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Neuroprotective Effects of Estrogen and Tamoxifen In Vitro

A Facilitative Role for Glia?

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Selective estrogen receptor modulators (SERMs) are steroidal or nonsteroidal compounds that can exhibit either estrogen-like agonistic effects or estrogen-antagonistic effects depending on the target tissue. While SERM actions in the breast, bone, and uterus have been well characterized, their effects in the brain are considerably less well understood. Previous work by our laboratory has demonstrated a beneficial effect of tamoxifen in the reduction of ischemic stroke damage in ovariectomized female rats. The present study utilized neuronal cell culture models to attempt to understand the mechanisms of tamoxifen-mediated neuroprotection. Neither physiologic doses of 17β-E_2 nor clinically therapeutic doses of tamoxifen directly protected GT1-7 neurons or purified cultures of rat cerebrocortical neurons from several forms of cell death. Reverse transcriptase polymerase chain reaction and Western blot analysis revealed that GT1-7 neurons possessed both estrogen receptor-α (ERα) and ERβ mRNA and protein, whereas purified embryonic rat cortical neurons only expressed appreciable levels of ERα transcript and protein, with little to no expression of ERβ. In contrast to the lack of protection in the purified neuronal cultures, both 17β-E_2 and tamoxifen significantly protected mixed glial/neuronal cortical cultures from cell death, suggesting that glia may facilitate 17β-E_2– and tamoxifen-mediated neuroprotection. Furthermore, astrocyte-conditioned media and exogenous transforming growth factor-β1, a documented astrocyte-derived cytokine, were shown to rescue purified cortical neurons from cell death. Together, these findings support a role for astrocytes in neuroprotection and raise the intriguing possibility that astrocytes may help mediate the neuroprotective effect of 17β-E_2 and tamoxifen.

Key Words: Selective estrogen receptor modulator; neuroprotection; cerebral cortex; tamoxifen; astrocyte; transforming growth factor-β1.

Introduction

Recent work by a number of laboratories has demonstrated that the ovarian steroid 17β-E_2 is neuroprotective against middle cerebral artery occlusion (MCAO), an animal model of acute ischemic stroke *(1–8)*. Additional work has suggested that 17β-E_2 may also delay the onset and ameliorate the symptoms of Alzheimer disease, although this is still somewhat controversial (see refs. *9* and *10* for reviews). Although potentially beneficial, 17β-E_2 also possesses certain disadvantages, which may limit its usefulness in clinical situations. For instance, the stimulatory effect of 17β-E_2 on the breast and uterus has been associated with an increased risk of cancer in these tissues. Because of such negative side effects, an intense interest in the development and potential therapeutic use of selective estrogen receptor modulators (SERMs) has emerged. SERMS are steroidal or nonsteroidal compounds that can exhibit estrogen-like agonistic or estrogen-antagonistic effects depending on several factors, such as the tissue it is acting on, the estrogen receptor (ER) types present in the tissue, and the milieu of coactivators and corepressors recruited to the receptor-DNA complex *(11)*.

Recent work by our laboratory demonstrated that clinically therapeutic doses (0.8–2.4 mg/[kg·d]) of the SERM tamoxifen significantly protected the ovariectomized adult female rat brain from ischemic stroke damage *(12)*. However, no protection was observed at 0.1 mg/(kg·d), which corresponds to a subtherapeutic dose of tamoxifen. The protective effect was largely observed in the cerebral cortex, although some protection was also observed in the striatum. The protective effect of tamoxifen was independent of changes in cerebral blood flow. Furthermore, pretreatment with tamoxifen was necessary to achieve protection, resembling the situation observed for protection of the brain by 17β-E_2 *(13)*. In addition to our findings, Kimelberg et al. *(14)* have reported that a 5 mg/kg dose of tamoxifen reduces ischemic damage in the brain of male rats following ischemia-reperfusion injury. These findings raise the intriguing possibility that a SERM may protect the brain of both genders

