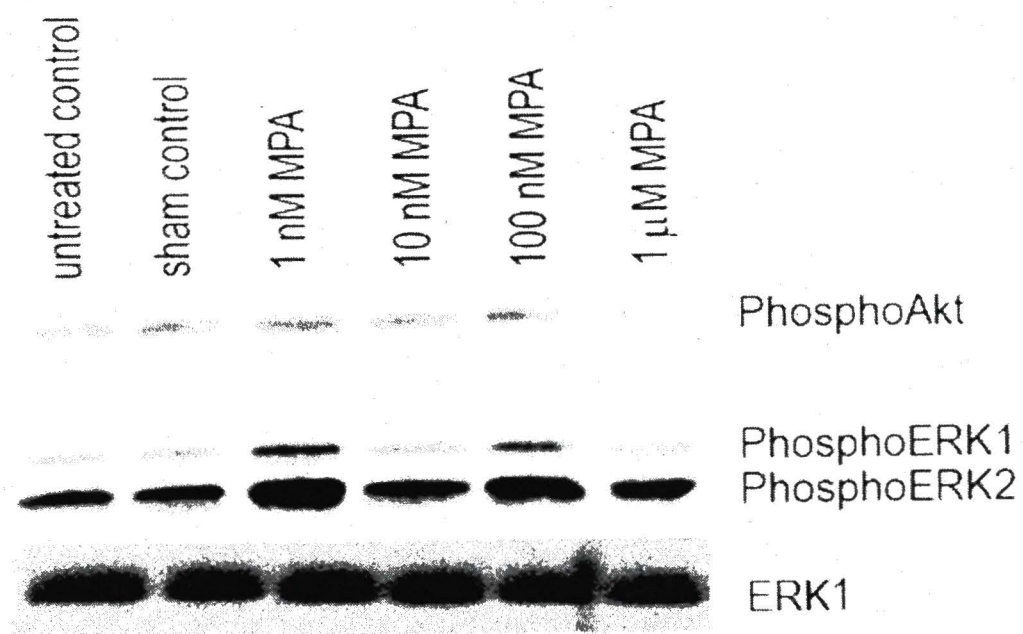Legend:
* - p < 0.05
** - p < 0.001
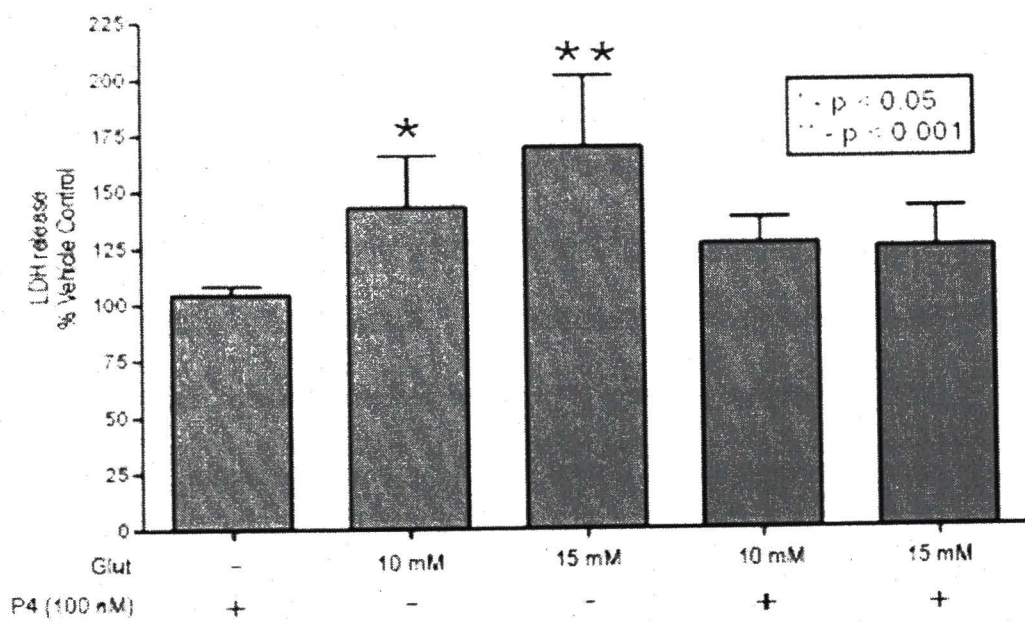
Figure 5. Medroxyprogesterone acetate fails to protect against glutamate-induced LDH release.

Cerebral cortical explants were pre-treated with MPA (100 nM) for 24 hr prior to the administration of L-glutamate (10 mM or 15 mM for 6 hr). MPA failed to prevent glutamate-induced LDH release. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr. treatment). The numerical values in the bar graphs represent the LDH values normalized to protein and vehicle treated controls. LDH release was expressed as a percentage of the vehicle control. The graph shown represents data averaged from two or three independent experiments. Data involving concentrations of 10 mM glutamate were performed in triplicate whereas data accrued for concentrations of 15 mM glutamate were performed in duplicate. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.05$ and **, $p < 0.001$).

Figure 6. Progesterone, but not MPA, elicits an increase in BDNF mRNA.

BDNF mRNA was assessed in cerebral cortical explants treated with progesterone (P4, 100 nM) or medroxyprogesterone acetate (MPA, 100 nM) for 18 hr. Using real time RT-PCR, we found that P4, but not MPA elicited an increase in BDNF mRNA. Statistical significance was determined using a t-test (*: $p \leq 0.05$). The graph shown represents data averaged three independent experiments.
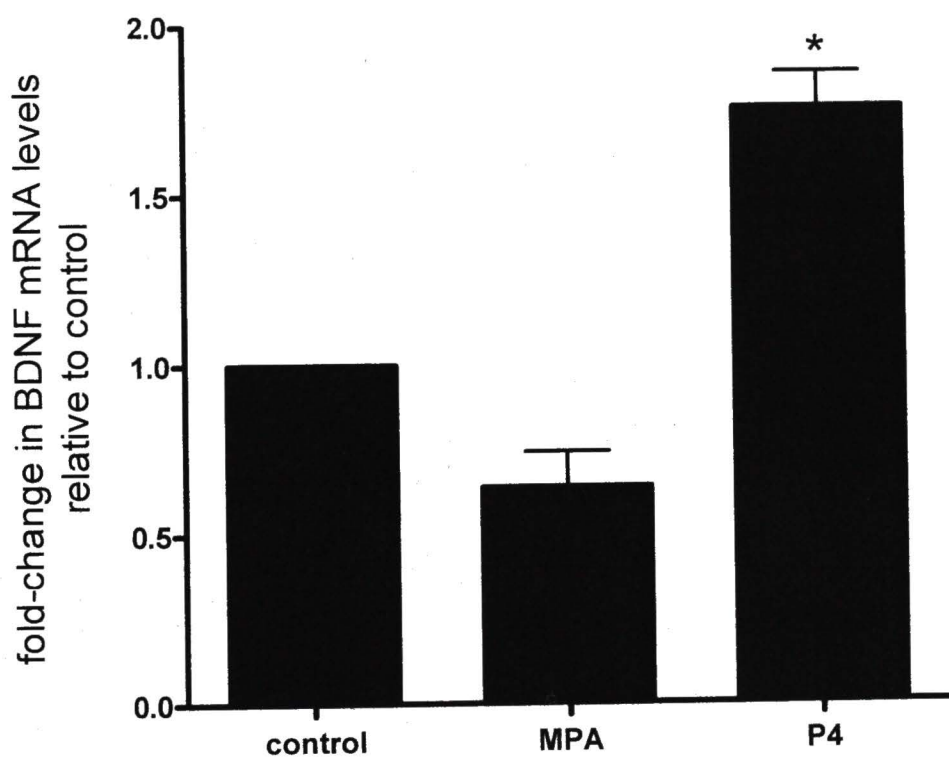
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Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN, 3rd, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J. 2003. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. Jama 289:2651-2662.

