Estrogen Receptors and Type 1 Metabotropic Glutamate Receptors Are Interdependent in Protecting Cortical Neurons against β-Amyloid Toxicity

Simona Federica Spampinato, Gemma Molinaro, Sara Merlo, Luisa Iacovelli, Filippo Caracci, Giuseppe Battaglia, Ferdinando Nicoletti, Valeria Bruno, and Maria Angela Sortino

Departments of Clinical and Molecular Biomedicine (S.F.S., S.M., M.A.S.) and Drug Sciences (F.C.), University of Catania, Catania, Italy; Istituto Neurologico Mediterraneo Neuromed Pozzilli, Pozzilli, Italy (G.M., G.B., F.N., V.B.); and Department of Physiology and Pharmacology, University of Rome Sapienza, Rome, Italy (L.I., F.N.)

Received June 10, 2011; accepted October 7, 2011

ABSTRACT

We examined the interaction between estrogen receptors (ERs) and type 1 metabotropic glutamate receptors (mGlu1 receptors) in mechanisms of neurodegeneration/neuroprotection using mixed cultures of cortical cells challenged with β-amyloid peptide. Both receptors were present in neurons, whereas only ERα but not mGlu1 receptors were found in astrocytes. Activation of 17β-estradiol (17βE2) protected cultured neurons against amyloid toxicity, and its action was mimicked by the selective ERα agonist, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) as well as by a cell-impermeable bovine serum albumin conjugate of 17βE2. The selective ERβ agonist, diarylpropionitrile (DPN), was only slightly neuroprotective. The mGlu1/5 receptor agonist, 3,5-dihydroxyphenylglycine (DHPG), was also neuroprotective against amyloid toxicity, and its action was abolished by the mGlu1 receptor antagonist, (3,4-dihydro-2H-pyran-2,3-biquinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone (JNJ 16259685). Neuroprotection by 17βE2 or PPT (but not DPN) and DHPG was less than additive, suggesting that ERα and mGlu1 receptors activate the same pathway of cell survival. More important, neuroprotection by 17βE2 was abolished not only by the ER antagonist fulvestrant (ICI 182,780) but also by JNJ 16259685, and neuroprotection by DHPG was abolished by ICI 182,780. ERα and mGlu1 receptors were also interdependent in activating the phosphatidylinositol-3-kinase pathway, and pharmacological blockade of this pathway abolished neuroprotection by 17βE2, DHPG, or their combination. These data provide the first evidence that ERα and mGlu1 receptors critically interact in promoting neuroprotection, information that should be taken into account when the impact of estrogen on neurodegeneration associated with central nervous system disorders is examined.

Introduction

Estrogens are neuroprotective in a variety of cellular and animal models, including cell cultures challenged with excitotoxins or other insults (Goodman et al., 1996; Singer et al., 1999; Harms et al., 2001; Cimarosti et al., 2005), models of focal or global brain ischemia (Simpkins et al., 1997; Dubal et al., 1998; Lebesgue et al., 2009), mice treated with the parkinsonian toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Bourque et al., 2009), and transgenic mice carrying mutations associated with Alzheimer’s disease (Carroll et al., 2007;Amtul et al., 2010). Estrogens are also effective in reducing β-amyloid toxicity in cultured neurons (Goodman et al., 1996; Chae et al., 2001; Marin et al., 2003; Sortino et al., 2004; Cordey and Pike, 2005), an established cellular model of Alzheimer’s disease. The classic estrogen receptors, named ERα and ERβ, are nuclear transcription factors that activate or repress gene expression (Nilsson et al., 2001). However, a large body of evidence suggests that neuroprotection is me-
iated by membrane ERs, which are able to induce rapid intracellular effects in response to estrogens (Micevych and Dominguez, 2009). In addition, a G protein-coupled receptor, GPR30, has been identified as an additional candidate membrane ER (Revankar et al., 2005; Thomas et al., 2005) and reported to also mediate estrogen neuroprotective effects against excitotoxicity (Gingerich et al., 2010). Membrane ERs trigger a variety of putative neuroprotective pathways, which include the mitogen-activated protein kinase (MAPK) pathway (Singer et al., 1999; Mize et al., 2003) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Honda et al., 2000; Harms et al., 2001; Cimarosti et al., 2005). The mechanism whereby membrane ERs activate the neuroprotective cascade is largely unknown.

