Dual Effect of 17β-Estradiol on NMDA-Induced Neuronal Death: Involvement of Metabotropic Glutamate Receptor 1

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Pretreatment with 10 nM 17β-estradiol (17βE2) or 100 μM of the metabotropic glutamate 1 receptor (mGlu1R) agonist, dihydroxyphenylglycine (DHPG), protected neurons against *N*-methyl-D-aspartate (NMDA) toxicity. This effect was sensitive to blockade of both estrogen receptors and mGlu1R by their respective antagonists. In contrast, 17βE2 and/or DHPG, added after a low-concentration NMDA pulse (45 μM), produced an opposite effect, *i.e.* an exacerbation of NMDA toxicity. Again this effect was prevented by both receptor antagonists. In support of an interaction of estrogen receptors and mGlu1R in mediating a neurotoxic response, exacerbation of NMDA toxicity by 17βE2 disappeared when cultures were treated with DHPG prior to NMDA challenge, and conversely, potentiation of NMDA-induced cell death by DHPG was prevented by pretreatment with 17βE2. Addition of calpain III inhibitor (10 μM), 2 h before NMDA, prevented the increased damage induced by the two agonists, an affect that can be secondary to cleavage of mGlu1R by calpain. Accordingly, NMDA stimulation reduced expression of the full-length (140 kDa) mGluR1, an effect partially reversed by calpain inhibitor. Finally, in the presence of NMDA, the ability of 17βE2 to stimulate phosphorylation of AKT and ERK was impaired. Pretreatment with calpain inhibitor prevented the reduction of phosphorylated ERK but had no significant effect on phosphorylated AKT. Accordingly, the inhibition of ERK signaling by U0126 (1 μM) counteracted the effect of calpain inhibition on 17βE2-induced exacerbation of NMDA toxicity. The present data confirm the dual role of estrogens in neurotoxicity/neuroprotection and highlight the role of the timing of exposure to estrogens. (***Endocrinology*** **153: 5940–5948, 2012)**

Estrogens have been shown to modulate fundamental functions in the central nervous system such as motor behavior, mood and mental state, pain sensitivity, and neuroprotection (1). A large body of preclinical data, obtained in different experimental models, both *in vitro* and *in vivo*, support a protective role for estrogens on reducing neuronal death: these include global or focal ischemia (2, 3), excitotoxic insults (4, 5), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration (6), and β-amyloid (Aβ) toxicity (7-11). It has been shown that estrogens prevent neuronal death, in different Aβ toxicity models, by modifying amyloid precursor protein metabolism toward the non amyloidogenic pathway (12), by increasing Aβ catabolism and clearance (13-15), and by counteracting Aβ insult either directly on neurons (reviewed in Ref. 16) or on glial cells (11, 17). Accordingly, in animal models of Alzheimer's disease, estrogen treatment prevents disease progression by reducing plaque formation as well as τ-hyperphosphorylation (18, 19).

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Abbreviations: Aβ, β-Amyloid; 10-DEBC, 10-[4-(N,N-diethylamino)butyl]-2-chlorophenoxazine hydrochloride; DHPG, dihydroxyphenylglycine; DIV, days *in vitro*; 17βE2, 17β-estradiol; ER, estrogen receptor; ERT, estrogen replacement therapy; ICI 182,780, 7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; JNJ, JNJ 16259685; LDH, lactate dehydrogenase; mGlu1R, metabotropic glutamate 1 receptor; NMDA, *N*-methyl-D-aspartate; p, phosphorylated; PI3K, phosphatidylinositol 3-kinase.

These observations led to predict a promising use of estrogens in neurodegenerative diseases. Accordingly, clinical studies demonstrated reduced cognitive impairment in women treated with estrogen replacement therapy (ERT) at the onset of menopause (20-23). However, the Women Health's Initiative, the largest randomized controlled trial on ERT outcomes, revealed that estrogen treatment caused cognitive decline and dementia in women older than 65 yr, at the time of initiation of ERT (24, 25). This controversy helped developing the critical period hypothesis (26), which suggests a positive estrogen activity in reducing cognitive decline associated with normal aging, when treatment starts at the beginning of menopause, or very early in the postmenopausal period. However, estrogen treatment has no effect, or is even harmful, when initiated decades after menopause onset. Identifying the temporal frame for estrogen treatment then became a primary goal for a successful treatment. *In vitro* experiments were performed to pursue this aim by exposing cultured hippocampal neurons to estrogens at different times during Aβ toxic insults (27). 17β-Estradiol (17βE2) was neuroprotective when given before and during the Aβ1-42 neurotoxic insult, whereas it exacerbated Aβ-induced neuronal death when given after the toxic stimulus, suggesting that estrogens may give rise to neuroprotective as well as neurotoxic effects.