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CHAPTER IV

ADDITIONAL DATA

Figure 1. Concentration response curve for L-glutamate in cerebral cortical explants.

Increasing concentrations of L-glutamate was placed onto the cerebral cortical explants slices. Cell death was assessed for six hours after the administration of L-glutamate using the CytoTox One- Homogenous Membrane Integrity Assay kit. The numerical values in the bar graphs represent the net LDH values normalized to protein and vehicle treated controls. Data accrued for concentrations from 0.01 through 10 mM glutamate were tested in cerebral cortical explants independently from the 15 mM glutamate tested.
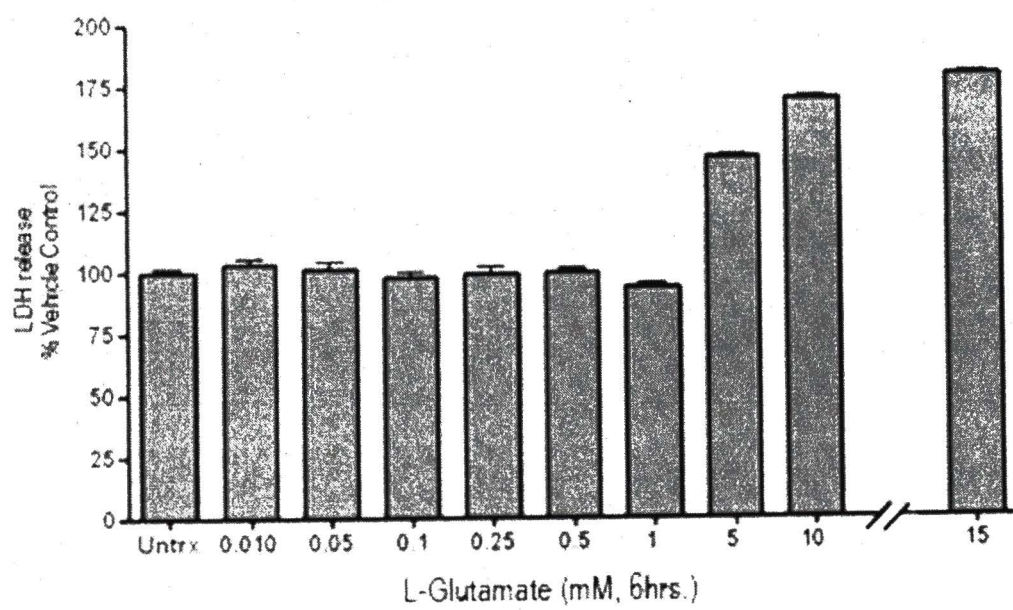
L-Glutamate (mM, 6hrs.)

Figure 2. Assessment of PARP cleavage resultant of L-glutamate exposure in cerebral cortical explants.

Cerebral cortical explants were treated with increasing concentrations (1, 5, or 10 mM) and increasing exposures (3, 6, or 16 hrs.) to L-glutamate. An assessment for the cleavage of Poly ADP-ribose polymerase was made with western analysis. Staurosporine (1.5 μM for 3 hrs.) serves as a positive control for PARP cleavage. A time and dose dependent increase in PARP cleavage with L-glutamate insult was observed.

Untreated | 1mM glu — 3 hrs. 6 hrs. 16 hrs. | 5mM glu — 3 hrs. 6 hrs. 16 hrs. | 10mM glu — 3 hrs. 6 hrs. 16 hrs. | Stauro

115 kDa

85 kDa
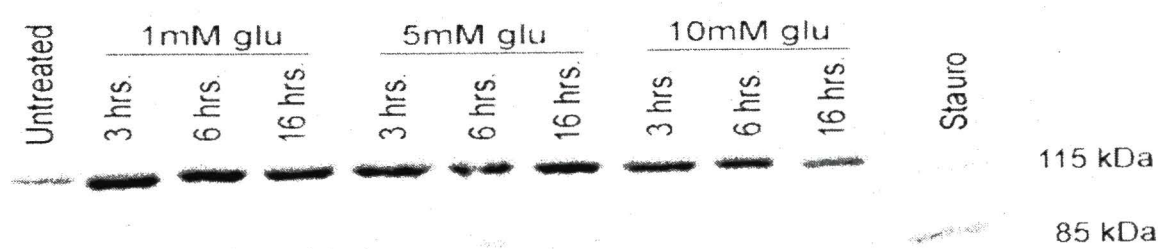
Figure 3. A 24 hour pre-treatment of 100 nM progesterone is effective in protecting cerebral cortical explants against glutamate-induced LDH release.

Cerebral cortical explants were pre-treated with P4 (100 nM) for either 24, or 48 hrs. prior to glutamate, or applied at the time of glutamate insult or 1 hour post-insult.. A 24 hr pre-treatment of P4 prevented L-glutamate induced LDH release, whereas a 48 hr pre-treatment was ineffective. Also, a co-application and 1 hr post-treatment displayed a trend in reducing glutamate – induced LDH release to baseline levels. The vehicle control group represents LDH levels associated with explants treated with the vehicle, 0.1% dimethylsulfoxide (DMSO), alone (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). Cell death was assessed six hours after the administration of L-glutamate using the CytoTox One- Homogenous Membrane Integrity Assay Kit. The numerical values in the bar graphs represent the net LDH values normalized to protein, and then normalized to vehicle treated controls. The graph shown represents data from two independent experiments. 24 and 48 hour treatments were repeated in duplicate whereas co-application and 1 hr. post-treatment groups were only evaluated once. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (*, $p < 0.05$).