It has long been known that membrane ERs can transactivate different classes of tyrosine kinase receptors, including epidermal growth factor receptors (Song et al., 2010) and type I insulin-like growth factor receptors (Marin et al., 2009; Varea et al., 2010). In addition, this mechanism of transactivation has been extended to metabotropic glutamate (mGlu) receptors, which are G protein-coupled receptors. Eight subtypes of mGlu receptors (mGlu1–mGlu8) have been described and divided into three groups on the basis of their amino acid sequence, pharmacological profile, and transduction pathways. Group I mGlu receptors (mGlu1 and mGlu5 receptors) are coupled to Gα subunits, and their activation leads to phosphoinositide (PI) hydrolysis with ensuing formation of inositol-1,4,5-trisphosphate and diacylglycerol. mGlu1 and mGlu5 receptors can also activate the MAPK and PI3K/Akt pathways (Chong et al., 2006; Ferraguti et al., 2008). Group II (mGlu2 and mGlu3) and Group III (mGlu4, mGlu6, mGlu7, and mGlu8) receptor subtypes are all coupled to Gαi proteins (for reviews, see Niswender and Conn, 2010; Nicolletti et al., 2011). A series of elegant studies have shown that membrane ERα receptors transactivate mGlu1 receptors in the hypothalamus (Dewing et al., 2007; Micevych and Mermelstein, 2008; Mermelstein, 2009; Dominguez and Micevych, 2010). For example, transactivation of mGlu1 receptors by ERα in hypothalamic astrocytes leads to the synthesis of neuroprogenestone, which is necessary for the estradiol-induced ovulatory surge of luteinizing hormone (Micevych and Sinchak, 2008b; Kuo et al., 2009). In hypothalamic neurons, stimulation of ERα by estradiol leads to internalization of both ERα and mGlu1 receptors, suggesting that the two receptors interact also in neurons (Dominguez and Micevych, 2010). In contrast, GPR30 does not seem to couple with mGlu1 receptor and to involve this receptor in modifying rapid intracellular Ca2+ signaling in astrocytes (Kuo et al., 2010).

mGlu1 receptors are linked to mechanisms of neurodegeneration/neuroprotection and can either amplify or attenuate neuronal death, depending on the cellular context and the experimental paradigm of neurodegeneration (Allen et al., 1999; Bruno et al., 1999; Nicolletti et al., 1999; Battaglia et al., 2001; Bruno et al., 2001a; Pellegrini-Giampietro, 2003; Pshenichkin et al., 2008; Scartabelli et al., 2008; Zhou et al., 2009; Emery et al., 2010).

We now report that activation of either ERα or mGlu1 receptors protects cortical neurons against β-amyloid toxicity and that the two receptors are interdependent in supporting neuronal survival. This is the first evidence that ERα and mGlu1 receptors interact in cortical neurons.

### Materials and Methods

#### Drugs and Reagents

17β-Estradiol (17βE2) (Sigma-Aldrich, St. Louis, MO), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), diarylpropionitrile (DPI), and fulvestrant (ICI 182,780) (all from Tocris Cookson Ltd., North Point, UK) were dissolved in ethanol. 3,5-Dihydroxyphenylglycine (DHPG) and (3,4-dihydro-2H-pyran)[2,3-b]quinolin-7-yl)(cis-4-methoxyacycloxyl)methanone (UNJ 162596865), both purchased from Tocris Cookson Ltd., were dissolved in dimethyl sulfoxide (Sigma-Aldrich), 10-[4-(N,N-diethylamino)butyl]-2-chlorophenoxazine hydrochloride (10-DEBC) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) (both from Tocris Cookson Ltd.) were dissolved in water, and BSA-conjugated 17βE2 (Sigma-Aldrich) was dissolved in 50% ethanol. β-Amyloid peptides Aβ1–40 and Aβ25–35 were obtained from Bachem (Bubendorf, Switzerland). Aβ1–42 was dissolved in dimethyl sulfoxide at an initial concentration of 5 mM, whereas Aβ25–35 was solubilized in water at a final concentration of 2.5 mM. All stock solutions were diluted in culture media as appropriate before use. [3H]Insitol (18 Ci/mmol) was purchased from GE Healthcare (Milan, Italy). Cell culture materials and all plastics, unless otherwise specified, were from Invitrogen (Carlsbad, CA) and Nalge Nunc International (Rochester, NY). All drugs were used at concentrations reported in the literature to be effective in the cellular system used. In the case of 17βE2 and DHPG, concentration-response studies were carried out in a preliminary phase to allow choice of the concentration to be used.

#### Primary Cell Cultures

All animal experimental procedures were performed in accordance with the directives of the Italian and European Union regulations for the care and use of experimental animals (DL116/ 92) and were approved by the Italian Ministry of Health.

Cortical glial cultures were prepared from the cortex of 1- to 3-day-old Sprague-Dawley rats (Harlan, Udine, Italy). After isolation of cortices and removal of meninges, cells were dispersed by mechanical and enzymatic dissociation using a solution of trypsin in Hank’s balanced salt solution, pH 7.4. Cells were plated onto 75-mm² flasks and maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml), at 5% CO2 and 37°C for 14 DIV.

Confluent cultures were shaken for 7 h at 37°C to remove microglia and oligodendrocytes and obtain a >90% pure astrocytic culture as assessed by GFAP staining. Astrocytes were replated at a density of approximately 1 to 2 × 105 cells/cm2 and used when appropriate confluence was reached.

Cultures of pure cortical neurons were obtained from rats at embryonic day 15 (Harlan), prepared according to a procedure described previously (Sortino et al., 2004). In brief, cortices were dissected in Ca2+/Mg2+-free buffer, pH 7.4, mechanically dissociated, and grown on multiwell vessels or 35-mm dishes precoated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich). Cultures were maintained in DMEM-F12 supplemented with the following components: penicillin (100 U/ml), streptomycin (100 µg/ml), at 5% CO2, and 37°C for 14 DIV. Mixed cortical cultures, containing both astrocytes and neurons, were obtained from rats at embryonic day 17 and grown onto 0.1 mg/ml poly-D-lysine-coated multiwell vessels. Cultures were maintained in minimal essential medium supplemented with penicillin (50 U/ml), streptomycin, (50 µg/ml), BSA (10 mg/ml), glucose (6 mg/ml), insulin (10 ng/ml), apotransferrin (10 ng/ml), putrescine (100 µM), glutamine (2 mM), selenium (30 nM), and progesterone (20 nM) (all from Sigma-Aldrich). Arabinoside cytoside (50 µM) was added 18 h after plating to reduce non-neuronal element proliferation and maintained for 72 h. Subsequent partial medium replacements were performed every 2 days. After 7 DIV, cultures were treated for the experiments. These conditions yield a pure neuronal culture as assessed by flow cytometry (Copani et al., 1999).

