A Combination Therapy of 17β-Estradiol and Memantine Is More Neuroprotective Than Monotherapies in an Organotypic Brain Slice Culture Model of Traumatic Brain Injury

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Abstract

Combination therapies are a promising therapeutic option for traumatic brain injury (TBI) owing to the clinical failure of monotherapy treatments, such as progesterone. Organotypic hippocampal slice cultures (OHSCs) from Sprague-Dawley rats were subjected to an in vitro TBI, and the neuroprotective effects of 17β-estradiol (E2) or memantine (MEM) monotherapies were quantified. Several combination treatments at different concentrations of both drugs were tested, with 100 pM of E2 and 10 μM of MEM statistically and significantly reducing cell death over either monotherapy when administered immediately after injury. This combination was also significantly neuroprotective when administered 1 h postinjury, possibly supporting future in vivo studies. Further, we hypothesized that this synergy could be the result of MEM blocking a potentially deleterious effect of E2, specifically E2 enhancement of N-methyl-D-aspartate (NMDA) currents. Evoked electrophysiological responses in OHSCs were potentiated by E2 treatment, whereas this potentiation was significantly reduced by MEM. In conclusion, a combination therapy of E2 and memantine was significantly more neuroprotective than both monotherapy treatments, and this synergy may be the result of MEM blocking a deleterious E2-mediated enhancement of NMDA receptors.

Key words: combination therapies; E2; electrophysiology; memantine; traumatic brain injury

Introduction

The pathology of traumatic brain injury (TBI) is complex and dynamic, with multiple secondary injury mechanisms causing delayed cell death for hours or days after the primary injury.1–3 These secondary injuries can lead to progressive neurodegeneration, often accompanied by permanent loss of sensory, motor, and/or cognitive function. Individual drugs, or monotherapies, which have been successful in experimental models, have failed clinically.4–7 Because of the complexity of interactions between secondary injury mechanisms, it is likely that multiple drugs targeting several secondary injury cascades or a single drug with multiple mechanisms of action will be required to provide neuroprotection after TBI.1

Although many monotherapies have demonstrated efficacy in animal models of TBI, none have been clinically effective (i.e., providing a benefit to patients, compared to those receiving a placebo).7 These failures could be owing to many factors, including lack of understanding of the underlying injury mechanisms, inadequate pre-clinical testing in multiple injury models, or because TBI initiates several secondary pathological cascades that a single drug cannot sufficiently reverse the negative outcome.1 The goal of a combination therapy is to combine two or more therapeutics with complementary or synergistic actions to increase the efficacy of TBI treatment to a level that is clinically relevant.1,3,8

17β-estradiol (E2) is the most potent naturally occurring form of estrogen and exerts a multitude of effects after binding to the estrogen receptor. After binding E2, the classical estrogen receptors translocate to the nucleus and act as transcription factors, which control the expression of many genes.9 E2 also binds cell-surface receptors, causing near immediate physiological effects.10,11 E2 is not without drawbacks, and some research suggests potentially negative effects, such as increased N-methyl-D-aspartate (NMDA) receptor (NMDAR) activation, which may be detrimental in the acute phase of TBI. On the positive side, reduced oxidative stress, anti-inflammation, improved vascular function, decreased apoptosis, and attenuated excitotoxicity are all benefits associated with E2 therapy postinjury.12–16 It is currently U.S. Food and Drug Administration (FDA)-approved for hormone replacement therapies. Although E2 has shown efficacy as a therapeutic compound in animal models of TBI, it has not translated clinically. There is currently a clinical phase II trial, RESCUE-TBI, testing the safety
and efficacy of Premarin IV (conjugated estrogens for injection), which has not released results.17

Memantine (MEM) is a noncompetitive, use-dependent NMDAR antagonist. It is FDA-approved to treat Alzheimer’s disease (AD) and is generally well tolerated. Although MEM, as with E2, has shown promise as a treatment for TBI in animal models, it has not translated clinically. Whereas excess glutamate and excitotoxicity are well-known secondary injuries post-TBI, the therapeutic window for NMDAR antagonists is short. Several studies have shown that NMDAR antagonists must be administered < 1 h postinjury to provide significant neuroprotection. In reality, therapeutics for TBI are rarely administered in this time frame. Because of this short therapeutic window, MEM and other NMDAR antagonists are not likely to be clinically significant treatments for TBI as monotherapies. However, these limitations do not exclude NMDAR antagonists as a component of a combination treatment that relies on synergistic actions between two drugs.