Glut (15 mM)	−	+	+	+	+	+
P4 (100 nM)	24 hr	−	48 hr	24 hr	co-app.	1 hr post.
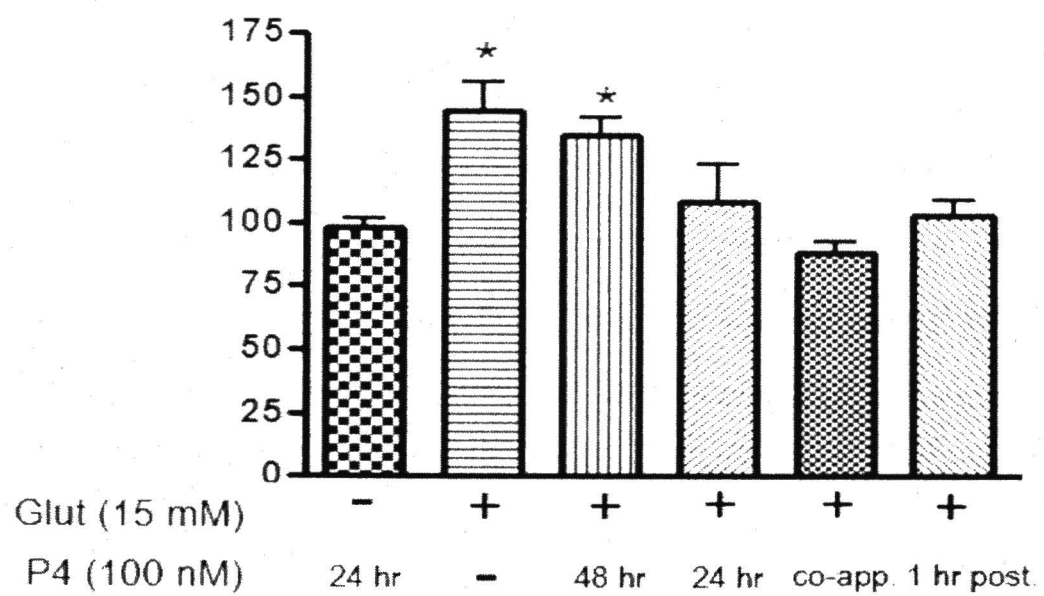
Figure 4. Progesterone does not protect against glutamate-induced LDH release in cultures derived from female mouse pups.

Cerebral cortical explants were pre-treated with P4 (100nM) for 24 hr prior to the administration of L-glutamate, 5 mM for 6 hr in female derived cultures. P4 did not prevent glutamate-induced LDH release. The vehicle control groups demonstrate LDH levels associated with explants treated with 0.1% dimethylsulfoxide (DMSO) (simulating a mock 24 hr. pretreatment, and a 6 hr treatment). Cell death was assessed six hours after the administration of L-glutamate using the CytoTox One- Homogenous Membrane Integrity Assay Kit. The numerical values in the bar graphs represent the net LDH values normalized to protein and vehicle treated controls. The graph shown represents data from two independent experiments. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (**, $p < 0.001$).

Figure 5. Progesterone receptor protein expression in cerebral cortical explants.

Cerebral cortical explants were harvested after 7 days *in vitro* (DIV). 50 µg of protein lysate was loaded onto a sodium dodecyl sulfate (SDS), 10% polyacrylamide gel. The membrane was transferred to a PVDF membrane and blocked with 3% bovine serum albumin (BSA) in 0.2% Tween containing TBS (TBS-T). The membrane was subsequently probed with PR(C-19): sc-538 antibody (Santa Cruz, 1:200 dilution). Antibody binding to the membrane was detected using a secondary antibody (goat anti-rabbit) conjugated to horseradish peroxidase (1:20,000) (Pierce, Rockford, IL) and visualized with the aid of the UVP imaging system, using enzyme-linked chemiluminescence. Cerebral cortical explants express both isoforms of the progesterone receptor as revealed by the two bands corresponding to the molecular weights of PR-A (94 kDa) and PR-B (114 kDa). These bands co-migrated with the PR bands observed in the positive control lane (T47D cell lysate).
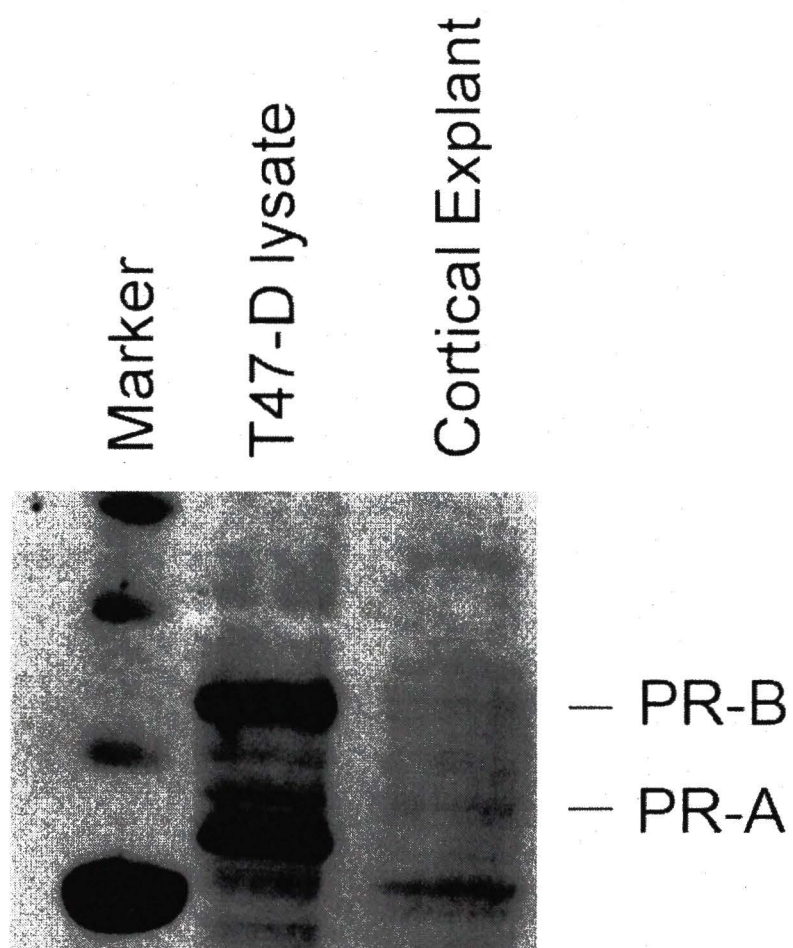
Figure 6. PR mRNA expression levels of cerebral cortical explants increase with increasing days *in vitro*.