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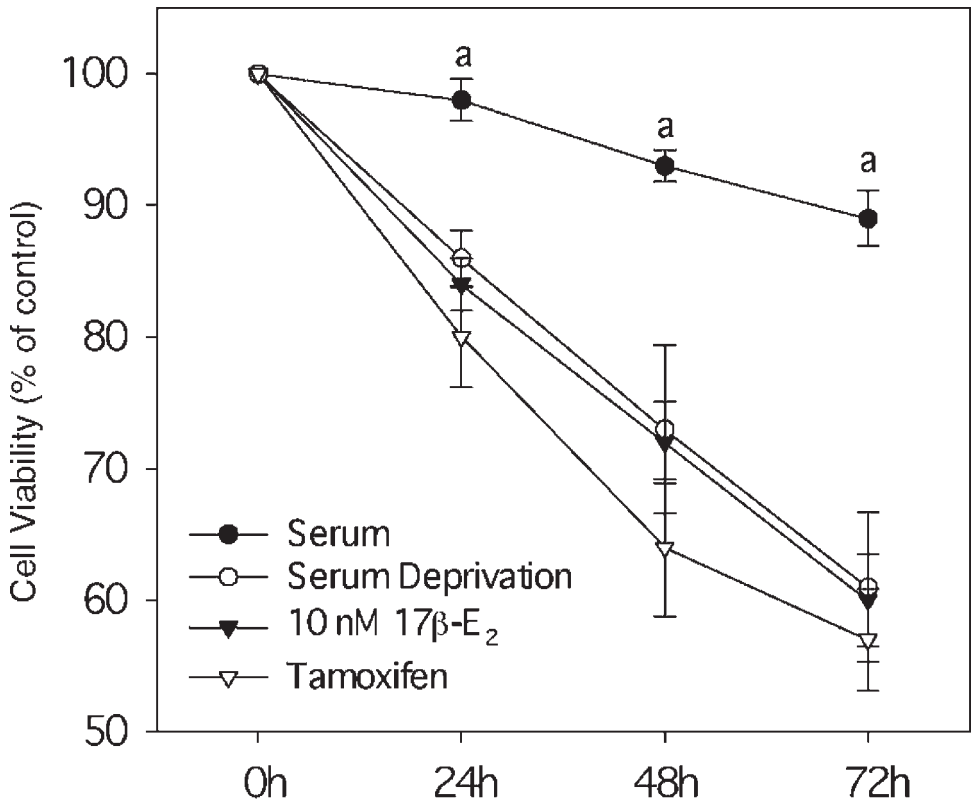

Fig. 1. Effect of 17β-E_2 and tamoxifen on cell death induced by serum deprivation in GT1-7 neurons. GT1-7 neurons were treated with either serum-free medium, or serum-free medium containing 10 n*M* 17β-E_2 or 1 μ*M* tamoxifen. Cell viability was assessed 24, 48, or 72 h following serum deprivation. Groups with different subscripts are significantly different from each other ($p < 0.05$).

against ischemic stroke damage. Additionally, Dluzen et al. *(15)* recently reported that tamoxifen can protect the rat striatum against 1-methyl-4-phenyl-pyridine (MPP+)-induced toxicity, a model for Parkinson disease. Together, these findings suggest that tamoxifen exerts a neuroprotective effect in the brain and adds to a growing list of reports of estrogen-like effects of tamoxifen in the central nervous system, such as the regulation of glucose transporters and insulin-like growth factor expression in the primate cerebral cortex *(16)*, glutamate receptor levels in the rat cerebral cortex *(17, 18)*, choline acetyltransferase expression in the rat basal forebrain *(19)*, and brain metabolism in humans *(20)*. Thus, there appears to be growing recognition that SERMs such as tamoxifen can exert estrogen-like actions in the brain, some of which may be beneficial.

Although recent literature suggests that both physiologic levels of 17β-E_2 and therapeutic concentrations of tamoxifen are neuroprotective, the cellular and molecular mechanisms underlying this effect are poorly understood. Work performed by several groups has demonstrated a direct neuroprotective effect of physiologic levels of 17β-E_2 against cell death in neuronal cultures *(21–24)*. However, an equal number of studies has only documented neuroprotection using high, pharmacologic levels of 17β-E_2, with physiologic doses failing to protect against cell death *(25–29)*. The protection by pharmacologic (micromolar) doses of 17β-E_2 was suggested to be owing to an antioxidant activity of 17β-E_2. Since there are considerable conflicting reports on protection in vitro by physiologic estrogen doses and the effect of tamoxifen has not been characterized, we performed studies to test their effects in vitro in several neuronal models, including GT1-7 neurons, purified cortical neurons, and mixed glial/neuronal cortical cultures.

Results

Lack of Neuroprotection by 17β-E_2 and Tamoxifen in GT1-7 Neurons

The first model used was the immortalized gonadotropin-releasing hormone (GnRH) (GT1-7) neuronal cell line, which is a commonly used neuronal cell line to study neuroprotection and mechanisms of neuronal cell death/survival. As shown in Fig. 1, withdrawal of serum from GT1-7 neurons resulted in a time-dependent decrease in cellular viability. A significant decrease in cell viability was observed within 24 h and a maximal effect was observed at 72 h of treatment —of which a nearly 40% reduction in cellular viability was noted, as compared to serum-treated controls. Treatment of serum-deprived GT1-7 neurons with either 10 n*M* 17β-E_2, which mimics a physiologic dose, or 1 μ*M* tamoxifen, which

approximates a clinically therapeutic dose, did not provide any significant protective effect (Fig. 1).

