

BRAIN

## Medroxyprogesterone acetate exacerbates glutamate excitotoxicity

JON NILSEN, ALISON MORALES, & ROBERTA DIAZ BRINTON

Department of Molecular Pharmacology and Toxicology and the Program in Neuroscience, University of Southern California, Pharmaceutical Sciences Center, Los Angeles, CA, USA

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### Abstract

We previously demonstrated that progesterone functions as a neuroprotective agent whereas medroxyprogesterone acetate (MPA; Provera<sup>®</sup>) does not. Moreover, MPA antagonized the neuroprotective and neurotrophic outcomes induced by 17 $\beta$ -estradiol (E<sub>2</sub>). Towards developing effective hormone therapies for protection against neurodegeneration, we sought to determine whether formulation, chemical features or prevention versus treatment mode of exposure affected the outcome of MPA treatment in survival of primary hippocampal neurons. Results of these analyses indicated that both crystalline MPA and a pharmaceutical formulation (Depo-Provera<sup>®</sup>) lacked neuroprotective efficacy, indicating that the effects were not dependent upon MPA formulation. Likewise, MPA in the prevention and treatment paradigms were equally ineffective at promoting neuronal survival, indicating that timing of MPA administration was not a factor. Further, the detrimental effects of MPA were not due to the presence of the acetate group, as medroxyprogesterone was as ineffective as MPA in promoting neuronal survival. Moreover, MPA pretreatment exacerbated neuron death induced by glutamate excitotoxicity as indicated by a 40% increase in neuron death determined by direct live/dead cell count and a commensurate increase in the number of positive cells by terminal deoxynucleotidyl transferase-mediated nick end-labeling. Collectively these results predict that the progestin formulation of hormone therapy will affect the vulnerability of the central nervous system to degenerative insults.

**Keywords:** Progestin, neuroprotection, estradiol, hormone therapy, neuron

### Introduction

Although the effect of hormone therapy (HT) on the central nervous system is an issue that has generated much debate, extensive basic science *in vitro* and *in vivo* analyses indicate a protective role of estrogen [1–5]. Neurological benefits of estrogen replacement therapy in humans include reversal of estrogen deficiency-induced memory dysfunction and reduced risk of Alzheimer's disease (AD) [6–10]. In contrast to the extensive body of knowledge regarding the role of estradiol actions in brain, the effects of progesterone on non-reproductive functions in brain have lagged behind. Of the limited studies performed on progestins some have reported no neuroprotective effect of progestins [11], whereas others have reported positive effects of progestin on cell survival [12–14].

Further limiting knowledge of the field is the dearth of studies directly comparing the effect of one progestogen with another. In directly comparing different progestins, we previously demonstrated that,

in contrast to progesterone, medroxyprogesterone acetate (MPA) completely antagonized the neuroprotective and memory mechanisms of estrogen [12,15]. Blockade of estrogen-inducible mechanisms of neuroprotection and cognition would be expected to obstruct estrogen protection against insults that culminate in AD, thereby increasing the risk for the disease. To develop more effective HT for the prevention of neurodegenerative disease, it is necessary to understand the pharmacology and chemical structure dependence of the neural effects of progestogens. Towards this goal the present study was designed to further explore the characteristics of MPA underlying its neural effects and how this affects neuronal survival. We examined if and how the formulation, chemical features and prevention versus treatment mode of exposure altered the response of primary hippocampal neurons to MPA. Results of the present study demonstrate that MPA by itself exacerbates neurotoxicity via increased apoptosis.

## Materials and methods

### Animals

Studies were approved by the University of Southern California Institutional Review Board for Animal Care. Timed-pregnant Sprague–Dawley rats purchased from Harlan (Indianapolis, IN, USA) were housed under controlled conditions of temperature (22°C), humidity and light (14 h light, 10 h dark) with water and food available *ad libitum*.