Cerebral cortical explants from postnatal day 2 mice were maintained in medium containing 2 nM estrogen for 3 and 7 days *in vitro*. There was a time-dependent increase of the PR mRNA expression with increasing days *in vitro*. Adult cortex and uterus were also harvested in order to compare the levels of PR to *in vivo* animals. Quantitative real-time PCR was used to determine the level of BDNF mRNA from total RNA isolated. BDNF mRNA levels were normalized for 18s rRNA levels. The results are representative of two independent experiments.
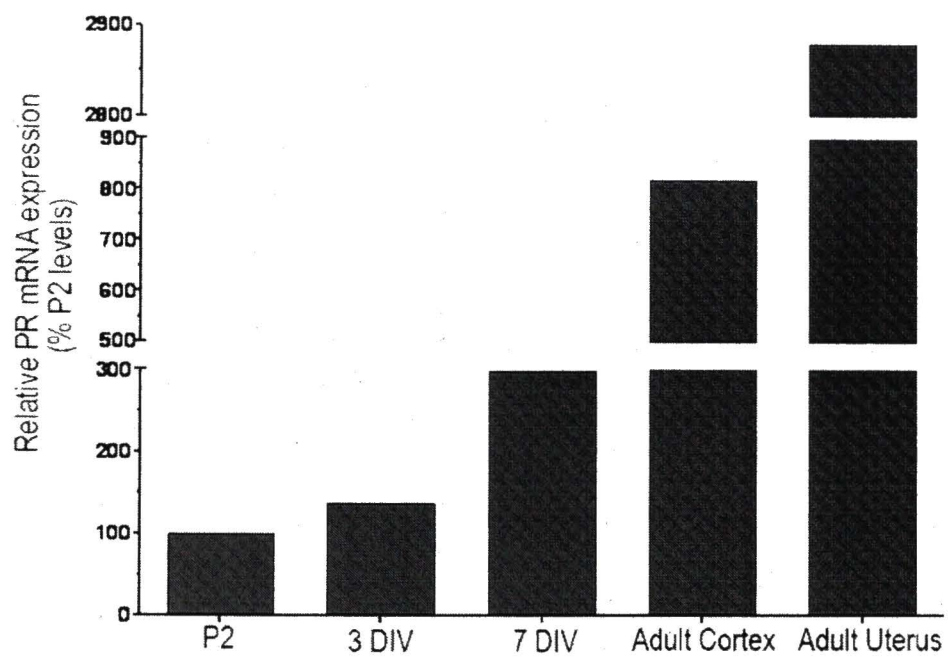
Illustration 1. Organotypic cerebral cortical explant (4X).

Cerebral cortical explants from P3 mice were maintained in CFF containing 2 nM estrogen for 6 days *in vitro*, followed by a 24 hr washout period. On day seven, the cultures were treated with the treatment of choice.
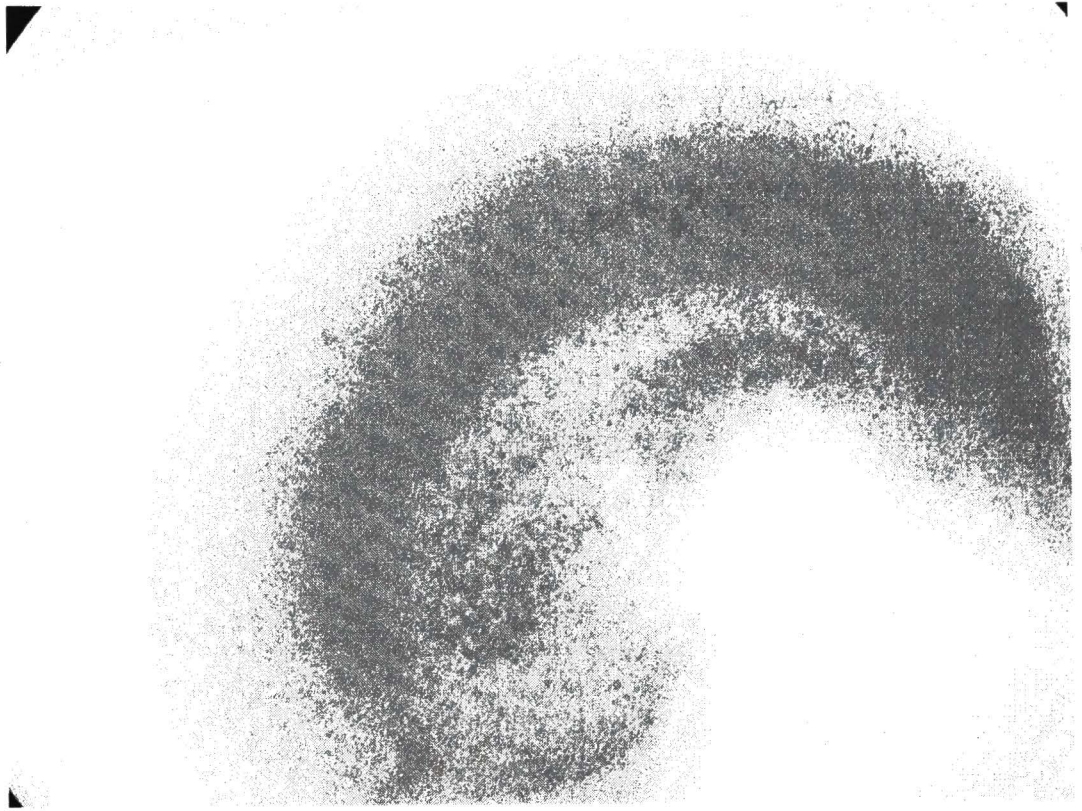
Illustration 2. Progesterone receptor domains.

The numbers denote the positions of the amino acids for each isoform proteins.

This figure was adapted from http://rex.nci.nih.gov/RESEARCH/basic/lrbge/PR.html.

| 1 | 165 | 556 | 642 | 687 | 933 |

NH_2— [AF1] [DBD] [Hinge] [LBD] —COOH

AF1 NLS_{HD} NLS_C AF2
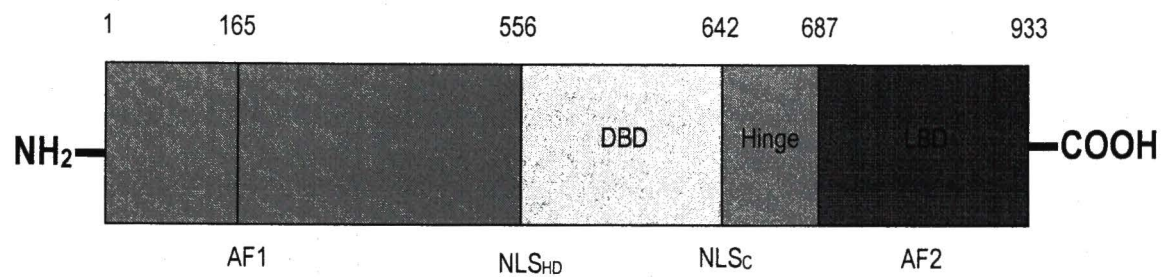
AF1,2 = activation function 1 or 2 domains
DBD = DNA binding domain
Hinge = hinge region
LBD = ligand (hormone) binding domain
NLSHD, NLSC = nuclear localization signals (hormone dependent and constitutive)

Illustration 3. Chemical structure of progesterone and medroxyprogesterone acetate.