The effects of estrogens in the central nervous system are mediated by classical intracellular as well as membrane estrogen receptors (ER). Membrane ERs have been shown to interact with other membrane receptors (28-30), including G protein-coupled receptors. In the past few years, the interaction between ERs and metabotropic glutamate receptors (mGluRs) has been described in different brain areas (31-33), and we have recently shown that, in cultured cortical neurons, 17βE2 reduces Aβ toxicity by transactivating mGluR1 (34). Similarly to ERs, mGluR1 also influences neuronal survival by inducing both neuroprotection and neurodegeneration (35, 36). The dual role of both ER and mGluR1 led us to investigate whether such receptor interaction also mediates a neurotoxic response. We here report that 17βE2, through mGluR1, exacerbates N-methyl-D-aspartate (NMDA)-induced neuronal death, an effect that is prevented by inhibition of calpain activity that occurs after NMDA activation.

Materials and Methods

Reagents

17βE2 (Sigma-Aldrich Co., St. Louis MO) and 7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) (Tocris Cookson Ltd., North Point, UK) were dissolved in ethanol. 3,5-Dihydroxyphenylglycine (DHPG), JNJ 16259685 (JNJ) (both from Tocris), and calpain inhibitor III (Calbiochem, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (Sigma); 2-methyl-6-(phenylethynyl)pyridine, 10-[4-(N,N-diethylamino)butyl]-2-chlorophenoxazine hydrochloride (10-DEBC) (both from Tocris); and *N*-methyl-D-aspartic acid, (NMDA; Sigma-Aldrich) were dissolved in water. All of the stock solutions were diluted in culture media as appropriate, before use.

Cell culture materials and all plastics, unless otherwise specified, were from Invitrogen (Carlsbad, CA) and Nunc (Rochester, NY). All drugs were used at concentrations reported in literature to be effective in the cellular system used.

Mixed cortical cultures

Cortical cultures containing both neurons and astrocytes were prepared from fetal mice at 16-18 d of gestation, as described previously (36). In brief, dissociated cortical cells were plated in 24/48-multiwell vessels or 35-mm dishes, precoated with 0.1 mg/ml poly-D-lysine (Sigma). Cultures were maintained in MEM-Eagles supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM), and glucose (21 mM). After 3-5 d *in vitro* (DIV), cultures were exposed to 10 μM cytosine arabinofuranoside for 1-3 d and then maintained in a medium identical with the plating medium but lacking fetal bovine serum. Subsequent partial medium replacements were carried out twice a week. Neuronal death was examined in cultures at 13-14 DIV.

Assessment of NMDA toxicity in culture

To induce excitotoxic neuronal death, cultures were exposed for 10 min to 45-90 μM NMDA at room temperature, in a solution containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 20 mM HEPES, and 15 mM glucose (pH 7.4). Afterward, cultures were extensively washed and incubated at 37 C for the following 20 h in MEM-Eagles medium supplemented with 6 mg/ml glucose. When present, drugs were either applied during the 2 h or 30 min preceding the NMDA pulse and then reapplied after the pulse or they were applied immediately after the pulse and maintained for the following 20 h.

Neuronal death was examined by Trypan blue staining (Sigma; 0.4% for 5 min) 20 h after the NMDA pulse. Stained neurons were counted from three random fields per well with phase contrast microscopy at a ×100 magnification. At least 80-100 cells/field were counted.

Lactate dehydrogenase (LDH) released into the medium was measured using the cytotoxicity detection kit (Roche, Basel, Switzerland).

Western blot

In selected experiments, cultures were exposed to the NMDA pulse, washed, incubated for 2 h, and then exposed to various treatments for 10 min before being processed for Western blot analysis.

Cultures were harvested in RIPA lysis buffer (Sigma) with the addition of Triton X-100 and a protease- and phosphatase-inhibitor cocktail mix. Proteins were quantitated by the Bradford protein assay Eighty micrograms of protein extract were separated by SDS-PAGE and transferred to nitrocellulose membranes using a Transblot semidry transfer cell (Bio-Rad Laboratories,