Mixed cortical cultures, containing both astrocytes and neurons, were obtained from rats at embryonic day 17 and grown onto 0.1 mg/ml poly-D-lysine-coated multiwell vessels. Cultures were maintained in minimal essential medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml), glucose (6 mg/ml), 10% FCS, 10% horse serum, and glutamine (2 mM) (all from Sigma-Aldrich). At 5 DIV FCS was removed from the medium, and cells were supplemented with 5 µM arabinoside cytoside for 72 h. Subsequent partial medium replacements were performed every 2 days. The cultures...
were used for experiments at 14 DIV. Mature cultures contained approximately 40% neurons.

Assessment of Neuronal Death in Mixed Cortical Cultures. Aβ1-42 and Aβ25-35 peptides were applied to serum-deprived mature mixed cortical cultures at 14 DIV. After 24 h, neuronal toxicity was examined by light microscopy and quantified after staining with trypan blue (0.4% for 5 min). Stained neurons were counted from three random fields/well. A variable number between 80 and 300 dead neurons per field were counted. All experiments were performed in the presence of the glutamate receptor antagonists dizocilpine maleate (MR 901; 10 μM) and 2,3-dihydroxy-6,7-dinitroquinoxaline (30 μM) to avoid endogenous glutamate toxicity.

Immunoblot Analysis. Astrocytes and neurons were harvested in radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) with the addition of Triton X-100 and a protease and phosphatase inhibitor cocktail mix (both from Sigma-Aldrich). Transfected HEK293 cells were rapidly rinsed in ice-cold PBS and solubilized in Triton X-100 lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β-glycerophosphate). Proteins were quantitated by the Bradford protein assay (Bradford, 1976). Eighty micrograms of protein extract were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using a Trans-Blot semidy transfer cell. After blocking in 1% nonfat dry milk, membranes were incubated with primary rabbit antibody anti-ERα (1:500; Millipore Corporation, Billerica, MA), rabbit anti-mGluR1 (1:750; Millipore Corporation), and rabbit anti-pAkt (1:750; Cell Signaling Technology, Danvers, MA), followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein loading was determined using anti-α-Actin (1:1000; Cell Signaling Technology). In selected experiments, the same membranes were then reblotted with anti-β-actin (Sigma-Aldrich) (not shown). Specific bands were detected by enhanced chemiluminescence using the Immobilon detection system (Millipore Corporation). Full-range rainbow markers (GE Healthcare) were used to assess the size of the band. Densitometric analysis of band intensity was performed with the aid of ImageJ software (http://rsbweb.nih.gov/ij/).

Coimmunoprecipitation. Neurons were harvested in radioimmunoprecipitation assay buffer, and the protein concentration was determined by the Bradford method (Bradford, 1976); for coimmunoprecipitation, 500 μg of proteins, in a final volume of 500 μl, were incubated for 1 h at 4°C in a rotating stirrer with 25 μl of rabbit serum to reduce nonspecific binding. Then 20 μl of protein G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) were added for 30 min at 4°C to remove endogenous antibodies. Samples were centrifuged (850 rpm for 5 min), and supernatants were retained. Rabbit anti-ERα (1:100) or rabbit anti-mGluR1 (1:100) was added to supernatants, and the mixture was placed in a rotating stirrer at 4°C for 7 h. The antibody-protein complex was adsorbed with 20 μl of protein G PLUS-Agarose in a rotating stirrer at 4°C for 10 h and then washed 5 times with a solution containing PBS and 1% Tween 20 (Sigma-Aldrich). Samples were run using SDS-polyacrylamide gel electrophoresis, with 4 to 15% gradient gels (Bio-Rad Laboratories, Milan, Italy) and transferred to nitrocellulose membranes. After blocking in PBS solution containing 2% nonfat milk and 0.1% Tween 20, membranes were incubated with primary rabbit anti-mGluR1 antibody (1:750) or rabbit anti-ERα (1:100), followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Detection of specific bands was performed with the Immobilon detection system.

Immunostaining. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and saturated with 3% BSA. Cells were then incubated with the following primary antibodies: rabbit anti-mGluR1 (1:75) and mouse anti-ERα (1:25; Santa Cruz Biotechnology, Inc.) overnight at 4°C; mouse anti-GFAP (1:300; Cell Signaling Technology) and mouse anti-MAP2 (1:120; Millipore Corporation) for 2 h at room temperature. For fluorescent immunodetection, the following fluorochrome-conjugated antibodies were used: Alexa Fluor 488 anti-mouse (1:300; Invitrogen, Carlsbad, CA) and anti-rabbit Texas Red (1:75; Santa Cruz Biotechnology, Inc.).