Progesterone
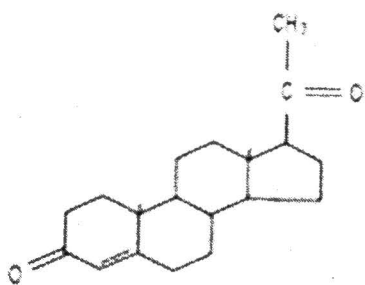
Medroxyprogesterone Acetate
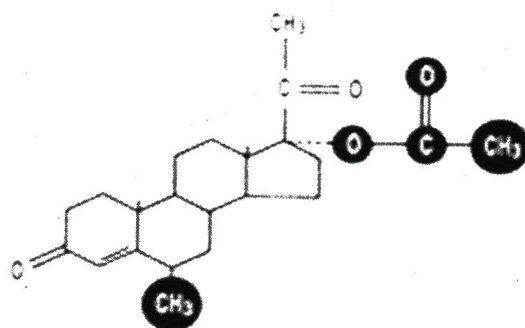
Illustration 4. A schematic view of the neurosteroidogenic pathway in oligodendrocytes, astrocytes, and neurons and their potential interactions between the cell types.

The dotted arrows denote a proposed pathway. This diagram was adapted from Zwain and Yen, 1999.

Cholesterol

Cholesterol

Astrocytes

P450scc

P450scc

Pregnenolone $\cdots\cdot\blacktriangleright$ Pregnenolone $\xrightarrow{\text{P450c17}}$ DHEA

3β-HSD

3β-HSD

3β-HSD

Progesterone ◄ Progesterone $\xrightarrow{\text{P450c17}}$ Androstenedione $\xrightarrow{\text{P450arom}}$ Estrone

Oligodendrocytes

17β-HSD

Estradiol $\xleftarrow{\text{P450arom}}$ Testosterone

Testosterone

Estrone

Estradiol

P450arom

Androstenedione $\xleftarrow{\text{P450c17}}$ Progesterone

3β-HSD

3β-HSD

Neurons

DHEA $\xleftarrow{\text{P450c17}}$ Pregnenolone $\xleftarrow{\text{P450scc}}$ Cholesterol

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Illustration 5. Schematic representation of progesterone's signaling pathways in the cerebral cortex.

Progesterone's protective effects are dependent on the activation of specific signal transduction pathways, but by itself, is not sufficient to result in neuroprotection since MPA also resulted in phosphorylation of ERK and Akt without an associated cytoprotective effect. However, P4 but not MPA, elicited the induction of BDNF synthesis, suggesting that a distinguishing mechanism between P4 and MPA, with respect to their neuroprotective effects, may be the ability to elicit the production of this neurotrophin. Collectively, I conclude that both signaling and neurotrophin production may be necessary for P4-induced neuroprotection.
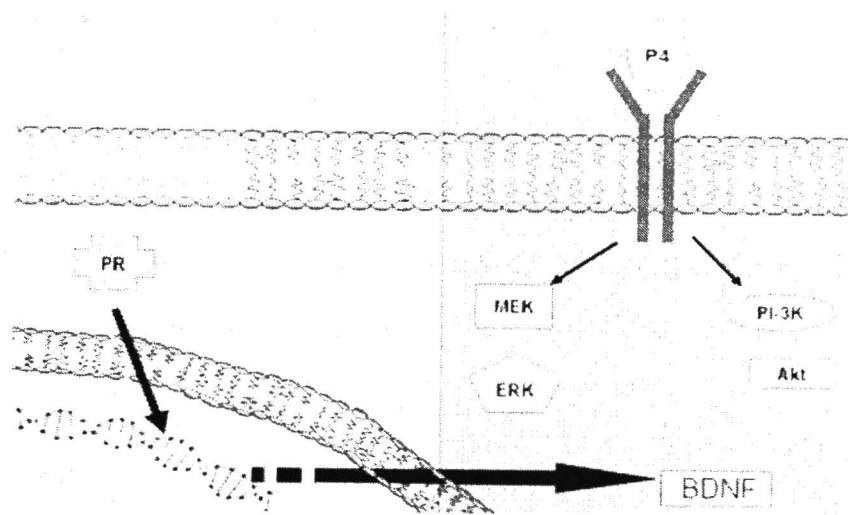
GENERAL DISCUSSION AND SUMMARY

The data presented in this dissertation demonstrate that while both P4 and MPA were capable of eliciting ERK phosphorylation, only P4 was neuroprotective. Further, P4's protective effects against glutamate toxicity were dependent on the activation of the ERK/MAPK pathway and PI3-K pathways. And finally, the data argue that P4's protective effects may also be mediated by the induction of the neurotrophin, BDNF. These data provide strong evidence that P4, and not MPA, may be the relevant progestin to use when considering using such hormones for the purposes of neuroprotection. And though MPA may be effective at reducing the uterotrophic effects associated with un-opposed estrogen treatment, it may be a poor choice when considering its role in the brain.

Why didn't the kinetics of ERK and Akt phosphorylation differ between the protective P4 and the non-protective MPA?

Differences in the temporal patterns of ERK phosphorylation have been associated with distinct cellular consequences (Marshall, 1995). For example, a rapid onset and limited duration of the phosphorylation of ERK protein has been associated with a differentiating or survival promoting signal. Whereas a delayed and persistent phosphorylation of ERK (lasting usually for more than 18 hrs.) has been correlated more with cellular death (Marshall, 1995). Thus, the pattern of ERK and/or Akt

phosphorylation elicited by P4, as compared to MPA, was pursued to explain postulated differences in the ability of these two compounds to serve as neuroprotective agents (as was evaluated in Aim 2). Though my data argue that both P4 and MPA elicited similar temporal patterns of ERK and Akt phosphorylation (Figure 2, Chapter 3), other potential differences between these hormones, in terms of their regulation of cell signaling, cannot be ruled out. For example, Nilsen and Brinton described a difference in the pattern of nuclear translocation of phosphorylated ERK protein in response to MPA and P4 in primary dissociated, neuron-enriched hippocampal neurons. Specifically, P4 induced the nuclear translocation of ERK, whereas MPA did not (Nilsen and Brinton, 2003). Similarly, it is possible that P4 and MPA may also have differences in their ability to influence the nuclear translocation of phosphorylated ERK in the cerebral cortex. Additionally, it is possible that the protective effects of P4 were mediated by prior conversion to its 5α-reduced metabolite, allopregnanolone, a potent allosteric enhancer of GABA-gated currents. Such a mechanism would be expected to counter glutamate-induced cytotoxicity. Since, MPA does not get metabolized to allopregnanolone (Ciriza et al., J. Neurobiol. 2006), it would be ineffective against excitotoxic insult.