In this study, we hypothesized that a combination treatment of E2 and MEM would work synergistically to provide better neuroprotection after a mechanical injury of organotypic hippocampal slice cultures (OHSs), compared to monotherapies. Further, we hypothesized that this synergy would be the result of MEM mitigating a possible negative side effect of E2, specifically potentiation of NMDAR currents. Our in vitro model of TBI is well established as an alternative to in vivo experiments and was an appropriate system to test a combination therapy, which requires several experimental groups.18 We tested three concentrations of each drug individually and followed with all possible combinations; a combination treatment of E2 and MEM significantly decreased cell death, compared to vehicle controls and monotherapies, in the cornu ammonis (CA)1 region of the hippocampus post-TBI. E2 has been shown to increase NMDAR conductance, and we hypothesized that MEM may reduce this effect and potentially improve neuroprotection by decreasing these effects.19 We showed increased Schaffer collateral-evoked responses in the CA1 region after the addition of E2 and that MEM significantly reduced this potentiation. These data suggest that the combination therapy of E2 and MEM warrants more detailed testing in vivo.

**Methods**

**Organotypic hippocampal slice cultures**

All animal procedures were approved by Columbia University’s Institutional Animal Care and Use Committee (New York, NY). OHSs were cultured as previously described.20–22 Briefly, Sprague–Dawley rat pups (P8–P10) were rapidly decapitated, and the hippocampus excised and placed in ice-cold Gey’s balanced salt solution (Life Technologies, Grand Island, NY), supplemented with d-glucose (4.5 mg/mL; Sigma-Aldrich, St. Louis, MO). Transverse sections (400 μm thick) were cut using a McIlwain tissue chopper (Ted Pella, Redding, CA) and then plated on poly-dimethylsiloxane membranes (Specialty Manufacturing, Saginaw, MI) previously coated with laminin (80 μg/mL; Life Technologies) and poly-l-lysine (320 μg/mL; Sigma-Aldrich) in custom-made stainless steel wells. Cultures were initially fed Neurobasal (Life Technologies) medium supplemented with B27 (Life Technologies), GlutaMAX (2 μM; Life Technologies), d-glucose (4.5 mg/mL; Sigma-Aldrich). Medium was changed every 2–3 days. E2 dipotassium salt and MEM hydrochloride (Sigma-Aldrich) were added at the concentrations indicated below, either immediately postinjury or with a delay of 1 or 2 h postinjury.

**In vitro model of traumatic brain injury**

After 10 DIV, OHSs cultured on silicone membranes were subjected to a moderate mechanical injury. The injury was induced by stretching the underlying silicone substrate to a predetermined strain at a predetermined strain rate to produce the desired tissue injury under motion control. Our well-established model produces a highly accurate and reproducible injury to OHSs. Tissue deformation was verified by image analysis of high-speed video (MotionPro; Redlake, Pasadena, CA) at 1000 frames per second.23 Lagrangian strain of the tissue was determined by calculating the deformation gradient tensor by locating fiduciary markers on the tissue slice before and at maximal stretch using custom MATLAB (MathWorks, Natick, MA) scripts.23

**Cell death assessment**

Quantification of cell death in OHS using the fluorescent vital stain, propidium iodide (PI; Life Technologies), has been described previously.21,24 Briefly, micrographs were taken before induction of injury and 4 days postinjury. Cultures were transferred to serum-free medium containing 75% MEM (Sigma-Aldrich), 25% HBSS (Sigma-Aldrich), GlutaMAX (2 μM; Life Technologies), d-glucose (4.5 mg/mL; Sigma-Aldrich) and supplemented with 5 μg/mL of PI for 30 min before imaging. Brightfield and PI images were acquired on an Olympus IX-80 fluorescent microscope fitted with a 175 W Xenon Arc lamp (PerkinElmer, Boston, MA). CoolSNAP ES camera (Photometrics, Tucson, AZ), with an excitation of 556–580 nm and an emission of 590–630 nm (PI exposure 2 sec, brightfield exposure 3 ms). Metamorph ( Molecular Devices, Sunnyvale, CA) image analysis software was used to outline the CA1, CA3, and dentate gyrus (DG) regions in the brightfield image, and these regions of interest (ROIs) were transferred to the PI images taken pre- and postinjury. Percentage cell death was determined as the area above a threshold in the PI fluorescent image divided by the total area of the ROI (i.e., CA1 region). The threshold was determined by the fluorescence level of uninjured, control slices imaged on the same day as injured experimental groups. OHSs with preinjury cell death > 5% in the ROI were discarded.