Lack of a Significant Protective Effect of 17β-E_2 and Tamoxifen in Purified Rat Cortical Neurons

Since GT1-7 neurons are an immortalized neuronal cell line, we next examined the protective ability of 17β-E_2 and tamoxifen in primary purified rat cortical neurons in vitro. Purified cortical neurons were pretreated for 24 h with a physiologic dose of 17β-E_2 (10 n*M*), a supraphysiologic dose of 17β-E_2 (100 n*M*), or a clinically therapeutic dose of tamoxifen (1 μ*M*). Three types of cell death inducers were used, glutamate, chemical hypoxia/ischemia, and camptothecin (an apoptotic-inducing agent). The models utilized recapitulate the molecular mechanisms of cell death following ischemic stroke. As shown in Fig. 2A–C, glutamate, chemical hypoxia/ischemia, and camptothecin induced significant cell death of the purified cortical neurons. Furthermore, similar to the situation observed with GT1-7 neurons, neither 17β-E_2 nor tamoxifen was able to significantly protect the purified cortical neurons from any of the cell death–inducing agents (Fig. 2A–C).

Expression Profile of ERα and ERβ in GT1-7 Neurons and Purified Rat Cortical Neurons

Since we observed no significant protective effect of 17β-E_2 and tamoxifen in either GT1-7 neurons or purified rat embryonic cortical neurons, we wished to determine the presence and pattern of ER subunit expression in the cells. To accomplish this aim, we utilized both reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis. As shown in Fig. 3A, both GT1-7 neurons and purified rat embryonic cortical neurons expressed the mRNA and protein for the classic ER, ERα. By contrast, whereas GT1-7 neurons strongly expressed both ERβ mRNA and protein, rat cortical neuronal cultures did not express ERβ mRNA and had little/no ERβ protein expression (Fig. 3B). As also shown in Fig. 3A,B, hypothalamus, a positive control, expressed both ERs, as expected.

Significant Neuroprotective Effect of 17β-E_2 and Tamoxifen in Mixed Glial/Neuronal Cultures

The lack of a significant protective effect in GT1-7 neurons and purified rat cortical neurons by 17β-E_2 and tamoxifen, while they are protective in vivo, could suggest a role for another cell type in mediating the protective effects. Thus, we next examined whether 17β-E_2 or tamoxifen would be protective in mixed glial/neuronal cortical cultures, which may better mimic the in vivo situation owing to the presence of multiple cell types. Cultures of rat cortical cells containing both neurons and glial cells were pretreated for 24 h with either 10 n*M* 17β-E_2 or 1 μ*M* tamoxifen, and then exposed to camptothecin (10 μ*M*) for a further 24 h. Camptothecin was chosen as the cell death agent because it has been shown to induce cell death in neurons, while glial cells are resistant. As shown in Fig. 4, both 17β-E_2 and tamoxifen