What does the model of cell death teach us about the cellular target of P4?

In order to assess the cytoprotective effects of the progestins, a model of cellular death was established. L-glutamate treatment was chosen as the insult to elicit cellular damage. Evaluation of other chemical means for mimicking age-related dysfunction

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within the cell including hydrogen peroxide, cyanide, and iodoacetic acid were also performed (data not shown). However, L-glutamate was chosen due to the ability to provide a more consistent damage within the explants described and the vast amount of research accrued using this particular insult for the evaluation of protection by steroids (Simonian and Coyle, 1996; Vajda, 2002).

As stated above, L-glutamate has been used in numerous cellular systems to promote excitotoxicity as well as oxidative stress. This insult mimics the decrease in cellular ATP levels, increased Ca^{2+} levels, and the generation of reactive oxygen species seen in aging or age-associated diseases (Michaelis, 1998). To establish the appropriate conditions to promote cell damage/death in explants, a concentration-response curve for L-glutamate's ability to elicit LDH release was first performed. The data showed that concentrations below 5 mM were relatively ineffective at inducing cell death (Figure 1, Appendix). Though other cell models typically see cell toxicity at lower concentrations of glutamate (20 – 50 μM), the explants seemed relatively resistant and required higher concentrations. This could be explained by the abundance of glia in the slice culture (organotypic explant) model. Glia can take up glutamate and convert it to the relatively non-toxic glutamine, and thus, serve as a "detoxicant" against glutamate insult.

To determine if the glutamate induced cellular death was occurring by an excitotoxic mechanism, we assessed if the glutamate-induced LDH release could be inhibited by pre-treatment of the cultures with the NMDA receptor antagonist, MK-801. We found that MK-801 completely prevented glutamate-induced cytotoxicity (Figure 3,

Chapter 2). As such, we suggested that the principle mode of cell death induced by glutamate at the concentration and duration of treatment used in the explant cultures was excitotoxic in nature. Accordingly, we argue that a possible target of P4's actions may be the NMDA receptor, and through modulation of the NMDA receptor function (or perhaps even receptor number (Cyr et al., 2000), could reduce the toxic consequences of glutamate. Supporting this hypothesis are data that describe the effect of ovariectomy and hormone replacement on the relative levels of NMDA receptors. Specifically, ovariectomy caused an overall increase in the number of NMDA receptors in the cerebral cortex while estradiol combined with progesterone, or progesterone alone decreased the NMDA binding density (Cyr et al., 2000). In contrast, however, El-Bakri et al. described that progesterone was without effect on NMDA receptor binding in the hippocampus (El-Bakri et al., 2004). Thus, regional differences may exist with regards to progesterone's ability to influence NMDA receptor number.

We recognize that the primary endpoint to assess cell damage/death, LDH release, does not distinguish between whether the mode of cell death is apoptotic or necrotic. To this end, I also evaluated if glutamate caused an increase in Poly (ADP-ribose) polymerase (PARP) cleavage in cerebral cortical explants. PARP is a nuclear enzyme activated by DNA strand breaks and is involved in DNA repair. PARP cleavage, often a result of caspase 3/7 activity, is a well-known marker of apoptotic cell death. The full length PARP protein and its cleavage products were assessed using western blotting, employing a PARP antibody (Cell Signaling: Cat no. 9542; 1:1000 dilution) that

recognizes both the full-length (116 kD) and cleaved PARP (89 kD). We found that glutamate treatment did, in fact, cause an increase in PARP cleavage (Figure 2, Appendix), suggesting that glutamate may cause cell damage/death in cortical explants at least in part, through an apoptotic mechanism. And by inference, P4 may protect against pro-apoptotic cascades triggered by glutamate administration.

How did my data contribute to or extend what was already known about the neurobiology of progesterone?

At the time the studies in my dissertation were originally conceived, there was little information on how P4 and MPA might differ in terms of their ability to regulate cell viability. Since then, however, data from the Brinton laboratory has offered two mechanisms by which P4 and MPA differ in terms of their neuroprotective effects. First, their laboratory described that MPA antagonized estrogen's protective effects, whereas P4 did not. Then, they showed that P4 promotes nuclear translocation of phosphorylated ERK, whereas MPA did not. It is important to note, however, that the data from the Brinton laboratory focused on primary neuron-enriched cultures derived from the hippocampus. In contrast, our data were obtained from studies performed in non-dissociated cultures (slice culture or organotypic explants) derived from the cerebral cortex. As such, my data extend the work of the Brinton lab by showing that progesterone is protective in the cerebral cortex. While this may have been argued *a priori* as an expected finding, it is worth pointing out that how hormones work in one

brain region may not necessarily be true in other brain regions. For example, estradiol induces an increase in the expression of progesterone receptors in the hypothalamus, whereas in the cerebral cortex, this relationship does not appear to hold true. Further, the ability of estrogen to normalize the ovariectomy-induced deficit in BDNF mRNA was seen primarily in the hippocampus, whereas estrogen was unable to completely restore BDNF levels to that of ovary-intact animals in the cerebral cortex (Singh et al., 1995). Thus, knowledge of how P4 is protective in the cerebral cortex is a new and important finding that had not been previously described.