Studies in Heterologous Expression Systems. HEK293 cells were cultured in DMEM supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were transfected in 10-mm dishes using 10 μl of Lipofectamine 2000 in OptiMEM medium and 18 μl of total cDNA as follows: 7.5 μg of mGlu1 receptor cDNA, 7.5 μg of ERα cDNA, and 3 μg of excitatory amino acid carrier 1 (EAAC1) cDNA. Transfections were performed for 4 h, and then cells were plated in culture medium in six-well plates, previously coated with 0.01% poly-L-lysine. With use of this procedure, approximately 80 to 85% of HEK293 cells are immunopositive to cotransfected green fluorescent protein. Experiments were performed 72 h after transfection and serum starvation of 16 to 18 h.

Measurement of Polyphosphoinositide Hydrolysis in Cultured Neurons. Cortical neuronal cultures were incubated overnight with [myo-3H]inositol (1 μCi/dish), washed in Krebs-Henseleit buffer containing 10 mM LiCl, and incubated for 30 min at 37°C under constant oxygenation. DHPG and 17βE2 were added and maintained for 30 min. Incubation was stopped by the addition of methanol-chloroform-water (1:1:1). After further addition of 300 μl of chloroform and 600 μl of water, samples were centrifuged at low speed to facilitate phase separation, and the upper aqueous phase was loaded into Dowex 1X8 columns for separation of [3H]inositol phosphate (InsP).

Statistical Analysis. Data shown are always the mean ± S.E.M. of three to six independent experiments, each run in triplicate. Data were analyzed by one-way analysis of variance followed by the Newman-Keuls test for significance. P < 0.05 was taken as the criterion for statistical significance.

Results

Expression of ERα and mGlu1 Receptors in Cortical Neurons and Astrocytes. Immunoblot analysis of ERα showed a band at approximately 66 kDa. mGlu1 receptor antibodies labeled a major band at 140 kDa, corresponding to receptor monomers. The ERα was detected in protein extracts from both pure cultures of cortical neurons and pure cultures of cortical astrocytes (Fig. 1a). In contrast, the mGlu1 receptor was found exclusively in pure cultures of cortical neurons (Fig. 1b). The cellular pattern of ERα and mGlu1 receptor expression was confirmed by immunocytochemical analysis performed in mixed cultures of cortical cells (the cultures used in toxicity studies). Double fluorescent immunostaining showed the expression of ERα in both neurons and astrocytes (expressing MAP2 and GFAP, respectively) (Fig. 1, c and d). In contrast, mGlu1 receptors were exclusively found in neurons (Fig. 1d). In pure cultures of cortical neurons, mGlu1 receptors were detected in immunoprecipitates with ERα antibodies. Coimmunoprecipitation was increased in cultures treated with 10 nM 17βE2 for 30 min (Fig. 1e). Likewise, a 30-min exposure to DHPG increased coimmunoprecipitation of ERα with mGlu1 receptor, suggesting that the two receptors functionally interact in cortical neurons and that activation of each receptor increases their coupling.

ERα and mGlu1 Receptors Are Interdependent in Protecting Cortical Neurons against β-Amyloid Toxicity. Mixed cortical cultures at 14 DIV were exposed to 100 nM Aβ1-42 for 24 h. Under these conditions, neuronal death, assessed by cell counting after labeling with the cell dye trypan blue, increased by 2- to 3-fold. Pretreatment with 10
nM 17βE2 for 30 min reduced Aβ1–42-induced neuronal death by approximately 30% (Fig. 2a). Identical results were obtained when cultures were challenged with a 25 μM concentration of a shorter fragment of β-amyloid, Aβ25–35, which rapidly forms toxic aggregates in cultures (Fig. 2b). 17βE2 was equally effective as a neuroprotectant when it was added 24 h before the addition of Aβ25–35 (Fig. 2b). Thus, 17βE2 was routinely applied 30 min before Aβ25–35 in all further experiments. A BSA-conjugated form of 17βE2 (100 nM), which is not cell-permeable, protected cortical neurons against Aβ25–35 toxicity to the same extent as free 17βE2 (Fig. 2c). This result suggested that the protective action of estrogen was largely mediated by membrane ERs. Mixed cultures of cortical cells were also treated with the mGlu1/5 receptor antagonist, DHPG. A 30-min pretreatment with DHPG (100 μM), produced a neuroprotective effect comparable with that observed with 10 nM 17βE2 or 100 nM 17βE2-BSA against Aβ25–35 toxicity (Fig. 3). Neuroprotection induced by DHPG plus 17βE2 was less than that predicted if the effects of the two drugs were additive (Fig. 3). To exclude the possibility that the effect of DHPG could involve the activation of mGlu5 receptor, experiments were repeated in the presence of the selective mGlu5 receptor antagonist, MPEP (1 μM; added to neuronal cultures 30 min before 17βE2 and DHPG). Although of reduced magnitude, the neuroprotective effect of 17βE2 and DHPG was still detected in the presence of MPEP, and the effects of the two drugs were not additive (Fig. 4).