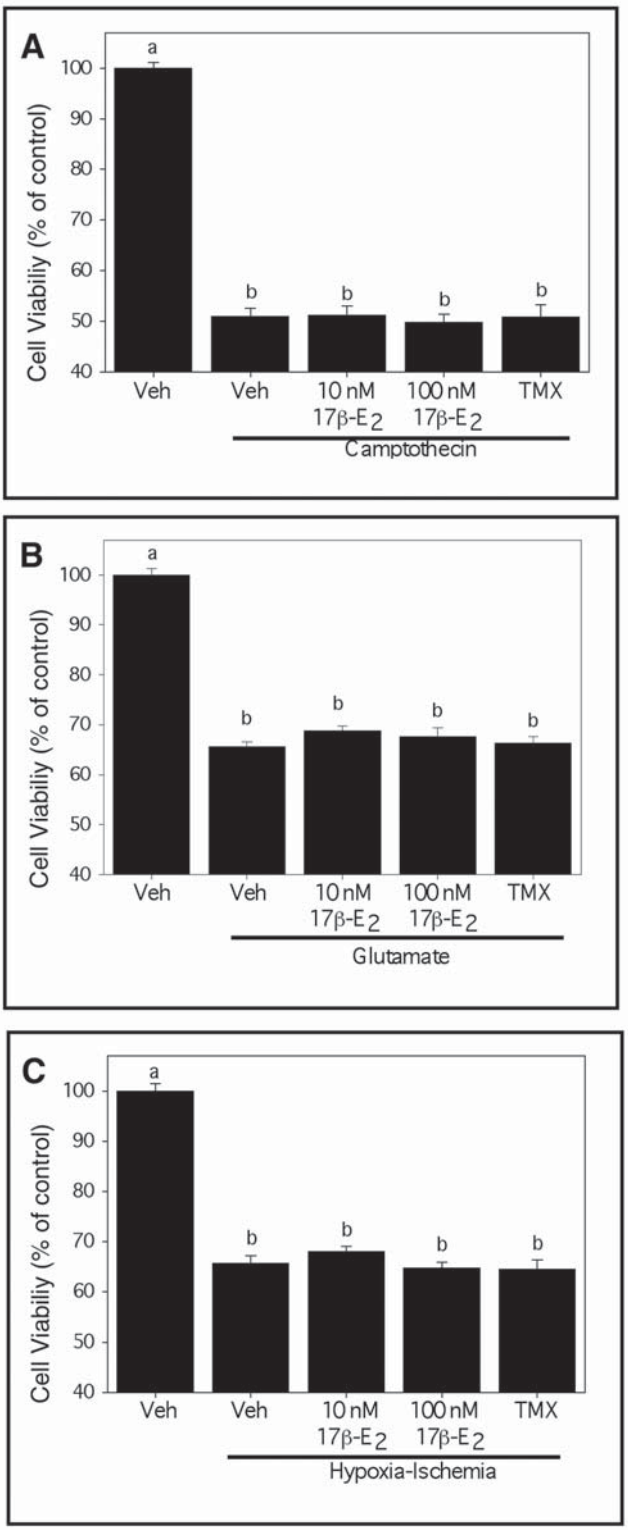

Fig. 2. Effect of 17β-E_2 and tamoxifen on cell death induced by glutamate (**A**), chemical hypoxia (potassium cyanide and 2-deoxyglucose) (**B**), and camptothecin (**C**) in purified primary rat embryonic cortical neurons in culture. Rat cortical neurons were pretreated with vehicle (Veh), 17β-E_2, or tamoxifen (TMX) 24 h prior to application of either glutamate (300 μ*M*), chemical hypoxia (potassium cyanide and 2-deoxyglucose, 1 and 2 m*M*, respectively), or camptothecin (10 μ*M*). Cell viability was assessed 24 h following addition of cell death inducers. Groups with different subscripts are significantly different from each other ($p < 0.05$).

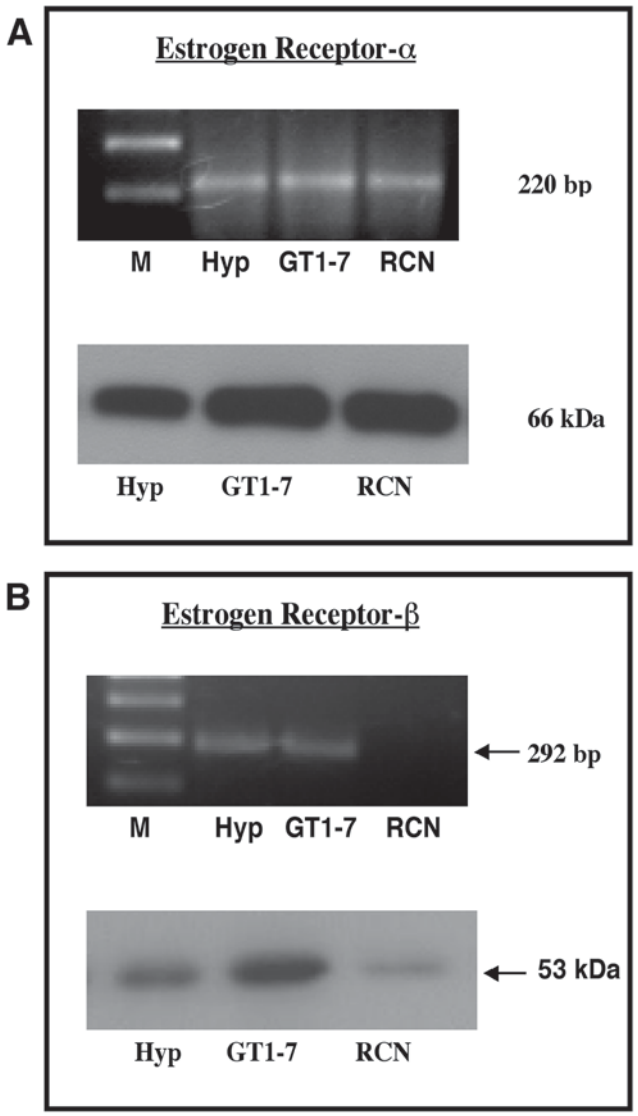

Fig. 3. RT-PCR (**A**) and Western blot (**B**) analysis of ER expression in GT1-7 neurons and in purified primary rat embryonic cortical neurons. Rat hypothalamus was included as positive controls for ERα and ERβ. Hyp, rat hypothalamus; GT1-7, immortalized GnRH (GT1-7) neuronal cell line; RCN, purified primary rat embryonic cortical neurons.