### Chemicals

All culture materials were purchased from Gibco BRL (Rockville, MD, USA). 17 $\beta$ -Estradiol (E<sub>2</sub>), medroxyprogesterone acetate (MPA) and medroxyprogesterone (MP) were purchased from Steraloids (Newport, RI, USA). Depo-Provera<sup>®</sup> was obtained from Pharmacia & Upjohn, Inc. (Peapack, NJ, USA). All chemicals were purchased from ICN (Costa Mesa, CA, USA) unless noted otherwise.

### Neuronal culture

Primary cultures of dissociated hippocampal neurons were obtained as described previously [16]. Briefly, hippocampi from the brains of embryonic day 18 (E18) rat fetuses were dissociated in 0.02% trypsin with fire-polished constricted Pasteur pipettes. Then 10<sup>6</sup> cells/ml were plated on polyethylenimine-coated, 24-well plates for biochemical assays and 10<sup>5</sup> cells/ml were plated on poly-D-lysine-coated gridded coverslips for survival counts or chamber slides for staining by terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL). Neurons were grown in Neurobasal medium (Gibco) supplemented with 10 mM NaHCO<sub>3</sub>, 5 U/ml penicillin, 5 mg/ml streptomycin and B27 supplement (Gibco). Cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10–12 days before use in experiments. This protocol routinely yields cultures of >98% pure neurons.

### Glutamate toxicity

Forty-eight hours before glutamate exposure, cultures were placed in fresh Neurobasal medium supplemented with 10 mM NaHCO<sub>3</sub>, 5 U/ml penicillin, 5 mg/ml streptomycin and B27 supplement with steroids or vehicle control (ethanol <0.0001%) as indicated. E<sub>2</sub>, MPA, MP and Depo-Provera<sup>®</sup> were all dissolved in ethanol to a stock solution of 1 mg/ml and further diluted in culture medium so that final concentration of ethanol was less than 0.0001%. Glutamate exposure was performed for 5 min at 37°C in buffer containing 2 mM KCl, 1 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1 mM NaHPO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

(HEPES), 10 mM glucose, 0.1 M NaCl and 200  $\mu$ M L-glutamic acid. Cultures were then washed and returned to fresh Neurobasal medium with and without steroids as indicated by post-treatment designation.

### Biochemical assessment of cell damage and survival

Overall neuronal injury was assessed 24 h after the start of glutamate exposure by quantitative measurement of lactate dehydrogenase (LDH) released into the culture medium, an index that is proportional to the total number of neurons damaged by excitotoxic exposure [17]. LDH activity of 80  $\mu$ l of culture medium was measured using the Cytotoxicity Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions and quantified by measuring the absorbance at 490 nm. Data are normalized against total LDH release as determined by treatment of cultures with Triton X-100 (0.1%). Data are presented as mean  $\pm$  standard error of the mean (SEM) for at least three independent experiments with at least four wells per condition per experiment.

After removal of sample medium for LDH determination, calcein AM (1.5  $\mu$ M) was added to the cultures and incubated at 37°C for 20 min. Calcein fluorescence, as a result of calcein AM cleavage by viable cells, was measured by excitation at 494 nm/emission at 517 nm to determine relative cell survival. Data are normalized against the average calcein fluorescence for untreated control neurons. Data are presented as mean  $\pm$  SEM for at least three independent experiments with at least four wells per condition per experiment.

### Morphological assessment of cell survival

Morphological neuronal survival analysis was performed as described previously [18]. Briefly, for analyses of neuronal survival, between 40 000 and 60 000 cells were seeded onto poly-D-lysine-coated (10  $\mu$ g/ml) 25-mm<sup>2</sup> coverslips with a grid size of 600  $\mu$ m<sup>2</sup> composed of 520 alphanumeric grids. Hippocampal neurons were treated with E<sub>2</sub> (10 ng/ml), MPA (10 ng/ml) or vehicle control. Neurons were counted after 48 h and grids with viable neurons were selected for study over the entire period of the experiment. Microscopic fields for analysis were randomly selected blind to the experimental condition. One hundred neurons per coverslip were selected for study and there were three coverslips per condition for a total of 300 neurons analyzed per condition per experiment. Following the initial count of steroid/vehicle-pretreated cultures, cultures were rinsed with warm (37°C) HEPES-buffered saline and exposed to excitotoxic glutamate (200  $\mu$ M) for 5 min, followed by another

wash, after which fresh medium was added. Neurons from the same grids were counted 24 h following the glutamate exposure. Neuronal viability was determined by three morphological criteria: the presence of smooth, round, phase-bright soma; possession of at least one or more neurites longer than the diameter of the cell body; and granulation-free neurites as described previously [19,20].