**Electrophysiology**

For electrophysiological recordings, an MEA60 system (Multichannel Systems, Reutlingen, Germany) was used. Microelectrode arrays (MEAs) were first made hydrophilic with gas plasma treatment and treated with nitrocellulose (Thermo Fisher Scientific, Waltham, MA) for slice culture adhesion. Slices were transferred from Biopore CM membranes (EMD Millipore, Billerica, MA) to the MEA and perfused with artificial cerebrospinal fluid (aCSF; 125 mM of NaCl, 3.5 mM of KCl, 26 mM of NaHCO3, 1.2 mM of KH2PO4, 1.3 mM of MgCl2, 2.4 mM of CaCl2, 10 mM of d-glucose [pH = 7.4]; Sigma-Aldrich) at 37°C and aerated with 95% O2/5% CO2, as previously described.25 Evoked responses were generated with bipolar, biphasic stimuli (a positive phase for 100 μs followed by a negative phase for 100 μs) applied to the Schaffer collateral axons using a programmable stimulator (STG2004; Multi Channel Systems, Reutlingen, Germany). A stimulus-response curve was generated for each slice culture, as previously described.25 The I50 stimulation current, the current that produced a half-maximal response from the tissue, for each slice was determined with a custom MATLAB script, and this current was used to stimulate the OHS at 1-min intervals. Only recordings from electrodes in the CA1 region that exhibited a steady baseline were used for analysis.26 After 20 min of stable baseline-evoked responses, slices were...
perfused with aCSF containing 100 pM of E2 for 20 min followed by 20 min of aCSF containing 100 pM of E2 and 10 μM of MEM. Data were analyzed with custom MATLAB scripts to determine the peak-to-peak values of the evoked response. Evoked responses were normalized to the average evoked response of the final 10 min of baseline recordings; the normalized average of the final 10 min of E2 alone versus with E2 and MEM were used for statistical analysis.

**Statistical analyses**

All data are represented as mean ± standard error of the mean. Statistical significance was determined using one-way analysis of variance, followed by post-hoc comparisons with the Tukey-Kramer’s adjustment in MATLAB (MathWorks); *p* values less than 0.05 were deemed significant.

**Results**

For all experimental groups, a moderate injury was induced (20.1 ± 0.1% strain, 16.8 ± 0.2/sec strain rate) that resulted in moderate, statistically significant cell death within the CA1 (31.5 ± 5.4%), CA3 (19.9 ± 6.6%), and DG (42.8 ± 7.0%) regions of the OHSCs, compared to uninjured controls (2.9 ± 0.9%, 0.5 ± 0.5%, 4.3 ± 2.9%, respectively), as measured by PI fluorescence. Although E2 and MEM are neuroprotective in several models of TBI, neither has been characterized in our model. Therefore, we first tested a range of concentrations of each drug postinjury.

17β-estradiol after mechanical injury of organotypic hippocampal slice cultures

In both the CA1 and CA3 regions, when administered immediately after mechanical injury, E2 significantly reduced cell death, compared to vehicle-treated controls only at 100 pM (CA1: 13.7 ± 4.7%; CA3: 4.1 ± 2.4%), but did not significantly reduce cell death at 1 (25.2 ± 8.3%, 14.5 ± 7.5%) or 10 nM (22.9 ± 7.0%) measured 4 days post-TBI (Fig. 1A, B). E2 had no significant effect on cell death in the DG at 100 pM, 1 nM, or 10 nM of E2 (36.2 ± 6.3%, 19.7 ± 9.8%, 36.3 ± 9.7%, respectively; Fig. 1C). We have previously shown a similar result in an in vitro model of oxygen-glucose deprivation, and these data suggest that physiological concentrations of E2 are more effective at reducing cell death, compared to supraphysiological concentrations postinjury.