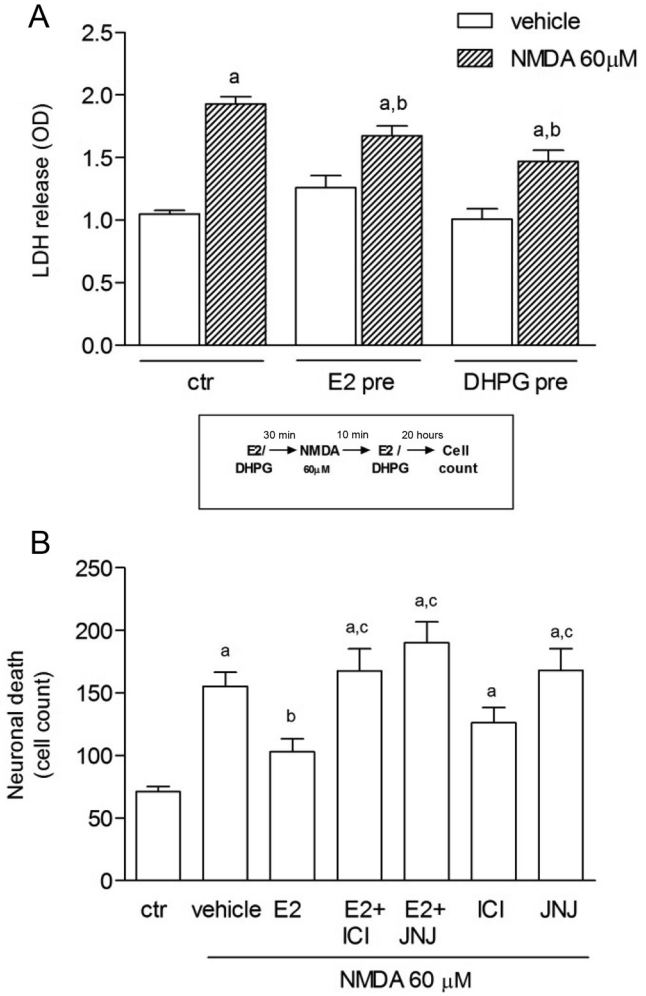

FIG. 1. Effect of pre-treatment with 17βE2 and DHPG on NMDA-induced toxicity. Mixed cortical cultures were treated with 10 nM 17βE2 (E2) or 100 μM DHPG for 30 min before a 10-min pulse with 60 μM NMDA. Neuronal death was assessed 20 h later by measurement of LDH release (A; f 22.44, *df* 17) and counting of cells that included trypan blue (B; f 10.22; *df* 123). When used, the ER antagonist ICI 182,780 (ICI; 1 μM) and the mGluR1 antagonist JNJ (100 nM) were added 30 min before 17βE2. Data are mean ± SEM of at least three independent experiments each run in triplicates. For cell counting, five to eight different fields per well were counted. Panel A: $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* respective control (b); panel B: $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA (b); $P < 0.05$ *vs.* 17βE2 + NMDA (c).

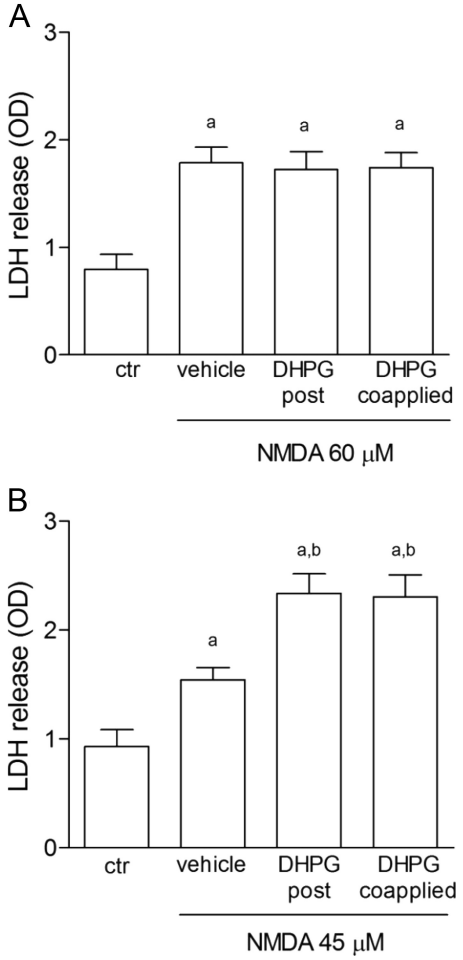

FIG. 2. Effect of DHPG on neuronal death induced by a 10 min-pulse with different NMDA concentrations. Mixed neuronal cultures were exposed to 45 (B) and 60 (A) μM of NMDA for 10 min. DHPG (100 μM) was coapplied during or added after the NMDA pulse and maintained for 20 h. Neuronal death was evaluated 20 h after pulse by assessing the release of LDH. Data are mean ± SEM of several independent determinations from three to four separate experiments. (In A, f 7.9; *df* 43; in B, F 17; *df* 20). $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA alone (b).

Hercules, CA). After blocking in 3% nonfat milk blocking solution, membranes were incubated with primary rabbit anti-mGluR1 (1:700; Millipore, Billerica, MA), rabbit antiphosphorylated (p) Akt (1:800; Cell Signaling Technology, Beverly, MA), rabbit anti-pERK (1:700; Cell Signaling Technology) followed by incubation with antirabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein loading was normalized using rabbit anti-Akt and anti-ERK (1:1000; Cell Signaling Technology) and mouse anti-β-actin (1:1000; Sigma). Specific bands were detected by enhanced chemiluminescence using the Immobilon detection system (Millipore). Full-range rainbow marker (GE Healthcare, Milan, Italy) was used to assess band size. Densitometric analysis of band intensity was carried out with the aid of the Image J software, developed by National Institutes of Health (Bethesda, MD) and in public domain.

Statistical analysis

Data shown are always mean ± SEM of three to six independent experiments each run in triplicates. Data were analyzed by one-way ANOVA followed by a Newman-Keuls test for significance or by two-way ANOVA and Bonferroni test for significance. $P < 0.05$ was taken as the criterion for statistical significance.