In another series of experiments, cultures were treated with 17βE2 or DHPG in the presence of the ER antagonist, ICI 182,780 (1 μM), or the selective mGlu1 receptor antagonist, JNJ 16259685 (100 nM). Both drugs were applied 5 min before 17βE2 or DHPG. As expected, treatment with ICI 182,780 abolished the protective activity of 17βE2 against Aβ25–35 neurotoxicity, whereas treatment with JNJ 16259685 abolished the neuroprotective activity of DHPG. It was unexpected, however, that ER receptor blockade with ICI 182,780 abolished neuroprotection by DHPG, and mGlu1 receptor blockade with JNJ 16259685 abolished neuroprotection by 17βE2 (Fig. 5, a and b). ERα specifically interacted with mGlu1 receptors because the selective ERα agonist, PPT (100 nM), mimicked the neuroprotective activity of 17βE2 and its action was blocked by the mGlu1 receptor antagonist, JNJ 16259685, whereas the ERβ selective agonist, DPN (1 nM), was only slightly neuroprotective and its action was insensitive to JNJ 16259685 (Fig. 6).  

ERα and mGlu1 Receptors Converge in Activating the Phosphatidylinositol-3-Kinase Pathway. Both mGlu1 receptors and ERα are known to activate the PtdIns-3-K/Akt pathway, a pathway that is characteristically linked to mechanisms of neuroprotection. Therefore, treatment with the Akt inhibitor, 10-DEBC hydrochloride (10 μM), abolished the neuroprotective effect of 17βE2 and DHPG (applied alone or in combination) in mixed cortical cultures challenged with Aβ25–35 (Fig. 7a). To examine whether ERα and mGlu1 receptors converge in activating the PtdIns-3-K/Akt pathway, we used pure cultures of cortical neurons. This result avoids the confounding effect produced by the stimulation of glial ERs in mixed cultures. Treatment of cultured cortical neurons with either 17βE2 (10 nM) or DHPG (100 μM) stimulated the PtdIns-3-K/Akt pathway, as detected by immunoblot analysis of phosphorylated Akt after 10 min of incubation (Fig. 7b). The effects of 17βE2 and DHPG on the PtdIns-3-K/Akt pathway were less than additive (Fig. 7b), and activation of ERα and mGlu1 receptors was again interdependent. Therefore, the ERα antagonist, ICI 182,780 abolished the activation of the PtdIns-3-K/Akt pathway produced by DHPG, whereas the mGlu1 receptor antagonist, JNJ 16259685, abrogated the action of 17βE2 (Fig. 7b). Both ICI 182,780 and JNJ 16259685 were devoid of any effect on their own (not shown). The study was extended to HEK293 cells expressing both ERα and mGlu1 receptors. Cells were coexpressing also the high-affinity glutamate transporter, EACC1, to limit the endogenous activation of mGlu1 receptors (Kanai et al., 1994). Both 17βE2 (10 nM) and the potent mGlu1/5 receptor agonist, quisqualate (200 μM), stimulated the PtdIns-3-K/Akt pathway in transfected HEK293 cells (Fig. 7, c and d). In this particular case, however, stimulation produced by the combined application of quisqualate and...
17βE2 was greater than that seen with either drug applied alone (Fig. 7c). Stimulation of pAkt produced by coadministration of 17βE2 and quisqualate was abrogated by pretreatment with ICI 182,780 and/or JNJ 16259685 (Fig. 7d). JNJ 16259685 inhibited Akt phosphorylation induced by 17βE2 and ICI 182,780 was also effective in reducing Akt phosphorylation induced by quisqualate (Fig. 7d). Finally, we examined whether ERα and mGlu1 receptors could also interact in stimulating polyphosphoinositide hydrolysis, which is the canonical signal transduction pathway activated by mGlu1 receptors (Ferraguti et al., 2008). Stimulation of PI hydrolysis produced by membrane ERα and mGlu1 receptors is required for the synthesis of neuroprogesterone in hypothalamic astrocytes (Micevych and Sinchak, 2008a; Kuo et al., 2009). DHPG (100 nM) substantially increased [3H]InsP formation (an indicator of PI hydrolysis) in cultured cortical neurons, whereas 17βE2 (10 nM) produced a slight stimulation of [3H]InsP accumulation without modifying the stimulation of PI hydrolysis by DHPG (Table 1). Both ICI 182,780 (1 μM) and JNJ 16259685 (100 nM) prevented the effect of 17βE2 and reduced stimulation of InsP formation induced by DHPG (Table 1).

**Discussion**

Membrane ERs have long been suggested to take part in the neuroprotective effect of estrogen against Aβ toxicity. Although several signaling pathways are involved, the issue of how membrane ERs signal is still debated. Transactivation of mGlu receptors by estrogen has been largely explored and
demonstrated to be involved in the control of sexual behavior in female rats (Dewing et al., 2007) and the regulation of progesterone synthesis by glia (Kuo et al., 2010). All these mechanisms appear to be mediated by the α subtype of ERs (Boulware et al., 2005; Kuo et al., 2010). We examined whether an interaction between ERα and mGlu1 receptors could be extended to mechanisms of neuroprotection in cortical neurons challenged with β-amyloid peptide. We found that ERα and mGlu1 receptors were colocalized in cultured cortical neurons, in agreement with previous studies showing a colocalization of the two receptors in hypothalamic or hippocampal neurons (Boulware et al., 2005; Dewing et al., 2007). Here, only ERα, but not mGlu1 receptors, could be detected in cortical astrocytes. This contrasts with the evidence that mGlu1 receptors are present in cultured hypothalamic astrocytes prepared from adult rats (Kuo et al., 2009). Developmental or regional differences in the expression of glial mGlu1 receptors may account for this discrepancy.