### TUNEL staining

Apoptotic cells were labeled 24 h after the start of glutamate exposure using the In Situ Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells were fixed for 30 min in 4% paraformaldehyde at room temperature. Cells were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate for 2 min at 4°C. The cells were then incubated with 50  $\mu$ l TUNEL reaction mixture per chamber at 37°C for 60 min in the dark. The slides were then coverslipped with Antifade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA, USA). Fluorescent images were acquired a Marianas inverted microscope system (Intelligent Imaging Innovations, Santa Monica, CA, USA). The threshold masking function of Slidebook software (Intelligent Imaging Innovations) was used to count apoptotic (TUNEL-positive) and total (DAPI) nuclei from four random fields per chamber with at least three chambers per condition per experiment. Data are normalized to control levels and presented as mean  $\pm$  SEM from three independent experiments.

### Statistical analysis

Statistically significant differences between groups were determined by analysis of variance followed by Newman-Keuls *post hoc* analysis.

## Results

*Neither medroxyprogesterone acetate nor Depo-Provera<sup>®</sup> is neuroprotective in vitro*

We had previously shown that a clinical preparation of MPA had detrimental effects on neuronal survival that were not apparent with the carrier vehicle [12]. However, there may have been an unrecognized interaction between the MPA and the compounds of the carrier in our *in vitro* system. Thus, we sought to determine if a clinical preparation of Depo-Provera<sup>®</sup> differed from crystalline MPA in neuroprotective efficacy. Primary hippocampal neurons were grown for 12 days prior to a 48-h exposure to E<sub>2</sub> (10 ng/ml) or progesterone (10 ng/ml) as positive control, MPA or Depo-Provera<sup>®</sup>. Following the steroid

pretreatment, neurons were exposed to excitotoxic glutamate (200  $\mu$ M) for 5 min and replaced with fresh medium. To test for any post-treatment effects differing from proactive adaptations, the following 24-h period occurred in the absence (Pretreat Only) or presence (Pre and Post Treat) of MPA and Depo-Provera<sup>®</sup>. Vehicle-treated control neurons exhibited low membrane damage and substantial cell survival as determined by LDH release and calcein imaging, respectively (Figure 1A and 1B). Exposure to glutamate resulted in a significant decline in cell survival 24 h later, with a 300% increase in LDH release (Figure 1A;  $p < 0.05$  vs. control) and a 50% reduction in calcein intensity (Figure 1B;  $p < 0.05$