exerted a significant protective effect against camptothecin-induced cell death in the mixed cortical cultures ($p < 0.01$ vs vehicle control). These findings contrast the results obtained in pure neuronal cultures, in which no significant protective effect of 17β-E_2 or tamoxifen was observed against camptothecin- induced cell death (see Fig. 2A).

Astrocyte Factors Protect Purified Cortical Neurons Against a Variety of Cell Death Inducers

The aforementioned findings suggested that glial cells might mediate or facilitate 17β-E_2– and tamoxifen-induced neuroprotection. Thus, we examined whether conditioned media from astrocytes could protect purified rat cortical neurons from camptothecin-, glutamate-, or chemical hypoxia/ischemia-induced cell death. The potential protective effect of transforming growth factor-β1 (TGF-β1), an astrocyte factor whose release has been shown to be enhanced by estrogen, was also examined. As shown in Fig. 5A–C, both astrocyte-conditioned media (ACM) and TGF-β1 were able to exert significant protection of purified cortical neurons from all three cell death–inducing agents. Pretreatment of cortical neurons with ACM protected against neuronal cell death owing to camptothecin exposure (Fig. 5A), glutamate excitotoxicity (Fig. 5B), and chemical hypoxia-ischemia (Fig. 5C). The protection elicited by ACM was approx 15–20%, as compared to vehicle-treated controls. Likewise, the astrocyte-derived factor, TGF-β1, yielded a similar level of protection to ACM from all forms of cell death tested (Fig. 5A–C).

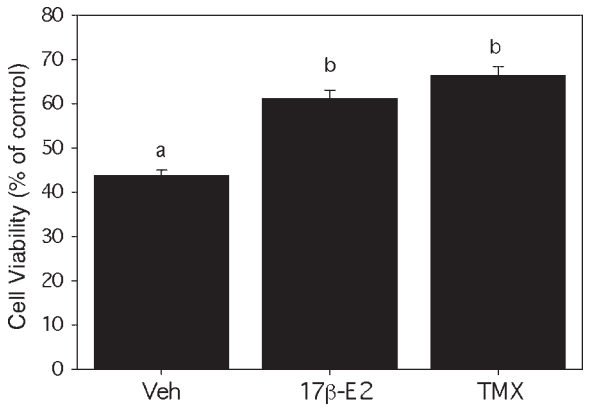

Fig. 4. Effect of 17β-E_2 and tamoxifen on cell death induced by camptothecin in mixed glial-neuronal cultures. Mixed cultures were pretreated for 24 h with 10 n*M* 17β-E_2 or 1 μ*M* tamoxifen (TMX), and then exposed to 10 μ*M* camptothecin for another 24 h prior to determination of cell viability. Groups with different subscripts are significantly different from each other ($p < 0.01$).

Discussion

Recent work by our laboratory has demonstrated that administration of clinically relevant doses of tamoxifen provides significant neuroprotection from MCAO-induced ischemic damage *(12)*. The protection elicited by tamoxifen was independent of vascular blood flow changes, implying that tamoxifen acts directly at the level of the brain to achieve protection from injury. Furthermore, the protective effect required pretreatment, suggesting that genomic mechanisms of action are important for neuroprotection. Although it is clear that tamoxifen protects the brain of both male and female rats from stroke injury in vivo, the molecular and cellular mechanisms of protection are still poorly understood. To gain a better understanding of the mechanisms of protection by 17β-E_2 and tamoxifen, cell and tissue culture models of neurons were employed.