My studies also partly addressed the therapeutic window in which P4 treatment would be protective. Given that P4 has even been demonstrated to be a useful post-injury treatment for patients with acute, ischemic and traumatic injuries of the brain and spinal cord (Gonzalez et al., 2004), the window of opportunity for P4 to protect explants against cellular damage was also of interest. The effect of three different pre-treatment paradigms was assessed: 48 hr-, 24hr-pretreatment, and co-application or post 1 hour treatment with the glutamate insult. We found that a 48 hr pre-treatment was ineffective in protecting cerebral cortical explants from glutamate induced cytotoxicity, whereas a 24 hr pre-treatment was optimal (Figure 3, Appendix). Co-application and the 1 hour post-treatment of progesterone after glutamate insult also display a trend towards the reduction of LDH release to baseline levels (n=1, therefore unable to test statistical significance). Although purely speculative, it is possible that a pre-treatment of 48 hours results in the down-regulation of cytoprotective progesterone receptors leading to the ineffectiveness

of progesterone treatment. Additionally, progesterone's ability to upregulate the expression of neuroprotective genes/proteins may be transient following a single treatment of the cultures with progesterone. Accordingly, I suggest that within 24hrs, the protective factors that mediate progesterone-induced protection are upregulated, but after 48 hrs, these same factors may have returned to basal levels. Consequently, since the cultures are not "prepared" any longer to deal with an insult after 48 hrs (due to return of the protective factors to baseline), progesterone is no longer protective. Yet another possibility is that the steroid is metabolized to an inactive, "non-protective" metabolite after 48 hrs in vitro, and as such, the lack of protection seen after 48 hrs is due to a reduction in the amount of bioactive protective progesterone. Such studies and hypotheses are being considered in the laboratory currently.

The other aspect of novelty associated with my dissertation, as alluded to above, is the use of organotypic explants. P4 has been demonstrated to be protective against a variety of insults in both glial and neuronal dissociated cell cultures. However within the brain *in vivo*, there exists an interaction of both glia and neurons. Therefore our experimental model of the organotypic explants is reflective of the complexity of the intact brain due to the retention of the cytoarchitecture within the slice which allows for the maintenance of cell-to-cell interactions and heterogeneity of cell types. Although the system within which our studies were undertaken is an *in vitro* model system, the retention of the cytoarchitecture (at least within the confines of the slice) and the presence of both neurons and glia within this model system render this experimental system closer

to the *in vivo* situation, and as such, may allow us to extrapolate our results to the *in vivo* situation more readily than if we were using dissociated, neuron-enriched cultures.

But the same properties that render the explant model appealing are the same properties that render limitations. For example, we acknowledge that, due to the complexity of the model, the cells responding to P4 may be neurons, glia or both. One way to address which cells are responding to progesterone include the use of immunocytochemistry. To some extent, such studies have been done in the Singh laboratory. For example, our laboratory has previously published that neurons are indeed responsive to P4, with regards to ERK phosphorylation, as assessed by immunohistochemistry coupled with confocal microscopy (Singh, 2001).

Another important aspect of the organotypic explant is that given its retention of many of the inter-cellular interactions, this model may also takes into account the ability of a cell to influence their surroundings (i.e., other cells). This is of relevance due to the fact that hormones such as estrogen can induce the *de novo* synthesis of P4 in astrocytes (Sinchak et al., 2003). Our model is thus able to account for the complexities of the neuronal and glial interactions in the brain and hence, may provide better insight in the manner of signaling towards neuroprotection by these progestagenic compounds *in vivo*. But as stated above, the very complexity of this model requires us to interpret our results with limitations, the most important of which is based on the fact that we cannot discriminate which cells in particular are responding or benefiting from progesterone treatment.

Additional interpretations of the studies that argued for the requirement of ERK1/2 in cytoprotection

P4 elicited ERK phosphorylation in a manner consistent with the promotion of survival-promoting signal in that it followed a rapid onset, but limited duration. This specific pattern of phosphorylation of ERK in other model systems has been associated with neuroprotection (Marshall, 1995). However, just because ERK phosphorylation was elicited in a temporal pattern consistent with neuroprotection did not necessarily demonstrate that it was a mechanism that mediated P4's protective effects. In order to demonstrate the requirement of the ERK1/2 (MAPK) pathway for P4-induced protection, cultures were pre-incubated with the MEK1/2 inhibitor, UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyalthio] butadiene) prior to P4 and glutamate treatment. At the time at which these experiments were performed, UO126 was thought to be a selective ERK1/2 inhibitor. However, we now know that UO126 inhibits not only ERK1/2, but Big MAP kinase 1 (BMK1) also known as ERK5, as well (Kamakura et al., 1999; Mody et al., 2001). Given relatively recent evidence that supports the role of ERK5 in neuroprotection (Suzaki et al., 2002), we suggest that P4's protective effects could also be mediated by ERK5. Accordingly, differences in the ability of P4 and MPA to protect may be related to the ability of P4, but not MPA, to increase the phosphorylation ERK5.

Our data also demonstrate that P4's protective actions require the activation of PI-3 kinase. PI-3 kinase activation causes the phosphorylation of Akt. Activated Akt is responsible for promoting cell survival through a multitude of mechanisms, including the phosphorylation of BAD. Singh (2001) demonstrated in the organotypic cerebral cortical explant model, that P4 (100 nM) elicits the phosphorylation of Akt. To evaluate the role of Akt in P4-induced protection, LY294002, a PI-3K inhibitor, was used. Previously, our lab had demonstrated that 15 μM LY294002 prevents the ability of P4 to phosphorylate Akt. This concentration of inhibitor chosen was based on the K_i of the compound for PI-3K (1.5 μM). Thus, we used this concentration to evaluate the involvement of PI-3 kinase in progesterone's protective actions, and found that P4's protective effects were dependent on the activation of PI-3 kinase.

Within this body of work, we demonstrated that P4 has a protective effect in cerebral cortical organotypic explants which is mediated via the MAPK and PI3K signal transduction pathways. Although it has been known that P4 can elicit the ERK and Akt pathways (Singh, 2001), this is the first observation demonstrating that P4 requires these two key effectors to protect against glutamate-induced LDH release in cortical explants. Our current data employ inhibitors of the MAPK and PI3K pathways, and demonstrate with the pharmacological inhibitors present, the ability of P4 to protect against glutamate-LDH release is attenuated.

Brain Derived Neurotrophic Factor (BDNF) as a possible mediator of progesterone's protective effects

BDNF is a neurotrophin known to play a role in the survival, differentiation, synaptogenesis and plasticity of neurons. Singh et al. published data in 1995 of observed differences in the levels of neurotrophins, particularly BDNF, in ovariectomized versus ovary-intact mice (Singh et al., 1995). Upon ovariectomy, BDNF levels declined in the hippocampus and cerebral cortex. In estrogen-replaced ovariectomized animals there was a partial recovery of BDNF in the hippocampus, although not to the extent of the intact animal. Within the cerebral cortex, however, the BDNF levels were still comparable to the ovariectomized animal. The inability of estrogen to restore the BDNF levels in the cerebral cortex may be due to the fact that progesterone, the other major ovarian hormone lost following ovariectomy, was not administered. This suggests that the deficient BDNF levels seen in the cortex could have been restored with the administration of P4. Therefore, we arrived at the hypothesis that P4 causes an increase in BDNF levels. In fact, as our results show progesterone treatment, at a concentration and duration consistent with its protective effects, did induce an increase in both BDNF mRNA (Figure 6, Chapter 2) and protein levels (Figure 7, Chapter 2).