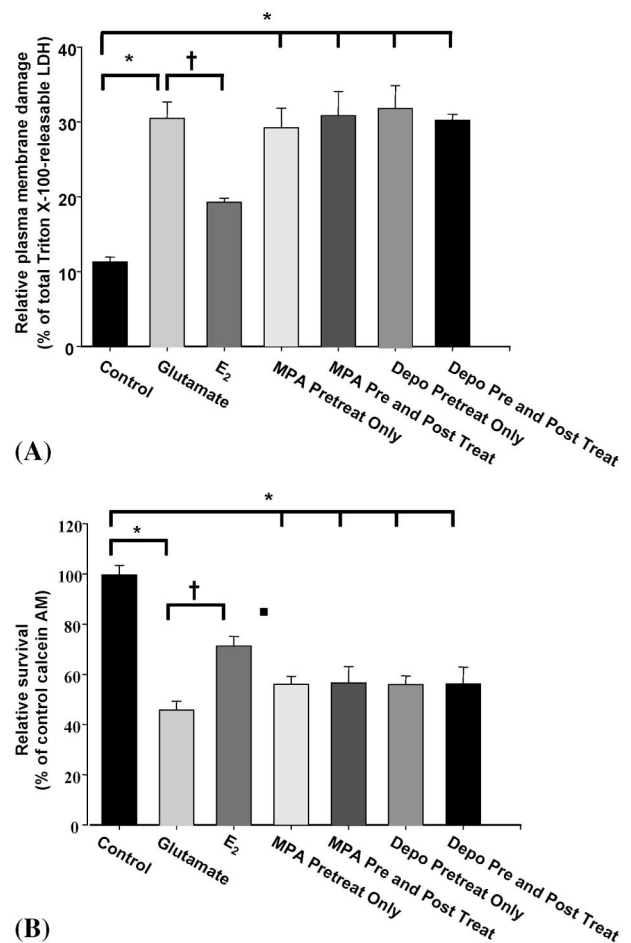


Figure 1. Medroxyprogesterone acetate and Depo-Provera<sup>®</sup> are ineffective at protecting against glutamate-induced neuronal death. Primary hippocampal neurons were treated with 10 ng/ml 17 $\beta$ -estradiol (E<sub>2</sub>), medroxyprogesterone acetate (MPA), Depo-Provera<sup>®</sup> (Depo) or vehicle control for 48 h prior to a 5-min exposure to glutamate (200  $\mu$ M) followed by a 24-h recovery in the presence (Pre and Post Treat) or absence (Pretreat Only) of continued steroid. (A) Cell damage was assessed by release of lactate dehydrogenase (LDH). (B) Cell survival was assessed by calcein AM imaging. Values are means with standard error of the mean shown by vertical bars ( $n = 4$ ). Mean values were significantly different: \* $p < 0.05$  versus control; † $p < 0.05$  versus glutamate alone.

vs. control). E<sub>2</sub> and progesterone pretreatment significantly increased cell survival 24 h following glutamate exposure, with a 50% and 52% reduction in the glutamate-induced increase in LDH release, respectively (Figure 1A;  $p < 0.05$  vs. glutamate alone), and a ~60% and 63% reduction in the glutamate-induced decline in calcein intensity, respectively (Figure 1B;  $p < 0.05$  vs. glutamate alone) (progesterone data not shown). Neither MPA nor Depo-Provera<sup>®</sup> prevented glutamate-induced LDH release (Figure 1A, Pretreat Only;  $p < 0.05$  vs. control) or decline in calcein intensity (Figure 1B, Pretreat Only;  $p < 0.05$  vs. control). Exposure to MPA or Depo-Provera<sup>®</sup> following glutamate exposure had no effect on the lack of neuroprotection by pretreatment of the compounds (Figure 1A and 1B, Pre and Post Treat;  $p < 0.05$  vs. control).

We next determined if the lack of neuroprotective effect was due to the core structure of MPA or if it was related to the acetate residue. To determine whether the non-acetated derivative MP lacked neuroprotection, primary hippocampal neurons were pretreated with MP for 48 h prior to an excitotoxic glutamate exposure (200  $\mu$ M for 5 min). Twenty-four hours later cell survival was assessed by calcein staining. As before, exposure to glutamate resulted in a significant decline in cell survival 24 h later, with a 50% reduction in calcein intensity (Figure 2;