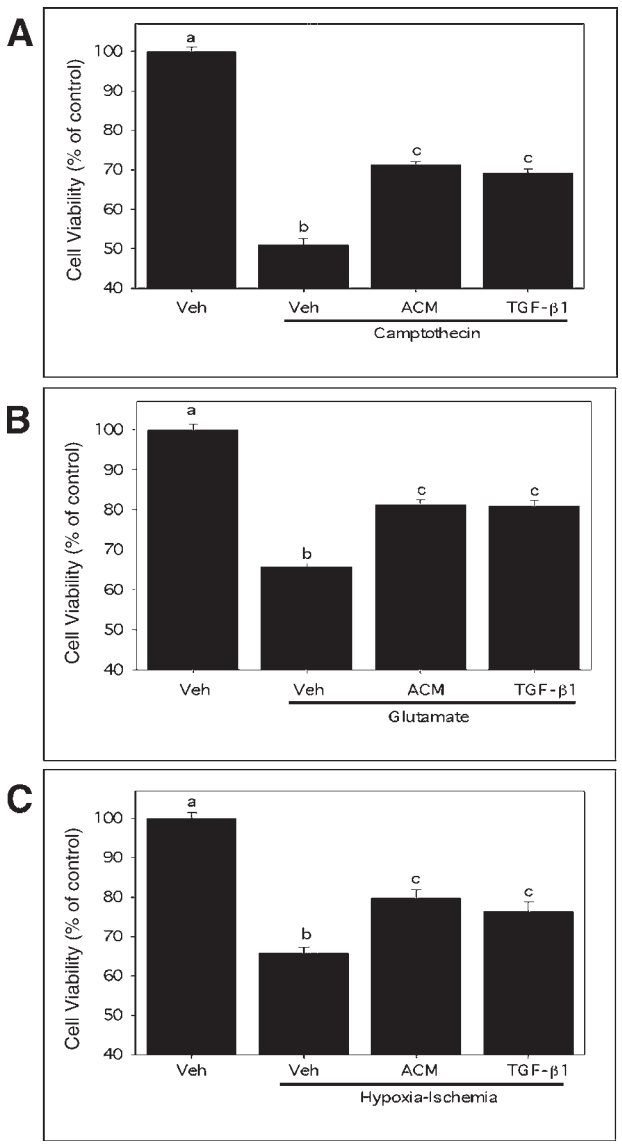

Fig. 5. Effect of ACM and TGF-β1 on cell death induced by glutamate (**A**), chemical hypoxia (potassium cyanide and 2-deoxyglucose) (**B**), and camptothecin (**C**) in purified primary rat embryonic cortical neurons in culture. Rat cortical neurons were pretreated with vehicle (Veh), ACM, or 10 ng/mL of TGF-β1 24 h prior to application of either glutamate (300 μ*M*), chemical hypoxia (potassium cyanide and 2-deoxyglucose, 1 and 2 m*M*, respectively), or camptothecin (10 μ*M*). Cell viability was assessed 24 h following addition of cell death inducers. Groups with different subscripts are significantly different from each other ($p < 0.05$).

In the present study, we failed to identify a neuroprotective effect of either physiologic levels of 17β-E_2 or therapeutic doses of tamoxifen, both of which protect in vivo, against serum deprivation–induced cell death in GT1-7 neurons. The failure of both compounds to protect was not owing to a lack of ERs, because GT1-7 neurons expressed both ERα and ERβ mRNA and protein. To determine whether the failure of estrogens to protect neurons was owing to the immortalized properties of GT1-7 neurons, purified primary cultures of rat cerebrocortical neurons were utilized. Interestingly, 24-h pretreatment with 17β-E_2 (at both physiologic and supraphysiologic doses) or tamoxifen (at a therapeutic dose) did not rescue the primary purified cortical neurons from cell death induced by glutamate, an excitotoxic model of cell death; potassium cyanide and 2-deoxyglucose, a model of ischemia/hypoxia; or camptothecin, a model of apoptotic neuronal cell death. It was important to test all three component/cell death pathways, because the protective effects of 17β-E_2 or tamoxifen may be component or pathway specific. However, regardless of the cell death pathway employed, neither 17β-E_2 nor tamoxifen was protective at the physiologic/therapeutic doses tested in the primary purified cortical neurons. Despite several reports documenting a protective effect of 17β-E_2 *(21–24)*, the present study and several others failed to observe such an effect *(25–29)*. These divergent results are not easily explained but may reflect differences in culture conditions, cell death paradigms, measurements of cellular viability, and treatment protocols used by the different laboratories.

An additional interesting observation of the study was the expression pattern of ERs that we observed in the purified primary embryonic rat cortical neurons. RT-PCR and Western blot analysis of the purified primary embryonic rat cortical neurons revealed high expression of ERα, but low to nondetectable levels of ERβ, which is in agreement with a recent report by Harms et al. *(25)*. This is quite interesting because the expression pattern that we observed in the purified rat embryonic neurons (strong ERα, low/no ERβ) is the opposite of the pattern reported in vivo in neurons in the adult rat cortex *(30)*. This developmental or in vitro–induced difference in ER expression may explain the lack of protection or weak effect of 17β-E_2 and tamoxifen in this in vitro model, as compared with the robust protection observed in vivo.