Results

Pretreatment with 17βE2 and DHPG protects neurons against NMDA excitotoxicity

Mixed cortical cultures at 14 DIV were exposed to 10 nM 17βE2 for 30 min before a brief pulse with NMDA (60

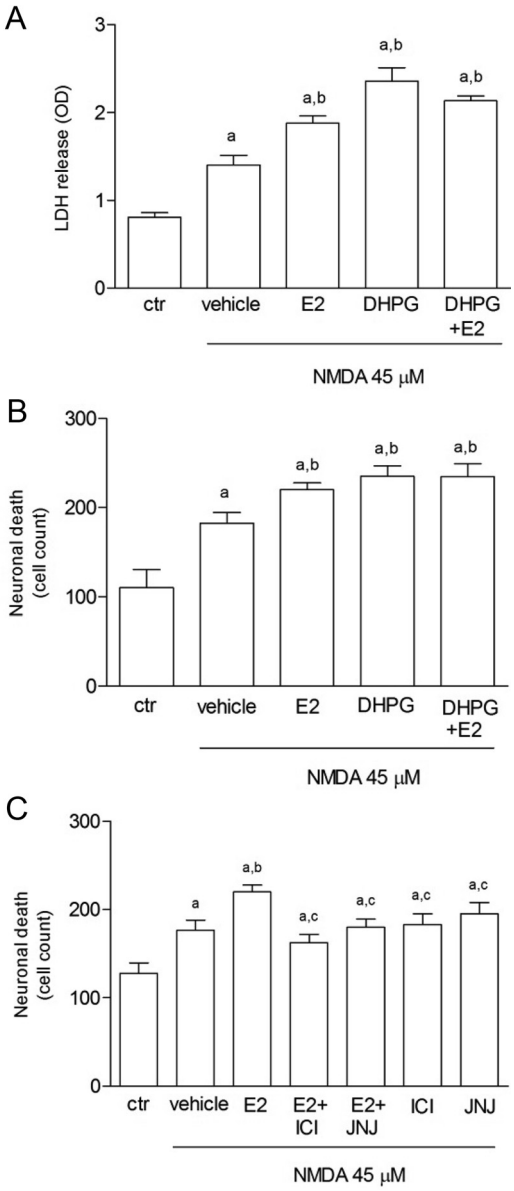

FIG. 3. Effect of 17βE2 and DHPG applied after the toxic insult on NMDA-induced neuronal death. Cortical cultures were exposed to NMDA (45 μM) for 10 min and then treated with 17βE2 (E2; 10 nM), DHPG (100 μM), or both compounds for 20 additional hours. When antagonists were used, either ICI 182,780 (ICI; 1 μM) or JNJ (100 nM), they were added 10 min before agonists. The release of LDH was used as an indicator of neuronal death (A) or trypan blue including cells were counted in five to eight different fields per well (B and C). In A, f 44.2; *df* 20; in B, 14.4; *df* 43; in C, f 6; *df* 62). In A, data are mean ± SEM of three independent determinations. $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA alone (b); $P < 0.05$ *vs.* NMDA + 17βE2 (C). ctr, Control.

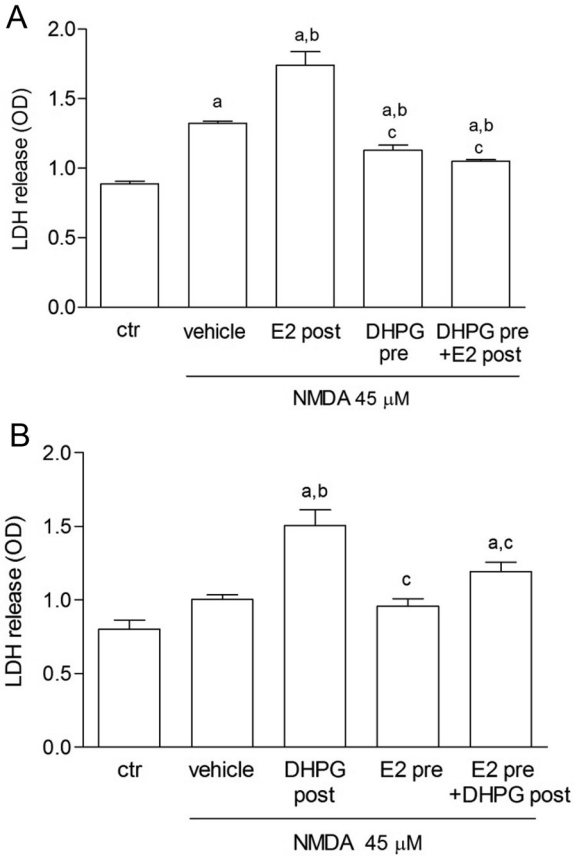

FIG. 4. Different effect of 17βE2 and DHPG added in sequence on NMDA-induced toxicity. Cultures were exposed to DHPG (100 μM; A) or 17βE2 (E2; 10 nM; B) for 30 min prior to a 10-min pulse with NMDA (45 μM) and subsequently treated with 17βE2 (A) or DHPG (B) for an additional 20 h. Data are mean ± SEM of three to five independent experiments. (In A, f 63.5; *df* 11; in B, f 13.8; *df* 18). In panel A, $P <$ 0.05 *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA (b); $P < 0.05$ *vs.* E2 after NMDA (E2 post) (c); in panel B, $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA (b); $P < 0.05$ *vs.* DHPG after NMDA (DHPG post) (c). ctr, Control.