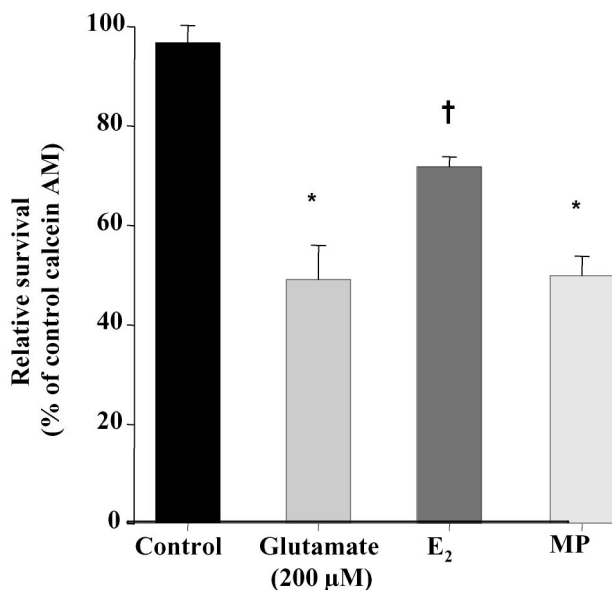


Figure 2. Medroxyprogesterone is ineffective at protecting against glutamate-induced neuronal death. Primary hippocampal neurons were treated with 10 ng/ml 17 $\beta$ -estradiol (E<sub>2</sub>), medroxyprogesterone (MP) or vehicle control prior to a 5-min exposure to glutamate (200  $\mu$ M) followed by a 24-h recovery in the continued presence of steroid. Cell survival was assessed by calcein AM imaging. Values are means with standard error of the mean shown by vertical bars ( $n = 4$ ). Mean values were significantly different: \* $p < 0.05$  versus control; † $p < 0.05$  versus glutamate alone.

$p < 0.05$  vs. control). E<sub>2</sub> pretreatment significantly increased cell survival 24 h after glutamate exposure, with a 60% reduction in the glutamate-induced decline in calcein intensity (Figure 2;  $p < 0.05$  vs. glutamate alone). MP was ineffective in preventing the glutamate-induced decrease in calcein intensity (Figure 2;  $p < 0.05$  vs. control).

#### *Medroxyprogesterone acetate exacerbates neuronal loss in response to glutamate excitotoxicity*

As the biochemical assays used above are estimations of cell survival based on membrane damage and cellular enzymatic activity, we determined neuron cell survival using direct cell counts to assess the effect of MPA on actual neuron survival. Primary hippocampal neurons grown on gridded coverslips were counted blind to the experimental condition prior to and 24 h after exposure to excitotoxic glutamate (200  $\mu$ M for 5 min). Under vehicle control conditions, 95% of neurons survived (Figure 3). Exposure of neurons to excitotoxic glutamate exposure resulted in 45% neuronal survival, representing a significant increase (52%) in neuronal death (Figure 3;  $p < 0.01$  vs. control). Pretreatment with E<sub>2</sub> (10 ng/ml) resulted in 82% survival rate 24 h after glutamate exposure, representing a significant attenuation (26%) of the glutamate-induced loss in neuron survival (Figure 3;  $p < 0.01$  vs. glutamate alone). In contrast, neurons pretreated with MPA prior to glutamate exposure exhibited 20% survival at 24 h post glutamate exposure, a survival rate significantly lower than that observed with glutamate alone (Figure 3;  $p < 0.001$  vs. control;  $p < 0.05$  vs. glutamate alone).

#### *Medroxyprogesterone acetate increases neuronal apoptosis in response to glutamate excitotoxicity*

To determine the mode of cell death induced by glutamate and exacerbated by MPA we assessed primary hippocampal neurons for apoptosis by TUNEL staining 24 h after excitotoxic glutamate exposure (Figure 4A). Primary hippocampal neurons grown on gridded coverslips were TUNEL stained followed by a quantitative analysis, blind to the experimental condition, of the number of TUNEL-positive cells prior to and 24 h after exposure to excitotoxic glutamate. Excitotoxic glutamate exposure (200  $\mu$ M for 5 min) resulted in significant increase (100%) in the percentage of TUNEL-positive neurons (Figure 4B;  $p < 0.05$  vs. control). Pretreatment with E<sub>2</sub> (10 ng/ml) significantly attenuated (35%) the glutamate-induced increase in TUNEL-positive cells (Figure 4B;  $p < 0.05$  vs. glutamate alone). In contrast, MPA pretreatment potentiated (140%) the glutamate-induced increase in TUNEL-positive cells (Fig. 4B;  $p < 0.01$  vs. control;  $p < 0.05$  vs. glutamate alone).