Our work also raises the question of whether in vitro models that include multiple cell types might not better reproduce the in vivo situation, as well as be more conducive for displaying protective effects. Along these lines, we observed a significant protective effect of 17β-E_2 and tamoxifen in mixed glial/neuronal cortical cultures, which was not observed in purified rat cortical neuronal cultures. Likewise, in contrast to the lack of a protective effect of 17β-E_2 and tamoxifen in purified neuronal cultures, low-dose estrogen treatment has been reported to be protective in organotypic cortical explant cultures, which have multiple cell types and a more preserved cellular/tissue architecture *(31,32)*. Taken together, these findings suggest that glial cells may influence neuronal survival under the control of estrogenic compounds. Of the nonneuronal cell types in the brain, the astrocyte has perhaps the greatest potential for mediating the neuroprotective effects of estrogen. In support of this contention, ablation of astrocytes in vivo has been shown to induce a significant decrease in neuronal survival *(33)*, and astrocytes have recently been shown to express ERs

both in vitro *(34–36)* and in vivo *(37,38)*. Recent work by our laboratory has suggested the presence of an estrogen-astrocyte-TGF-β pathway that we hypothesize may have implications in mediating the neuroprotective effects of estrogen in the brain *(34,39,40)*. In support of a neuroprotective role of astrocytes, the present study shows that pretreatment of purified cortical neurons for 24 h with ACM significantly reduced the cell death induced by all three models of cell death tested. Likewise, pretreatment with exogenous TGF-β1, a factor that we have demonstrated to be produced and released by astrocytes *(34)*, similarly protects the purified cortical neurons from cell death. Intriguingly, both 17β-E_2 and tamoxifen, which failed to protect purified cortical neurons, protected in the glial-neuronal mixed cultures at nearly the same magnitude as the rescue elicited by ACM and TGF-β1. Further work is ongoing in our laboratory to determine whether TGF-β1 from astrocytes, or other astrocyte-active factors, mediates the protective effects of 17β-E_2 and tamoxifen in mixed cortical cultures.

In conclusion, the present study utilized cell and tissue culture models of neuronal function to study estrogen and tamoxifen-induced mechanisms of neuroprotection. However, it is still unclear whether these findings will apply in vivo. Significant protection by estrogen and tamoxifen was observed in mixed glial/neuronal cortical cultures but not purified cortical neurons. Conditioned media from astrocytes and the astrocyte-derived growth factor TGF-β1 protected the purified cortical neurons from various cell death paradigms, demonstrating that astrocytes can protect neurons. Purified rat embryonic cortical neurons in culture were shown to have a significantly different pattern of ER expression than that reported in vivo in adult cortical neurons, which may explain the lack or weak effect of estrogen/tamoxifen in this model compared with the robust protective effect observed in vivo. As a whole, these studies suggest a possible intermediary role for astrocytes in estrogen and tamoxifen protection, which requires further study.

Materials and Methods

Reagents and Chemicals

All cell culture reagents, sera, and media were purchased from Invitrogen (Carlsbad, CA). 17β-E_2 and tamoxifen were purchased from Sigma (St. Louis, MO).

GT1-7 Neuronal Cultures

GT1-7 neurons (a gift from Dr. Pamela Mellon, University of California, San Diego) were cultured in 75-cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. On reaching 80% confluency, cells were recovered using 0.05% trypsin–0.53 m*M* EDTA and replated in 24-well plates at 1×10^5 cells/well. Cells were cultured until reaching 60% confluency, at which time they were used for treatments.

Primary Cortical Neuronal Cultures

Primary cultures of rat cerebrocortical neurons were isolated from rat pups at gestational d 18 (E18) (Sprague-Dawley, Holtzman, Madison, WI), according to the method of Brewer et al. *(41)*. Pregnant female rats were sacrificed on E18 and pups were collected by cesarean section. Embryos were decapitated, the brains were carefully removed, and the cerebral cortex was dissected into ice-cold phosphate-buffered saline (PBS). Cerebral cortices were then mechanically dissociated by trituration using a fire-polished Pasteur pipet. Following centrifugation at 1000*g* for 10 min, cells were resuspended in Neurobasal medium and plated at 6×10^5 cells/well in poly-D-lysine-coated 24-well plates. Neurons were cultured in Neurobasal medium containing 2% B27 supplement, 0.5 m*M* glutamine, 25 μ*M* glutamic acid, and 1% penicillin-streptomycin at 37ºC in a humidified 95%O_2–5%CO_2 atmosphere. Medium was partially replenished twice per week with plating medium lacking glutamic acid. All studies were performed between 8 and 10 d in vitro. This protocol yields cultures that are >95% pure neurons, as assessed by neuron-specific enolase immunostaining *(41)*.