Memantine after mechanical injury of organotypic hippocampal slice cultures

MEM monotherapy administered immediately postinjury significantly reduced cell death at 10 μM (CA1: 8.2 ± 1.9%; CA3: 0.2 ± 0.1%; DG: 0.7 ± 0.3%; Fig. 1A–C), the highest concentration tested, compared to vehicle-treated controls in all regions. In the CA3 and DG region, 1 μM of MEM significantly reduced cell death (4.3 ± 1.7% and 18.1 ± 4.3%, respectively), but did not in the CA1 (21.0 ± 4.3%). MEM did not significantly reduce cell death when administered at 100 nM (CA1: 21.0 ± 1.9%; CA3: 8.5 ± 2.4%; DG: 22.1 ± 7.4%) in any region.

**Combination treatments significantly decreased cell death**

In the CA1, eight of the nine concentration combinations administered immediately postinjury significantly reduced cell death, compared to vehicle-treated, injured controls (Fig. 2A). Cell death was significantly reduced post-treatment with a combination of 100 nM of MEM + 100 pM of E2 (13.6 ± 4.8%), 1 μM of MEM + 100 pM of E2 (6.9 ± 2.6%), 10 μM of MEM + 100 pM of E2 (2.6 ± 1.7%), 100 nM of MEM + 1 nM of E2 (13.3 ± 4.0%), 1 μM of MEM + 1 nM of E2 (6.4 ± 3.0%), 10 μM of MEM + 1 nM of E2 (9.8 ± 5.4%), 1 μM of MEM + 10 nM of E2 (10.5 ± 4.0%), and 10 μM of MEM + 10 nM of E2 (8.2 ± 2.5%). Only the combination of 100 nM of MEM + 10 nM of E2 (17.5 ± 3.3%) did not significantly reduce cell death, compared to injured controls. The combination of 10 μM of MEM + 100 pM of E2 was also significantly more neuroprotective than treatment at any concentration with either E2 or MEM alone.

![Fig. 1](image-url)
FIG. 2. Combination therapies were significantly more neuroprotective than monotherapies. Several of the combination therapies were significantly more neuroprotective, compared to vehicle treatment; \( ^* p < 0.05 \), compared to vehicle treatment. The combination of 100 nM of E2 and 10 \( \mu \)M of memantine was significantly more neuroprotective than either monotherapy for the CA1 region; \( ^* p < 0.05 \), compared to 100 nM E2 monotherapy; \( ^* p < 0.05 \), compared to 10 \( \mu \)M memantine monotherapy. Solid lines represent mean cell death for all regions after vehicle treatment; large dashed lines represent mean cell death for CA1 and CA3 after 100 nM E2 treatment and for DG after 1 nM E2 treatment; small dashed lines represent mean cell death for all regions after 10 \( \mu \)M memantine treatment. All \( n \geq 8 \).

In the CA3, only three of the nine combinations administered immediately postinjury significantly reduced cell death, compared to vehicle controls (Fig. 2B). These combinations were 10 \( \mu \)M of MEM + 100 nM of E2 (1.9 \pm 1.8%); 1 \( \mu \)M of MEM + 10 nM of E2 (0.34 \pm 0.53%); and 10 nM of MEM + 10 nM of E2 (0.1 \pm 0.1%). The combinations of 100 nM of MEM + 100 pM of E2 (2.1 \pm 1.8%); 1 \( \mu \)M of MEM + 100 pM of E2 (1.3 \pm 0.9%); 100 nM of MEM + 1 nM of E2 (3.3 \pm 1.3%); 100 nM of MEM + 10 nM of E2 (3.6 \pm 1.6%); 1 \( \mu \)M of MEM + 1 nM of E2 (3.2 \pm 3.0%); 10 \( \mu \)M of MEM + 1 nM of E2 (0.8 \pm 0.6%) did not significantly reduce cell death.