Others have also shown that ovarian steroids may elicit an increase in BDNF levels. For example, combined estrogen and progesterone treatment of ovariectomized rats was found to increase BDNF mRNA and protein levels in different regions of the brain (Gibbs, 1999). Moreover, physiological changes in the estrous cycle were also

found to result in the fluctuations of BDNF mRNA (50- 40%) in the hippocampus (Gibbs, 1998). However, these studies did not address the role of progesterone by itself on BDNF induction.

A caveat to our observation that total cellular protein is increased in response to progesterone is derived from the knowledge that neurotrophins, such as BDNF, are synthesized as precursors and cleaved to produce a mature and pro-hormone. The pro-neurotrophin is proteolytically cleaved to produce the mature protein by extracellular proteases. Pro-BDNF is thought to elicit pro-apoptotic pathways whereas the mature peptide may be involved in more survival promoting signaling. There is also evidence that BDNF has the ability to be secreted and thus may act as a signaling molecule. The pro-BDNF protein has a relatively higher binding affinity towards p75^{NTR} whereas the mature cleavage product binds preferentially to the TrkB receptor (Dechant, 2001). Preferential activation of the p75^{NTR} receptor has been postulated to favor the induction of apoptotic cascades, via activation of p75^{NTR} and sortilin (Teng et al., 2005), whereas activation of the TrkB receptor mediates survival pathways (Lu et al., 2005). Ongoing studies in the Singh laboratory are aimed at addressing whether the effects of progesterone on total cellular BDNF reflect a change in a particular species of BDNF (i.e., pro- versus mature-BDNF).

AD patients have been observed to have lower amounts of BDNF mRNA levels in the cortex and hippocampus (Holsinger et al., 2000). Particularly in the nucleus basalis of AD patients there is a 50% decrease in the amount of BDNF mRNA as when

compared to controls (Fahnestock et al., 2002). In the AD patient, an up to 40% reduction has been observed in the pro-BDNF levels of the parietal cortex as observed using western blot techniques (Fahnestock et al., 2002; Michalski and Fahnestock, 2003). Thus, the ability of progesterone to induce BDNF may have therapeutic value for treating/preventing such neurodegenerative diseases as Alzheimer's disease.

We also determined whether MPA altered BDNF levels. Preliminarily, it would appear that MPA may inhibit BDNF mRNA levels in slice cultures of the cerebral cortex (Figure 7, Chapter 3). Such a dichotomy in the regulation of BDNF could provide an additional mechanism that explains the ability of P4 to protect and MPA's inability to do so. Additional studies are underway to explore this effect further.

Male and Female Differences

Within my dissertation work, an observation was made in which progesterone protected cerebral cortical explants derived from male pups (Figure 1, Chapter 2) but not female pups (Figure 4, Appendix). This was somewhat surprising, given that estrogen elicits ERK phosphorylation in cultures derived from male and female mice equally (Setalo G, Singh M and Toran-Allerand, unpublished data).

Gender differences with respect to the protective effects of hormones are not without precedent. For example, it is described that male derived neurons are more sensitive to nitrosative stress and excitotoxicity than female neurons. In contrast, however, female derived neurons were preferentially sensitive to etoposide and

staurosporine- induced apoptosis than male derived neurons (Du et al., 2004). Also, a meta-analysis involving 8 studies and 20 variables after TBI illustrated that women fared worse than men in 85% of the variables studied (Farace and Alves, 2000). A year after the injury, it was also demonstrated that females hospitalized with TBI have a greater risk for higher disability and community integration (Wagner et al., 2000). Female patients whom have incurred severe TBI have a smaller oxidative damage load than males for a given excitotoxic or ischemic insult (Wagner et al., 2004). Further studies will also need to be done to address the underlying mechanisms observed in regards to P4's ability to protect against glutamate toxicity in male derived cultures whereas the protection observable in female derived cultures was marginal.

I am unaware of any gender differences observed at such an early age (perinatal) with respect to hormone-induced neuroprotection. Among the possible explanations for this gender difference include relative diffences in the expression of the specific progesterone receptors involved in P4's protective actions. Such studies are currently being done in the Singh laboratory.

CONCLUSIONS AND FUTURE DIRECTIONS

The results of the WHI trials concluded that hormone therapy is detrimental to post-menopausal women. However, this was contrary to the large amount of data accrued in basic research that supported P4 as a protective factor. There thus appeared to be a gap in knowledge, such that the field was unable to explain the differences observed between basic and more recent clinical research. Therefore the main goal of this dissertation was to ascertain the cellular and molecular differences of P4 and MPA on cellular viability after insult with glutamate in organotypic explants of the cerebral cortex. Our results are supportive of other data published in *in vitro* models demonstrating P4's effectiveness to protect against glutamate insult whereas MPA was not. Moreover, our data argue for the involvement of both the MAPK and PI-3K pathways in P4's protective actions, and suggests that a potentially relevant mechanism that distinguishes the effect of P4 and MPA is the ability to elicit an increase in BDNF levels.

As suggested above, other experiments to further my data include the determination of whether the protective effects of P4 are mediated by either glial or neuronal populations, or require both. These experiments would shed light on the involvement of one population towards the promotion of cellular viability or the interaction of both populations for a common end.

We also showed that a correlation existed between the ability of P4 to promote cell viability and its ability to elicit an increase in BDNF levels in organotypic explants of the cerebral cortex (Illustration 5, Appendix). Whether the changes in BDNF are a reflection of a change in pro- versus mature BDNF protein will require further experimentation. The findings within our experimental model will further our understanding of how P4 mediates its protective effects during injurious events and prevents disease pathologies in the brain.

Also, to determine if the involvement of P4's ability to protect is dependent upon BDNF, the TrkB inhibitor, K252a, could be used. Therefore if progesterone has the ability to protect against cytotoxicity in the presence of K252a, it could be concluded that the ability of progesterone to protect against cytotoxicity is not mediated by BDNF induced activation of the TrkB receptor.

The Women's Health Initiative studies were terminated prematurely due to the increase in certain health risks associated with hormone therapy. The data generated here have furthered our understanding of the neurobiology of P4, and in my view, will aid in developing safer and more effective hormone therapies for postmenopausal women. This work demonstrates unequivocally that there are differences in P4 and MPA in their ability to protect against excitotoxic cellular death. This work thus further begs the questions as to whether MPA, the current progestin of choice, should continue to be used in hormone therapies.

Collectively, this dissertation, in addition to previous studies, demonstrates that not all progestins are created equal.

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