For all models of cell death, culture medium was removed on d 8 in vitro, and cells were pretreated with culture medium containing vehicle, 17β-E_2, or tamoxifen. For glutamate-induced excitotoxic cell death, glutamate exposure (300 μ*M*) was carried out for 15 min at room temperature in buffer containing 2 m*M* KCl, 1 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 1 m*M* NaH_2PO_4, 4.2 m*M* $NaHCO_3$, 12.5 m*M* HEPES, 10 m*M* glucose, and 0.1 *M* NaCl, as previously described *(21)*. Cultures were then gently washed in PBS and returned to culture medium for an additional 24 h, at which time cell death was measured. For the apoptotic cell death model, cultures were exposed to 10 μ*M* camptothecin, a DNA topoisomerase I inhibitor, after pretreatment with vehicle, 17β-E_2, or tamoxifen. Cell death was assessed 24 hours after administration of camptothecin. Camptothecin specifically induces apoptosis in neuronal cultures, since glial cells are less sensitive to this agent *(42)*. For the ischemia/hypoxia model of cell death, following pretreatment, medium was replaced with medium containing 1 m*M* potassium cyanide and 2 m*M* 2-deoxyglucose for 24 h *(43)*.

Mixed Glial/Neuronal Cultures

Cortical cell cultures were prepared as described previously *(44)*, with minor modifications. Briefly, E18 rat cerebral cortices were dissected and plated in 24-well plates at 5×10^5 cells/well using a plating medium of Neurobasal medium containing 10% FBS, 2% B27 supplement, 0.5 m*M* glutamine, and antibiotics. Cultures were kept at 37ºC in a humidified 5% CO_2 incubator. After 2 d in vitro, nonneuronal cell division was halted by exposure to cytosine arabinoside (5 μ*M*) in Neurobasal medium. This procedure results in cultures containing 15–20% glial cells *(44)*. Cultures were used after 8–10 d in vitro.

Primary Rat Astrocyte Cultures

Primary astrocyte cultures were obtained from the cerebral cortex of 2-d-old rats (Sprague-Dawley) by the method of McCarthy and de Vellis *(45)*, as previously published by our laboratory *(34)*. For collection of conditioned medium, astrocytes were seeded in six-well plates at 4×10^5 cells/well and grown to ~80% confluency. One milliliter of serum-free Neurobasal medium was added to each well and incubated for 24 h. Conditioned medium was then removed and stored at −80ºC until use.

RT-PCR and Western Blot Analysis

RT-PCR for ERs was performed as previously described by our laboratory *(34)*. For detection of ERα and ERβ isoforms, the following primers were utilized, as previously described *(34)*: ERα: FP 5'-AGTCCTGGACAAAGATC AACGA-3' and reverse primer of 5'-ATGAAGACGATGA GCATCCAG-3' with a product length of 220 bp; ER-β: Forward primer of 5'-AATGCTCACACGCTTCGAG-3' and reverse primer of 5'-AACTTGGCATTCGGTGGTAC-3' product length of 292 bp. For ERα and ERβ protein measurements, Western blot analysis was performed as described previously by our laboratory *(34)* using an anti-ERα polyclonal antibody (H-184; Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-ERβ polyclonal antibody (Y-19; Santa Cruz Biotechnology).

Cell Viability Measurements

Cell viability was estimated with the MTT assay. Following treatments, 50 µL of 5 mg/mL of MTT in phenol red–free RPMI-1640 medium was added to each culture well and incubated for 4 h at 37ºC. MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT, making it a sensitive assay of cell viability. Following incubation, formazan crystals were solubilized by the addition of 500 m*M* 0.04*M* HCl in isopropanol to each well. Two hundred milliliters of cell suspension/sample was transferred to a 96-well plate, and absorbance was determined using a plate reader (Labsystems Multiskan MCC/340) at 540 nm using a reference wavelength of 690 nm. All readings were standardized to the control treatment group, which represented 100% viability.

Statistical Analyses

The effect of different treatments was analyzed using a one-way analysis of variance followed by Tukey test. For all groups, $n \geq 5$ and experiments were repeated in triplicate to verify results. The results were expressed as means ± SEM. A p value of <0.05 was considered significant.

Acknowledgments

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