All nine drug combinations administered immediately postinjury significantly reduced cell death, compared to vehicle controls, in the DG (Fig. 2C). Cell death was significantly reduced post-treatment with a combination of 100 nM of MEM + 100 pM of E2 (11.3 \pm 7.9%); 1 \( \mu \)M of MEM + 100 pM of E2 (8.1 \pm 5.3%); 10 nM of MEM + 100 pM of E2 (3.8 \pm 3.6%); 100 nM of MEM + 1 nM of E2 (6.8 \pm 3.9%); 1 \( \mu \)M of MEM + 1 nM of E2 (2.5 \pm 1.2%); 10 nM of MEM + 1 nM of E2 (1.8 \pm 0.9%); 100 nM of MEM + 10 nM of E2 (5.9 \pm 1.7%); 1 \( \mu \)M of MEM + 10 nM of E2 (5.1 \pm 3.3%); and 10 nM of MEM + 10 nM of E2 (0.2 \pm 0.1%).

The most effective combination treatment was neuroprotective when administration was delayed postinjury

The most effective combination treatment (100 pM of E2 + 10 \( \mu \)M of MEM) was tested for its efficacy when administered at 1 or 2 h postinjury. Because full media changes were used to administer the combination therapy at 1 and 2 h postinjury, time-matched controls that received a vehicle medium change at the same time point were also quantified. There was no significant difference (CA1: \( p = 0.83 \); CA3: \( p = 0.99 \); DG: \( p = 0.42 \)) in cell death between cultures receiving vehicle treatment at 1 (CA1: 30.9 \pm 9.5%; CA3: 12.5 \pm 4.3%; DG: 27.3 \pm 8.8%) or 2 h (CA1: 34.1 \pm 10.3%; CA3: 12.4 \pm 5.8%; DG: 17.4 \pm 8.0%) postinjury within a given region. Because they were not significantly different, the vehicle groups were combined (CA1: 32.8 \pm 6.9%; CA3: 12.4 \pm 3.6%; DG: 21.6 \pm 5.9%). At 1 h postinjury, the combination treatment significantly reduced cell death (CA1: 18.6 \pm 4.5%; CA3: 0.3 \pm 0.1%; DG: 29.8 \pm 1.2%) in all regions, whereas treatment at 2 h postinjury did not significantly reduce cell death (CA1: 38.5 \pm 5.9%; CA3: 10.1 \pm 5.1%; DG: 24.9 \pm 9.6%; Fig. 3) in any region.

**Memantine significantly reduced 17\(^{\beta}\)-estradiol-mediated potentiation of evoked responses**

After 20 min of perfusion with 100 pM of E2 in naive cultures, amplitude of evoked responses in the CA1 region increased by 29.4 \pm 5.5% (Fig. 4B) above baseline amplitude. After perfusion of E2 alone, addition of MEM to aCSF in the continued presence of E2 significantly decreased evoked responses to 7.6 \pm 2.9% above baseline amplitude (Fig. 4C).

**Discussion**

Post-TBI, the primary mechanical stimulus initiates a cascade of biological events leading to secondary injuries.1–28 Cell death by secondary injuries can continue for hours or longer after the primary injury.1–3 These ongoing cascades leading to delayed cell death provide a potential temporal window for therapeutic intervention to slow, stop, or repair damaged cells otherwise destined to die and may contribute to improved survival and decreased long-term disabilities.

Although intense research into TBI therapeutics continues, no clinically relevant monotherapy treatments have emerged to abrogate delayed cell death and improve outcome.1 Pre-clinical studies have provided many potential monotherapies that have shown benefit post-TBI in experimental models. One explanation for a lack of clinical translation may be that monotherapy...
treatments focus primarily on one aspect of a very complex injury and may be too limited to show clinical efficacy. For example, some of the secondary injuries that occur post-TBI include ischemia, hypoxia, edema, excitotoxicity, inflammation, and free radical formation, among others.  