μM for 10 min). Drugs were then removed and neuronal damage was evaluated 20 h later. Under these conditions, NMDA induced a marked increase of neuronal death that was attenuated by pretreatment with 17βE2, as assessed by LDH release (Fig. 1A) and cell counting of trypan blue stained cells (Fig. 1B). Stimulation of mGluR1 by the group 1 mixed agonist DHPG (100 μM), in the presence of the mGlu5 receptor selective antagonist 2-methyl-6-(phenylethynyl)pyridine (1 μM), produced a similar protective effect (Fig. 1A). The protective effect of 17βE2 on NMDA-induced neuronal death was prevented by preincubation with the estrogen receptor antagonist ICI 182,780 (1 μM; added 30 min before 17βE2) and, more surprisingly, blocked also by the mGlu1 receptor antagonist JNJ (100 nM; Fig. 1B). Both antagonists *per se* were devoid of a significant protective activity (Fig. 1B).

17βE2 and DHPG exacerbate NMDA-induced neuronal death when added after the excitotoxic stimulus

The protective effect of DHPG was not detectable any more when the drug was added to neuronal cultures after, or in combination with, the NMDA pulse (60 μM for 10 min; Fig. 2A). A similar effect was observed with a higher NMDA concentrations (90 μM; not shown). However,

100 μM DHPG added after (or at the same time of) a low concentration NMDA pulse (45 μM for 10 min), which *per se* induced only a mild neuronal damage, caused an exacerbation of the excitotoxic effect (Fig. 2B). 17βE2 behaved similarly, inducing a potentiation of neuronal death, when added after the NMDA pulse (45 μM, Fig. 3). The magnitude of the effect of DHPG and 17βE2 was comparable, when assessed as both LDH release (Fig. 3A) and cell counting (Fig. 3B). When the two drugs were added together, no additivity was observed (Fig. 3, A and B). Both ER antagonist ICI 182,780 (1 μM) and mGluR1 antagonist, JNJ (100 nM) prevented exacerbation of neuronal death induced by 17βE2 but were, *per se*, devoid of any effect (Fig. 3C). To substantiate the close interaction between ER and mGluR1, pretreatment with DHPG (100 μM, added 30 min before NMDA pulse) prevented the exacerbation of NMDA toxicity induced by treatment with 17βE2 (10 nM), added throughout the 24 h after the NMDA pulse, *i.e.* during the development of neuronal damage (Fig. 4A). Similarly, increased neuronal death observed when DHPG was added after NMDA pulse was prevented by pretreatment with 10 nM 17βE2 30 min before NMDA exposure (Fig. 4B).

Calpain-mediated cleavage of mGlu1 receptor contributes to exacerbation of NMDA toxicity by 17βE2

mGluR1 has been reported to undergo calpain-mediated changes in its conformation after NMDA activation, a phenomenon that may impact on its downstream signaling (37). On these bases, the ability of 17βE2 and DHPG to increase NMDA toxicity was assessed in the presence of 10 μM calpain III inhibitor, added 120 min before pulsing neurons with NMDA. This calpain III inhibitor concentration was chosen because it did not produce any toxic effect, in contrast to higher concentrations tested (up to 50 μM; not shown). Under these conditions, exacerbation of NMDA toxicity by 17βE2, DHPG, or both was totally prevented (Fig. 5A). In an attempt to correlate calpain action with the function of mGluR1, its expression and signaling were tested. As expected, a 10-min pulse with 45 μM NMDA reduced the expression of the full-length mGlu1 receptor (~140 kDa; Fig. 5B) 2 h after the pulse. This reduction was partially prevented by pretreatment with 10 μM calpain III inhibitor, added 2 h before the NMDA pulse (Fig. 5B). The 17β-E2 treatment did not modify the cleavage of mGluR1 observed after NMDA stimulation or its partial rescue by calpain III in-