Addition of 17βE2 attenuated β-amyloid toxicity in mixed cortical cultures, as expected (Pike et al., 2009). The effect of 17βE2 was mimicked by the ERα-selective agonist PPT, whereas pharmacological stimulation of ERβ with DPN caused only a slight protective effect. Addition of the mixed mGlu1/5 receptor agonist, DHPG, also caused neuroprotection to an extent similar to that seen with 17βE2. To dissect the specific contribution of mGlu1 and mGlu5 receptors in neuroprotection, we used an antagonist-based approach by combining DHPG with JNJ 16259865, which blocks mGlu1 receptors, or with MPEP, which blocks mGlu5 receptors. Neuroprotection was abolished by JNJ 16259865 and only slightly reduced by MPEP, suggesting that activation of mGlu1 receptors largely mediated the action of DHPG. The role of group I mGlu receptors in mechanisms of neurodegeneration/neuroprotection is controversial. Activation of mGlu1/5 receptors may cause amplification of neurotoxicity or protection, depending on the experimental paradigm of neuronal death, the nature of the insult, the exposure time to receptor agonists/antagonists, and the origin and composition of the cell culture (for reviews, see Nicoletti et al., 1999; Bruno et al., 2001b). Baudry and his associates (Xu et al., 2007) have found that mGlu1 receptors protect neurons via the activation of the PtdIns-3-K pathway, but they become neurotoxic if cleaved by calpain in response to Ca²⁺ influx mediated by N-methyl-D-aspartate receptor activation. Here, activation of mGlu1 receptors was entirely neuroprotective, perhaps because the endogenous excitotoxic component of β-amyloid toxicity was eliminated by a cocktail of ionotropic glutamate receptor antagonists (see Materials and Methods). We were surprised to observe full interdependence between ERα and mGlu1 receptors in causing neuroprotection. Therefore, neuroprotection by 17βE2/PPT and DHPG was less than additive, and, more important, neuroprotection by 17βE2/PPT was blocked by the mGlu1 receptor negative allosteric modulator, JNJ 16259865, and neuroprotection by DHPG was blocked by the ER antagonist, ICI 182,780. It is remarkable that the slight neuroprotection by the ERβ agonist, DPN, was insensitive to mGlu1 receptor blockade. The absence of glial mGlu1 receptors in our cultures suggests that the interdependence between ERα and mGlu1 receptors did not involve mechanisms of receptor cross-talk occurring in astrocytes. However, we cannot exclude the possibility that activation of glial ERα leads to the secretion of paracrine factors that interact with neuronal mGlu1 receptors in promoting neuroprotection. This would explain our previous finding that the medium of cultured astrocytes treated with estrogen protects pure neuronal cultures against β-amyloid toxicity.
We favor the hypothesis that ER$\alpha$ and mGlu1 receptors directly interact in cortical neurons (where they colocalize), and their combined activation is required to signal neuroprotection. This interaction involves $G_q$-mediated signaling as demonstrated by increased Ins$P_3$ formation after activation of both receptors and prevention of this effect in the presence of antagonists for the ER$\alpha$ or mGlu1 receptor. Although $G_\beta_\gamma$ mediates the ER$\alpha$-induced neuroprotective effect (Dominguez et al., 2009), $G_q$-mediated signaling has also been linked to membrane ER activation in astrocytes (Chaban et al., 2004). Our data, however, support the hypothesis that membrane ERs are not themselves G protein-coupled receptors but rather use mGlu1 receptor to signal, as previously suggested (Micevych et al., 2009; Meitzen and Mermelstein, 2011). MAPK signaling is known to participate in the neuroprotective effect of estrogen. However, in our conditions, increased phosphory-