Combination treatments are a promising alternative to monotherapies for treatment of TBI. A similar approach has proven successful in other diseases, such as acquired immune deficiency syndrome (AIDS) for which a 65% decrease in human

**FIG. 3.** Combination of $100 \text{pM}$ of E2 and $10 \mu M$ of memantine was neuroprotective when delivered 1, but not 2, h post-injury. Because full media changes were used to administer the combination therapy at 1 and 2 h postinjury, time-matched controls that received a vehicle medium change at the same time point were also quantified. There was no significant difference (CA1: $p = 0.83$; CA3: $p = 0.99$; DG: $p = 0.42$) in cell death between cultures receiving vehicle treatment at 1 (CA1: $30.9 \pm 9.5\%$; CA3: $12.5 \pm 4.3\%$; DG: $27.3 \pm 8.8\%$) or 2 h (CA1: $34.1 \pm 10.3\%$; CA3: $12.4 \pm 5.8\%$; DG: $17.4 \pm 8.0\%$) postinjury within a given region. Because they were not significantly different, the vehicle groups were combined on a per-region basis. All $n \geq 6$; *$p < 0.05$, compared to vehicle treatment.

**FIG. 4.** Memantine reduced E2-induced potentiation in the CA1 region of the hippocampus. (A) An organotypic hippocampal slice culture on a microelectrode array. Oval indicates CA1 electrodes, which were included in analysis. White stars represent Schaffer collateral stimulating electrodes. (B) Representative evoked response from a single electrode after (1) 20 min of $100 \text{pM}$ E2 potentiation and (2) 20 min of $100 \text{pM}$ of E2$+10 \mu M$ of memantine (offset by 50 ms for clarity). (C) Memantine significantly reduced E2-induced potentiation of evoked response amplitude.
immodeficiency virus/AIDS–related deaths occurred between 1995 and 1998 with the introduction of combination therapies.30 In 2008, a workshop organized by the National Institute of Neurological Disorders and Stroke brought together clinicians and scientists from a variety of disciplines to identify promising combination treatments for TBI.31 It was recommended that potential combination therapies should “combine agents with complementary targets and effects (e.g., mechanisms and time-points), rather than focusing on a single target with multiple agents.” To that end, we chose to test two FDA-approved drugs with very different physiological effects, but that may have complementary mechanisms of action to produce a synergistic effect.

**Estrogen as a monotherapy**

Estrogen is an important steroid hormone that has also been implicated in many physio- and psychological changes. The most potent, naturally occurring form of estrogen, E2, has been investigated as a neuroprotective therapy for TBI.31–33 An early study by Emerson and colleagues showed improved biochemical and cognitive outcomes with post-TBI (fluid percussion) injection of E2.34 Neuroprotection was dependent on gender, and only male rats significantly improved postinjury. Similarly, Roof and Hall found that 100% of female rats, compared to 75% of male rats, survived an impact-acceleration closed-head injury and suggested that endogenous estrogen may have been neuroprotective.35 In addition to TBI research, E2 is well known to be neuroprotective in models of ischemia.27,36–38 In our hands, E2 was neuroprotective only at a low, physiological dose of 100 pM and only in the CA1 and CA3 regions. In contrast, cell death was not significantly reduced in the DG with E2 alone.

**Memantine as a monotherapy**

MEM is an FDA-approved, noncompetitive NMDAR antagonist, which is prescribed primarily for AD, but is also used to treat Parkinson’s disease and senile dementia.30,40 It is a low-affinity, use-dependent inhibitor that does not affect learning and memory formation through long-term potentiation as other, more-potent NMDAR inhibitors can.42,43 Because its antagonism is use dependent, its effects are greater when higher concentrations of receptor agonists are present, which may target the drug’s actions to injured areas, particularly in the acute phase post-TBI. These properties alleviate the deleterious side effects of other NMDAR antagonists, such as MK-801, which produce hallucinations and psychosis as a result of potent blockade of synaptic NMDARs.44

The neuroprotective effects of NMDAR antagonists, including MK-801 and MEM, have been well documented in many different models of central nervous system injury over the past 20 years.45,46 As a monotherapy, MEM is a potent inhibitor of glutamate-induced excitotoxicity.47 We found that MEM was neuroprotective at the highest dose tested (10 μM) in the CA1, CA3, and DG. However, MEM has been reported to have a limited role in reducing excitotoxicity post-TBI. In rodents, increased glutamate postinjury is short lived (<1 h), and therefore a monotherapy of MEM may not be clinically relevant given delays in transportation to a trauma facility.9 In fact, NMDARs may be dysfunctional 1 h postinjury, and it has been suggested that enhancement of NMDAR signaling with d-cycloserine may be neuroprotective.48 In agreement with these results, clinical trials of NMDAR antagonists as monotherapies have all failed.49 Recognizing the limitations of MEM as treatment of post-TBI excitotoxicity, we chose to combine MEM with E2 as a direct complement to a potentially negative effect of E2 treatment.