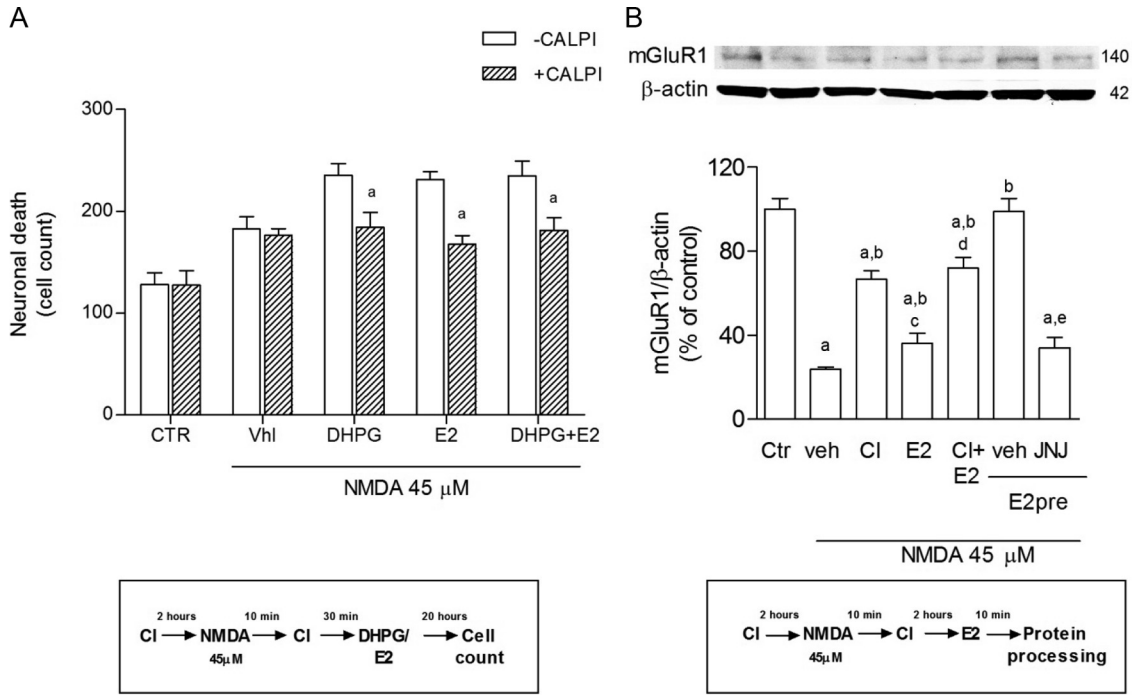

FIG. 5. Effect of calpain inhibition on 17ßE2 and DHPG effect on NMDA toxicity. Cultures were pretreated with calpain inhibitor III (CI; 10 μM), 2 h before NMDA pulse and then treated with 17βE2 (10 nM), DHPG 100 μM, or both for an additional 20 h before evaluating neuronal death by cell counting (A). A two-way ANOVA and Bonferroni *post hoc* test reveal a significant interaction, $P < 0.05$ (a), of calpain inhibitor (CI) treatment on 17βE2, DHPG, and 17βE2 + DHPG effect on NMDA-induced neuronal death. In B, the expression of mGluR1 after treatment with NMDA, NMDA + CI, and NMDA + CI + 17βE2. A representative blot is shown. $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA (b); $P < 0.05$ *vs.* CI + NMDA (c); $P < 0.05$ *vs.* 17βE2 + NMDA (d); $P < 0.05$ *vs.* 17 βE2 pretreatment + NMDA (e). A schematic diagram of the experimental paradigm is shown below each panel.

hibitor (Fig. 5B). In contrast, pretreatment with 10 nM 17β-E2 prevented NMDA-induced mGlu1 receptor cleavage, an effect antagonized by JNJ (100 nM) (Fig. 5B).

Signaling pathways initiated by mGluR1 activation were then tested. For this purpose, neurons were exposed to NMDA pulse, and after recovering for 2 h, they were challenged with 10 nM 17βE2 for 10 min. When the calpain III inhibitor was used, it was added 2 h before the NMDA pulse. 17βE2 induced enhanced phosphorylation of both AKT (Fig. 6A) and ERK (Fig. 6B), but the effect