Fig. 7. Involvement of the PtdIns-3-K/Akt pathway in the neuroprotective effect of 17$\beta$E2 and DHPG. a, mixed cortical cultures were treated with the Akt/PKB inhibitor 10-DEBC (10 $\mu$M), 30 min before treatment with 10 nM 17$\beta$E2, 100 $\mu$M DHPG, or a combination of the two drugs. Ap$_{25-35}$ was then added for additional 24 h and neuronal death was evaluated by cell counting after trypan blue staining. Data are the mean $\pm$ S.E.M. of three independent experiments, each run in triplicate. *, $p < 0.05$ versus untreated control; §, $p < 0.05$ versus 17$\beta$E2 alone; o, $p < 0.05$ versus respective treatment in the absence of 10-DEBC. b, Western blot analysis of Akt phosphorylation induced in pure cortical neurons by a 10-min exposure to 10 nM 17$\beta$E2, 100 $\mu$M DHPG, or both. When the antagonists ICI 182,780 (1 $\mu$M) and JNJ 16259685 (100 nM) were used, they were added 5 min before the agonists. A representative blot is shown, and bars are the mean $\pm$ S.E.M. of at least three determinations. *, $p < 0.05$ versus untreated control; §, $p < 0.05$ versus each agonist alone; o, $p < 0.05$ versus either agonist in the absence of antagonists. c and d, representative Western blot analysis of Akt phosphorylation in HEK293 cells transiently transfected with ER$\alpha$, mGlu1 receptor, and EAAC1 and exposed to 10 nM 17$\beta$E2, 200 $\mu$M quisqualate, or both for 10 min. ICI 182,780 (1 $\mu$M) and JNJ 16259685 (100 nM) were added 5 min before the agonists. Bars are the mean $\pm$ S.E.M. of three experiments. *, $p < 0.01$ versus untreated control; o, $p < 0.05$ versus either agonist alone or in combination; §, $p < 0.05$ versus quisqualate or 17$\beta$E2 alone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H]InsP$_3$ Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 9.4</td>
</tr>
<tr>
<td>DHPG (100 $\mu$M)</td>
<td>186.1 ± 20.7*</td>
</tr>
<tr>
<td>17$\beta$E2 (10 nM)</td>
<td>138.0 ± 8.2*</td>
</tr>
<tr>
<td>DHPG + 17$\beta$E2</td>
<td>175.0 ± 9.0*</td>
</tr>
<tr>
<td>DHPG + JNJ</td>
<td>131.0 ± 9.9**</td>
</tr>
<tr>
<td>DHPG + ICI</td>
<td>117.5 ± 4.6*</td>
</tr>
<tr>
<td>17$\beta$E2 + JNJ</td>
<td>93.9 ± 8.0**</td>
</tr>
<tr>
<td>17$\beta$E2 + ICI</td>
<td>104.9 ± 11.7**</td>
</tr>
<tr>
<td>JNJ (100 nM)</td>
<td>116.7 ± 3.7</td>
</tr>
<tr>
<td>ICI (1 $\mu$M)</td>
<td>99.4 ± 2.9</td>
</tr>
</tbody>
</table>

JNJ, JNJ 16259685; ICI, fulvestrant.
* $p < 0.05$ versus control.
** $p < 0.05$ versus respective treatment in the absence of the antagonist.

(Sortino et al., 2004; Carbonaro et al., 2009). We favor the hypothesis that ER$\alpha$ and mGlu1 receptors directly interact in cortical neurons (where they colocalize), and their combined activation is required to signal neuroprotection. This interaction involves $G_\alpha$-$\beta_\gamma$ mediated signaling as demonstrated by increased Ins$P_3$ formation after activation of both receptors and prevention of this effect in the presence of antagonists for the ER$\alpha$ or mGlu1 receptor. Although $G_q$-$G_\beta_\gamma$ mediates the ER$\alpha$-induced neuroprotective effect (Dominguez et al., 2009), a $G_q$-mediated signaling has also been linked to membrane ER$\alpha$ activation in astrocytes (Chaban et al., 2004). Our data, however, support the hypothesis that membrane ERs are not themselves G protein-coupled receptors but rather use mGlu1 receptor to signal, as previously suggested (Micevych et al., 2009; Meitzen and Mermelstein, 2011). MAPK signaling is known to participate in the neuroprotective effect of estrogen. However, in our conditions, increased phosphory-

TABLE 1
Effect of 17$\beta$E2 and DHPG in the presence and absence of antagonists on [3H]InsP$_3$ formation in cultured cortical neurons

Data are the mean $\pm$ S.E.M. of three to eight independent experiments.
loration of extracellular signal-regulated kinase by 17βE2 was not affected by pretreatment with JNJ 16259865 (not shown), suggesting that this signaling pathway is not primarily involved after coupling of the two receptors. Activation of ERα or mGlu1 receptors is also known to induce neuroprotection via the PtdIns-3-K pathway (Honda et al., 1998; Harms et al., 2001; Ferraguti et al., 2008). Here, ERα and mGlu1 receptors were interdependent in activating the PtdIns-3-K pathway in pure neuronal cultures, and the PtdIns-3-K block, 10-DEPC, prevented neuroprotection by 17βE2 or DHPG alone or in combination in mixed cultures. To examine whether this form of interdependence was related to the cellular context, we performed a series of experiments in recombinant cells expressing both ERα and mGlu1 receptors. Data obtained in recombinant cells diverged from those seen in cortical cultures. In HEK293 cells, 17βE2 and DHPG showed additive effects in activating the PtdIns-3-K. In addition, when both receptors were activated at the same time, stimulation of the PtdIns-3-K pathway was abrogated by either ICI 182,780 or JNJ 16259865; in contrast, when only one receptor was activated by the respective agonist, the response was only partially reduced by the antagonist of the other receptor (for example, the action of DHPG was only slightly reduced by ICI 182,780 and vice versa). Thus, in recombinant cells, ERα and mGlu1 receptors became interdependent only if activated at the same time with the respective agonists, whereas interdependence could not be demonstrated when only one of the two receptors was activated in cortical neurons. The most likely explanation is that all native-type α ERs are functionally coupled to mGlu1 receptors in cortical neurons, whereas coupling involves only a fraction of the two receptor populations in recombinant cells (i.e., under conditions of overexpression). Perhaps, when both receptors are activated at the same time in recombinant cells, the “coupled receptors” saturate the signaling mechanisms, thus unmasking the interdependence. When only one receptor is activated, then the “uncoupled receptors” largely contribute to the activation of the PtdIns-3-K pathway. It is also possible that the different behavior of native versus recombinant receptors reflects differences in the expression of scaffolding proteins or in the extracellular levels of endogenous agonists between neurons and HEK293 cells (for example, the amount of endogenous glutamate is kept low by the expression of the EAAC1 transporter in HEK293 cells). Of interest, a brain region specificity in estradiol-induced activation of different mGlu receptors has been reported, and it seems to depend on as yet unidentified factors rather than on the lack of expression of mGlu receptors in selected areas (Grove-Strawser et al., 2010).