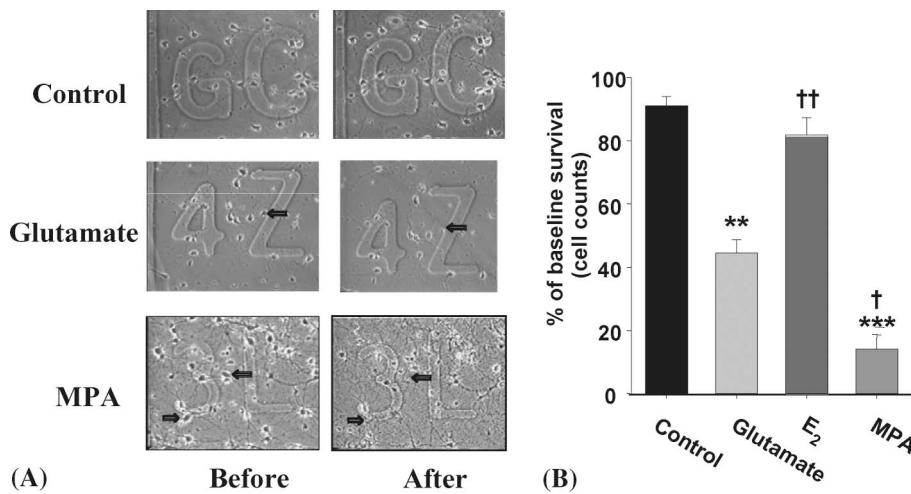


Figure 3. Medroxyprogesterone acetate potentiates glutamate-induced neuronal cell loss. Primary hippocampal neurons were treated with 10 ng/ml 17 $\beta$ -estradiol (E<sub>2</sub>), medroxyprogesterone acetate (MPA) or vehicle control prior to a 5-min exposure to glutamate (200  $\mu$ M) followed by a 24-h recovery in the continued presence of steroid. Images were acquired prior to and 24 h after glutamate exposure. (A) Representative images of control, glutamate and MPA cultures. Arrows point to cells alive in the left panel and dead in the right panel following glutamate exposure. (B) Values are means with standard error of the mean shown by vertical bars ( $n=4$ ). Mean values were significantly different: \*\* $p < 0.01$  versus control; \*\*\* $p < 0.001$  versus control; † $p < 0.05$  versus glutamate alone; †† $p < 0.01$  versus glutamate alone.

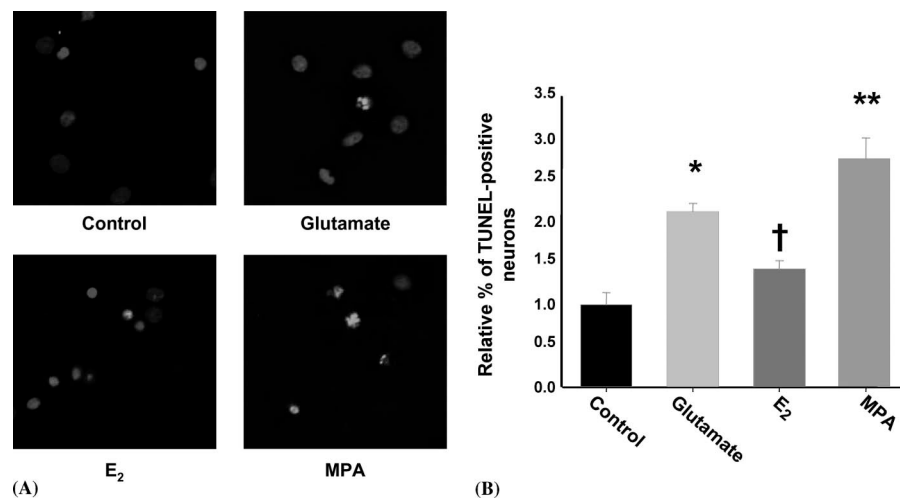


Figure 4. Medroxyprogesterone acetate potentiates glutamate-induced neuronal apoptosis. Primary hippocampal neurons were treated with 10 ng/ml 17 $\beta$ -estradiol (E<sub>2</sub>), medroxyprogesterone acetate (MPA) or vehicle control prior to a 5-min exposure to glutamate (200  $\mu$ M) followed by a 24-h recovery in the continued presence of steroid. (A) Neuronal apoptosis was assessed by TUNEL staining (green) and total neuron number was determined by DAPI counterstain (blue). (B) Values are means with standard error of the mean shown by vertical bars ( $n=20$  neurons). Mean values were significantly different: \* $p < 0.05$  versus control; \*\* $p < 0.01$  versus control; † $p < 0.05$  versus glutamate alone. TUNEL, terminal deoxynucleotidyl transferase-mediated nick end-labeling; DAPI, 4',6-diamidino-2-phenylindole.