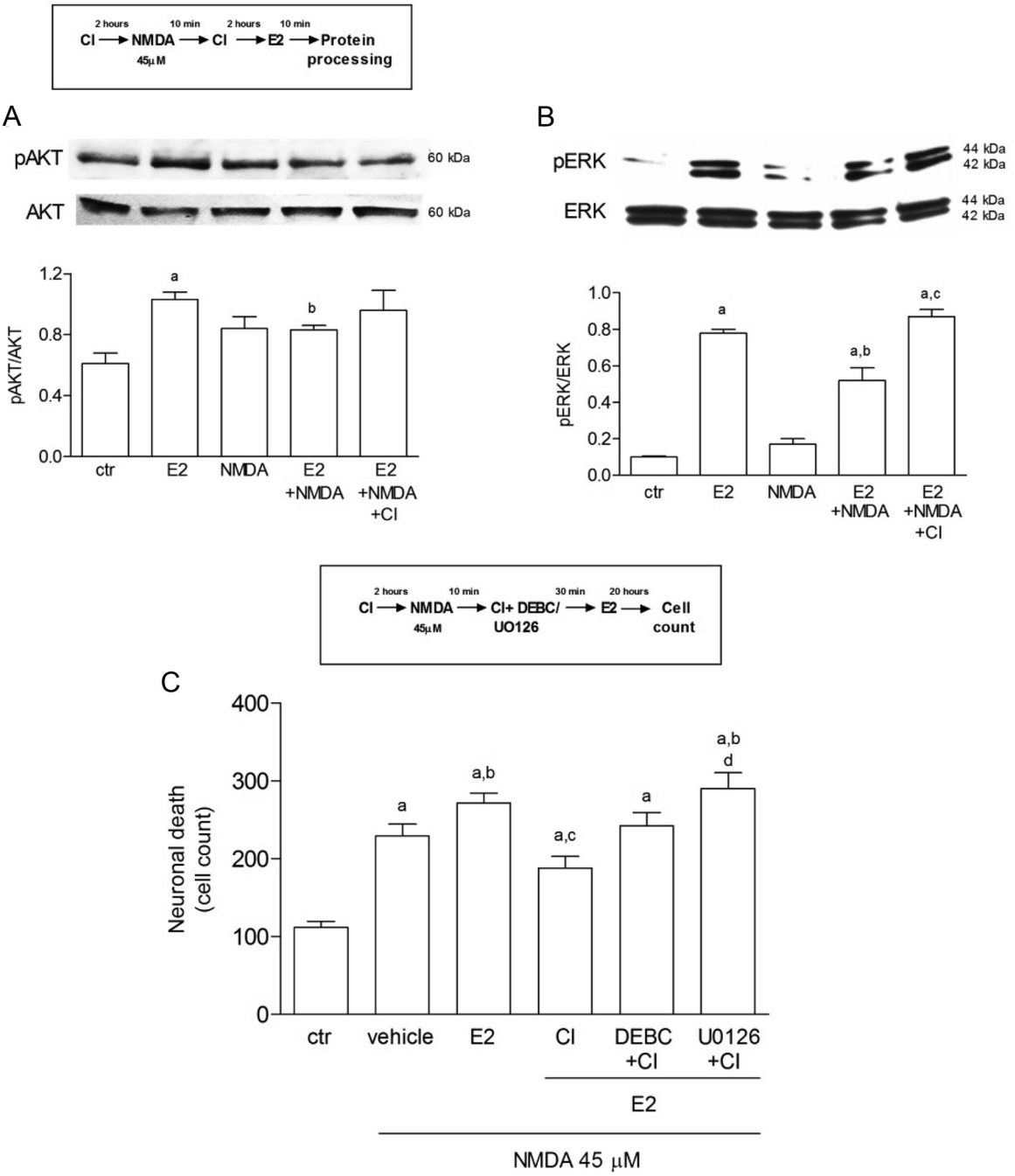

FIG. 6. Effect of calpain inhibition on signaling pathways activated by 17βE2. Cultures were exposed to calpain inhibitor III (CI; 10 μM) 2 h before NMDA pulse (45 μM for 10 min) and let develop the excitotoxic damage for 2 h before a 10-min exposure to 17βE2 (E2, 10 nM). This experimental paradigm is summarized in the diagram above panel A. Proteins were then extracted and processed for pAKT (A) or pERK (B) expression. In panel C, a 10-min exposure to 17βE2 was preceded by a 10-min treatment with the AKT inhibitor 10-DEBC (DEBC, 10 μM) or the MAPK inhibitor U0126 (1 μM), (experimental paradigm reported above panel C). Data are mean ± SEM of four to six independent determinations (a and b). In panel A, $P < 0.05$ *vs.* control (ctr; a) and $P < 0.05$ *vs.* 17βE2 (b). In panel B, $P < 0.05$ *vs.* control (ctr; a), $P < 0.05$ *vs.* 17βE2 (b) and $P < 0.05$ *vs.* NMDA + 17βE2 (c). In panel C, $P < 0.05$ *vs.* untreated control (a); $P < 0.05$ *vs.* NMDA (b); $P < 0.05$ *vs.* NMDA + 17βE2 (c); $P < 0.05$ *vs.* NMDA + 17βE2 + CI (d) (f 25.8; *df* 71).

was reduced after NMDA pulse. Pretreatment with the calpain III inhibitor rescued the ability of 17βE2 to phosphorylate ERK (Fig. 6B) but did not significantly modify the reduced AKT phosphorylation (Fig. 6A). Accordingly, the prevention of 17β-E2 effect by the calpain III inhibitor was precluded by pretreatment (for 10 min) with the MAPK inhibitor U0126 (1 μM), but was not significantly modified by preexposure to the AKT phosphorylation inhibitor 10 DEBC (10 μM; Fig. 6C).

Discussion

The present data demonstrate that 17βE2 acts through mGluR1 to exacerbate NMDA-induced neurotoxicity. Such an effect is present only when 17β-E2 treatment follows the excitotoxic insult and likely requires calpain-induced cleavage of mGluR1. These data are consistent with previous observations ascribing a key role to the interaction between ERs and mGluR in estrogen signaling and, more specifically, with our recent report showing that 17βE2, through ERα, transactivates mGluR1 to protect cortical neurons against Aβ-induced neuronal death (34). However, in striking contrast with our recent data showing an involvement of both receptors in neuroprotection, we here report that ERα and mGluR1 cooperate also to exacerbate neuronal damage.

Indeed, such a dual role for estrogens has already been reported, with prevention or potentiation of neuronal damage when estrogens are given before or after the neurotoxic challenge, respectively (27). Interestingly, the contradictory effect observed following activation of ER somehow recapitulates what observed with mGluR1, whose stimulation results in either enhancement or attenuation of excitotoxic neuronal death, depending on experimental conditions (36).

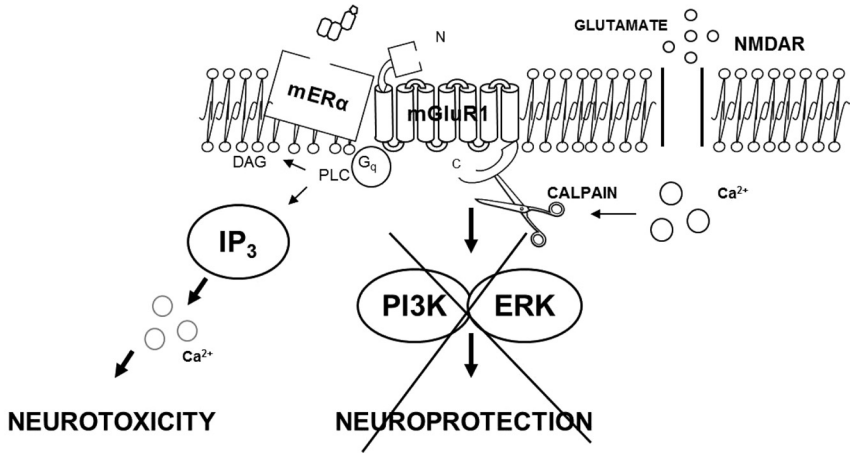

FIG. 7. Schematic model of the suggested interaction between membrane ERα and mGluR1 after an excitotoxic insult that, as previously reported (37), causes calpain-mediated cleavage of mGluR1.