**Potential synergistic effects of 17β-estradiol and memantine**

E2 induces numerous downstream events, which have the potential to simultaneously affect multiple therapeutic targets post-injury. Although many studies have confirmed that E2 is neuroprotective, a few have also noted potentially negative side effects of treatment. Some of these potentially negative side effects may include increased oxidative stress, inflammation, and excitotoxicity.50 Short-term treatment with E2 has been shown to increase excitatory postsynaptic potentials and enhance long-term potentiation through potentiation of N-methyl d-aspartate receptor subtype 2B (NR2B)-containing NMDAR receptors.10–51 Others have shown that activation of typically extrasynaptic, NR2B-containing NMDARs may lead to cell death, whereas activation of synaptic NMDARs containing the NR2A subunit may support neuronal survival.52 We hypothesized that efficacy of E2 may have been diminished by enhancement of extrasynaptic, NR2B-containing NMDAR receptors and that coadministration of an inhibitor that favors NR2B-containing receptors could increase the benefit of E2 treatment without blocking its other beneficial effects.

MEM was chosen for the current study because it acts primarily on NR2B-containing, extrasynaptic NMDARs, potentially blocking the negative effect of E2.53 In general, NR2B subunit-specific inhibitors tend to have a better therapeutic profile in many animal models when compared to traditional NMDAR antagonists, such as MK-801.53 In particular, previous studies of ifenprodil and edrophil, both NR2B-specific inhibitors, significantly reduced NMDA-induced release of acetylcholine. MEM had a similar effect, whereas the more potent NMDAR inhibitor, MK-801, was less effective. We suggest that a combination therapy of E2 and MEM may have synergistic effects, with memantine reducing the potentially lethal increase in activation of NR2B-containing NMDARs caused by E2.

We found that many of the combination treatments of E2 and MEM resulted in a significant reduction in cell death, compared to vehicle controls. Of particular interest, some combinations of drug concentrations that were not neuroprotective by themselves became significantly neuroprotective when combined (e.g., 100 nM of MEM + 10 nM of E2 in all regions). One combination significantly reduced cell death in the CA1 region more than either of the most effective monotherapies (100 pM of E2 + 10 μM of MEM). These results suggest that ineffective monotherapy doses of both drugs, when combined, have a beneficial and synergistic effect post-TBI, and those combinations may warrant further research.

**Combination treatment of traumatic brain injury**

Our study is the first to show that the combination of E2 and MEM significantly reduced cell death in a model of TBI. We have also shown that this neuroprotection was significantly more effective than either E2 or MEM alone and have proposed and tested a potential mechanism for this synergy. This is a promising result for a novel TBI therapy that may warrant in vivo follow-up studies.

Although the combination of E2 and MEM reduced cell death in our model of TBI, it has yet to be tested in vivo and faces many challenges before clinical testing. The most neuroprotective combination of E2 and MEM significantly reduced cell death up to 1 h
postinjury, which may limit its clinical relevance. Because of in-
formed consent issues, it is difficult to enter patients into clinical
trials before 6 h. The lowest concentrations of E2 tested was
100 pM, which corresponds to physiological levels; however, lower
concentrations, as part of a combination therapy, could be tested in
future studies. Our in vitro model of TBI lacks effects owing to
blood circulation, perfusion, and the blood–brain barrier among
others in vivo. Testing the combination in higher-order animals of
different age and sex as well further understanding the mechan-
istic link between E2 and MEM neuroprotection are also needed.
The pharmacological consequences of combining drugs are an
important and difficult hurdle that requires detailed pharmacoki-
netic studies. These studies should optimize the dose, route of ad-
ministration, timing, and evaluate the mechanism of action.
Nevertheless, our study suggests a potential combination therapy
for acute treatment of TBI.

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Author Disclosure Statement

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