In conclusion, our data provide the first demonstration that ERα and mGlu1 receptors interact in neurons to produce neuroprotection against β-amyloid toxicity. The possibility that the two receptors act together opens new perspectives in the modulation of neuronal function by estrogen and offers novel insights into the variable and controversial role ascribed to both ERs and mGlu1 receptors in neuroprotection.

**Authorship Contributions**

**Participated in research design:** Battaglia, Nicoletti, Bruno, and Sortino.

**Conducted experiments:** Spampinato, Molinaro, Merlo, Iacovelli, and Caraci.

**Performed data analysis:** Spampinato and Merlo.

**Wrote or contributed to the writing of the manuscript:** Nicoletti and Sortino.

**References**


Boulware MI, Boulware JA, W unclear factors rather than on the lack of expression of the PtdIns-3-K pathway. It is also possible that the action of DHPG was only slightly reduced by ICI 182,780 and vice versa. Thus, in recombinant cells, ERα and mGlu1 receptors became interdependent only if activated at the same time with the respective agonists, whereas interdependence could not be demonstrated when only one of the two receptors was activated in cortical neurons. The most likely explanation is that all native-type α ERs are functionally coupled to mGlu1 receptors in cortical neurons, whereas coupling involves only a fraction of the two receptor populations in recombinant cells (i.e., under conditions of overexpression). Perhaps, when both receptors are activated at the same time in recombinant cells, the “coupled receptors” saturate the signaling mechanisms, thus unmasking the interdependence. When only one receptor is activated, then the “uncoupled receptors” largely contribute to the activation of the PtdIns-3-K pathway. It is also possible that the different behavior of native versus recombinant receptors reflects differences in the expression of scaffolding proteins or in the extracellular levels of endogenous agonists between neurons and HEK293 cells (for example, the amount of endogenous glutamate is kept low by the expression of the EAAC1 transporter in HEK293 cells). Of interest, a brain region specificity in estradiol-induced activation of different mGlu receptors has been reported, and it seems to depend on as yet unidentified factors rather than on the lack of expression of mGlu receptors in selected areas (Grove-Strawser et al., 2010).

In conclusion, our data provide the first demonstration that ERα and mGlu1 receptors interact in neurons to produce neuroprotection against β-amyloid toxicity. The possibility that the two receptors act together opens new perspectives in the modulation of neuronal function by estrogen and offers novel insights into the variable and controversial role ascribed to both ERs and mGlu1 receptors in neuroprotection.

**Authorship Contributions**

**Participated in research design:** Battaglia, Nicoletti, Bruno, and Sortino.

**Conducted experiments:** Spampinato, Molinaro, Merlo, Iacovelli, and Caraci.

**Performed data analysis:** Spampinato and Merlo.

**Wrote or contributed to the writing of the manuscript:** Nicoletti and Sortino.

**References**


Boulware MI, Boulware JA, W unclear factors rather than on the lack of expression of the PtdIns-3-K pathway. It is also possible that the action of DHPG was only slightly reduced by ICI 182,780 and vice versa. Thus, in recombinant cells, ERα and mGlu1 receptors became interdependent only if activated at the same time with the respective agonists, whereas interdependence could not be demonstrated when only one of the two receptors was activated in cortical neurons. The most likely explanation is that all native-type α ERs are functionally coupled to mGlu1 receptors in cortical neurons, whereas coupling involves only a fraction of the two receptor populations in recombinant cells (i.e., under conditions of overexpression). Perhaps, when both receptors are activated at the same time in recombinant cells, the “coupled receptors” saturate the signaling mechanisms, thus unmasking the interdependence. When only one receptor is activated, then the “uncoupled receptors” largely contribute to the activation of the PtdIns-3-K pathway. It is also possible that the different behavior of native versus recombinant receptors reflects differences in the expression of scaffolding proteins or in the extracellular levels of endogenous agonists between neurons and HEK293 cells (for example, the amount of endogenous glutamate is kept low by the expression of the EAAC1 transporter in HEK293 cells). Of interest, a brain region specificity in estradiol-induced activation of different mGlu receptors has been reported, and it seems to depend on as yet unidentified factors rather than on the lack of expression of mGlu receptors in selected areas (Grove-Strawser et al., 2010).

In conclusion, our data provide the first demonstration that ERα and mGlu1 receptors interact in neurons to produce neuroprotection against β-amyloid toxicity. The possibility that the two receptors act together opens new perspectives in the modulation of neuronal function by estrogen and offers novel insights into the variable and controversial role ascribed to both ERs and mGlu1 receptors in neuroprotection.

**Authorship Contributions**

**Participated in research design:** Battaglia, Nicoletti, Bruno, and Sortino.

**Conducted experiments:** Spampinato, Molinaro, Merlo, Iacovelli, and Caraci.