In the mixed cell culture we have used, stimulation of ERα and mGluR1, before addition of a moderately toxic concentration of NMDA, caused reduction of neuronal death. This effect was comparable, and, more intriguingly, it was not modified when the two agonists, 17βE2 and DHPG, were added simultaneously. In addition, the neuroprotective effect of 17βE2 was prevented by the blockade of mGluR1 with JNJ. These results are consistent with our recent report demonstrating that mGluR1 is involved in the neuroprotective effect of 17βE2 against Aβ toxicity (34) and find support in the interaction occurring between the two receptor types in neurons, as shown by coimmunoprecipitation studies (34). ERα seems to be the receptor subtype involved in such mechanism as suggested by others' (38) and our own (34) observations. A similar receptor cooperation occurs also when ERs and mGluR1 are stimulated following NMDA toxic insult, a condition that results in exacerbation of neuronal damage. The potentiating effect of 17βE2 in this experimental paradigm is in fact similar to that induced by DHPG and is prevented by the blockade of mGluR1 by its selective antagonist. Furthermore, stimulation of each receptor before the NMDA pulse impedes exacerbation of neuronal damage induced by treatment with the other agonist, after NMDA. This observation strongly supports the close interaction between ER and mGluR1, and it further sustains the suggested involvement of mGluR1 in the action of 17βE2. Based on the present data, it seems reasonable to conclude that 17βE2, upon binding to membrane ER engages mGluR1 and exploits its signaling pathway, with a variable response, depending on preexisting neuronal status.

Of note, it has been suggested that following an excitotoxic stimulus, mGluR1 undergoes calpain-mediated truncation, causing the loss of the C-terminal tail of the receptor that becomes no longer able to signal through the neuroprotective phosphatidylinositol 3-kinase (PI3K)/AKT pathway (37). Consistent with this finding, we here report that exposure to NMDA reduces expression of full-length mGluR1 that is partially restored by inhibition of calpain activity. mGluR1 internalization in these conditions is unlikely, due to the short-term exposure to NMDA and the absence of a receptor ligand. It appears particularly interesting that cleavage of mGluR1 by NMDA is not present any more when neurons are preexposed to 17βE2. This may suggest that involvement of mGluR1 in 17βE2 effect causes conformational changes that do not allow cleavage of the recep-

tor. Accordingly, this effect of 17βE2 is reduced by the pharmacological blockade of mGluR1.

To strengthen the role of mGluR1 receptor cleavage, treatment with calpain III inhibitor reduces the exacerbation of NMDA toxicity induced by either 17βE2 or DHPG. To analyze downstream signaling pathways involved, we first focused our attention on the PI3K/AKT pathway. Stimulation of AKT phosphorylation by 17βE2 is reduced after NMDA exposure, but the recovery of 17βE2 response in the presence of calpain inhibitor is not clear and consistent over experiments. This may be related to the presence of contaminating glia, also sensitive to 17βE2 stimulation. In contrast, the ability of 17βE2 to stimulate ERK phosphorylation, which is impaired after NMDA treatment, is rescued by addition of calpain inhibitor. Coupling of mGluR1 stimulation to ERK phosphorylation occurs independently of PI3K/AKT activation (39) but equally involves the adaptor protein homer (40, 41). Thus, it is plausible that cleavage of mGluR1 by calpain modifies the receptor function so that it cannot signal anymore through PI3K/AKT but also through the ERK pathway. In this respect, the correlation between the mGluR1 receptor and ERK signaling in neuroprotection has been ascertained (42).

In conclusion, 17βE2 exhibits the dual ability to protect and to exacerbate NMDA-induced toxicity depending on the time of treatment. Both these effects rely on the ability of ER to couple to mGluR1 and use its signaling pathway. Under basal conditions, pretreatment with 17βE2 results in neuroprotection, whereas after an excitotoxic insult, exposure to 17βE2 causes potentiation of neurotoxicity. The latter effect, observed also with the mGluR1 agonist DHPG, may be secondary to calpain-mediated cleavage of mGluR1 that cannot couple anymore to PI3K/AKT and ERK pathways. Thus, receptor signaling will work only through the phospholipase C-induced Ca^{2+} increase, generating primarily a neurotoxic effect. A schematic diagram of the proposed model is reported in Fig. 7. The results reported here provide an experimental correlation to the unresolved issue of the use of estrogens as neuroprotective agents and further underline the importance of neuronal conditions at the time of estrogen exposure and consequently the choice of timing in the correct use of the hormone.

Acknowledgments

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