## Discussion

The predominant effects of synthetic progestins, such as MPA, on the brain remain controversial and the short- and long-term impact of co-administration of progestins with estrogen is unclear. Results of the present study indicate that MPA exacerbates glutamate-induced neurotoxicity in primary hippocampal neural cultures. This effect is in stark contrast to the protective effects of E<sub>2</sub> and progesterone [12]. Results reported herein and those reported previously

[12,21] are consistent with the recent finding that MPA co-administration blocks estrogen exposure reduction in infarct volume in striatal brain regions [22], in contrast to the protective effect of progesterone observed previously by the same group [23].

Although both the biochemical assays and the direct morphological assessment of neuronal survival indicate a lack of protection by MPA, only with the latter approach were we able to demonstrate the potentiation of neuronal loss. The biochemical assays used above are estimations of cell survival based on

membrane damage and cellular enzymatic activity and may not reflect the true extent of neuronal death. Furthermore, the sensitivity of the biochemical assays is limited by assessment of the signal from whole cell populations at a single time point following toxicity, decreasing the signal-to-noise ratio. This is in contrast to the morphological assessments, which assay a distinct population of cells prior to and following toxic insult.

The data indicate that lack of neuroprotection by MPA was not dependent upon formulation, chemical features or prevention versus treatment mode of exposure. This lack of neuroprotective efficacy is shared by crystalline MPA and clinical preparations of MPA. Structural analysis of the synthetic progestin, by comparing the neuroprotective efficacy of MPA and the deacetylated MP, indicated that the detrimental neural effects of MPA are not due to the inclusion of the acetate group *per se*, indicating that the perturbation of the progestogenic effect is related to the core MP structure rather than strictly to the addition of a side chain bulk to the steroid structure.

The present study suggests that the formulation of HT could potentially affect its therapeutic efficacy for the prevention of age-related neurodegenerative disease. Epidemiological analyses indicate that women have a greater risk of developing AD than their age-matched male counterparts [24]. In addition, the sparing effect against ischemic brain injury and its related mortality present in the premenopausal female [25] is greatly diminished following menopause or ovariectomy [26], suggesting that the sex-dependent increased risk for neurological damage is due, in part, to the deprivation of ovarian hormones in postmenopausal women. Ovariectomy, as well as menopause, results in a precipitous decline not only of estrogen but also of progesterone, which may contribute to the deficits observed and be equally important in attenuating or delaying the onset of neuropathological changes associated with aging.

These results have much broader implications encompassing the impact of progestins on estrogen-mediated effects in other tissues. The Women's Health Initiative (WHI) [27], the Heart and Estrogen/Progestin Replacement Study (HERS) [28] and the Postmenopausal Estrogen/Progestins Intervention (PEPI) trial [29] reported no protection or adverse effects of the hormones on women's health including breast cancer and coronary heart disease, in addition to stroke and AD. Although the PEPI trial compared different HT regimens, it along with the WHI and HERS trials all utilized the same HT formulation: conjugated estrogens (Premarin<sup>®</sup>) with MPA (Provera<sup>®</sup>). Other studies that used different hormone formulations have found mixed effects of estrogen/progestin use on breast cancer [30] and coronary heart disease [31]. In fact, MPA, but not progesterone, antagonized estradiol protection

against coronary artery vasospasm in rhesus monkeys [32]. These data together with the divergent effects of MPA and progesterone on infarct volume [22,23] and those of the present report predict that the effect of the progestin could be a critical component determining the therapeutic benefit of HT for the prevention of neurodegenerative disease.

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