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Estrogen receptors and type-1 metabotropic glutamate receptors are interdependent in protecting cortical neurons against β-amyloid toxicity

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Abbreviations: AD, Alzheimer's Disease; mGlu1, type-1 metabotropic glutamate receptors; ERs, estrogen receptors; 17βE2, 17-β-estradiol; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; DPN, diarylpropionitrile; DHPG, 3,5,-dihydroxyphenylglycine; MAPK, mitogen activated protein kinase; PtdIns-3-K, phosphatidylinositol 3-kinase; ICI 182,780, 7a,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol; 10-DEBC, 10-[4'-(N,N-Diethylamino)butyl]-2-chlorophenoxazine hydrochloride; PI, polyphosphoinositide; MPEP, 2-methyl-6-(phenylethynyl)pyridine; Ara-C, Arabinoside cytoside; EEAC1, excitatory amino acid

carrier 1; BSA, albumin from bovine serum; GFAP, glial fibrillary acidic protein; MAP2, anti-microtubule associated protein; ERK, extracellular signal regulated kinase.

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 mGlu1a receptors, were found in astrocytes. Addition of 17-β-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 BSA 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, JNJ16259685. 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, ICI182,780, but also by JNJ16259685, and neuroprotection by DHPG was abolished by ICI182,780. 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, an information that should be taken into account when examining the impact of estrogen on neurodegeneration associated with CNS disorders.

Introduction

Estrogens are neuroprotective in a variety of cellular and animal models, including cell cultures challenged with excitotoxins or other insults (Cimarosti et al., 2005; Goodman et al., 1996; Harms et al., 2001; Singer et al., 1999), models of focal or global brain ischemia (Dubal et al., 1998; Lebesgue et al., 2009; Simpkins et al., 1997), mice treated with the parkinsonian toxin, 1-methyl-4-phneyl-1,2,3,6-tetrahydropyridine (Bourque et al., 2009), and transgenic mice carrying mutations associated with Alzheimer's disease (AD) (Amtul et al., 2010; Carroll et al., 2007). Estrogens are also effective in reducing β-amyloid toxicity in cultured neurons (Chae et al., 2001; Cordey and Pike, 2005; Goodman et al., 1996; Marin et al., 2003; Sortino et al., 2004), an established cellular model of AD. The classical 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 mediated by membrane ERs, which are able to induce rapid intracellular effects in response to estrogens (Micevych and Dominguez, 2009). More recently, 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 mediate also 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 (Mize et al., 2003; Singer et al., 1999), and the phosphatidylinositol 3-kinase (PtdIns-3-K)/Akt pathway (Cimarosti et al., 2005; Harms et al., 2001; Honda et al., 2000). The mechanism whereby membrane ERs activate the neuroprotective cascade is largely unknown.

It has long been known that membrane ERs can trans-activate 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). More recently, this mechanism of trans-activation has been extended to metabotropic glutamate (mGlu) receptors, which are G-protein coupled receptors. Eight subtypes of mGlu receptors (mGlu1 to mGlu8) have been

described and divided into three groups on the basis of their amino acid sequence, pharmacological profile, and transduction pathways. Group-I subtypes (mGlu1 and mGlu5 receptors) are coupled to G_q, and their activation leads to polyphosphoinositide (PI) hydrolysis with ensuing formation of inositol-1,4,5-trisphosphate and diacylglycerol. mGlu1 and mGlu5 receptors can also activate the MAPK and PtdIns-3-K 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 Gi/Go proteins reviewed by (Nicoletti et al., 2011; Niswender and Conn, 2010). A series of elegant studies have shown that membrane ERα receptors trans-activate mGlu1 receptors in the hypothalamus (Dewing et al., 2007; Dominguez and Micevych, 2010; Mermelstein, 2009; Micevych and Mermelstein, 2008). For example, trans-activation of mGlu1 receptors by ERα in hypothalamic astrocytes leads to the synthesis of neuroprogesterone, which is necessary for estradiol-induced ovulatory surge of luteinizing hormone (LH) (Kuo et al., 2009; Micevych and Sinchak, 2008b). 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 Ca^{++} signalling 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; Battaglia et al., 2001; Bruno et al., 2001a; Bruno et al., 1999; Emery et al., 2010; Nicoletti et al., 1999; Pellegrini-Giampietro, 2003; Pshenichkin et al., 2008; Scartabelli et al., 2008; Zhou et al., 2009).

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

17-β-Estradiol (17βE2) (Sigma-Aldrich, St. Louis, MO), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), diarylpropionitrile (DPN), 7a,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) (all from Tocris Cookson Ltd, North Point, UK) were dissolved in ethanol. 3,5-Dihydroxyphenylglycine (DHPG) and JNJ 16259685 (JNJ), both purchased from Tocris, were dissolved in dimethyl sulfoxide (DMSO, Sigma); 10-[4'-(N,N-Diethylamino)butyl]-2-chlorophenoxazine hydrochloride (10-DEBC) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) (both from Tocris) were dissolved in water; BSA-conjugated 17β-E2 (Sigma) was dissolved in 50% ethanol. β-Amyloid peptides $A\beta_{(1-42)}$ and $A\beta_{(25-35)}$ were obtained from Bachem, Feinchemikalien AG (Bubendorf, Switzerland). $A\beta_{(1-42)}$ was dissolved in DMSO at an initial concentration of 5 mM whereas $A\beta_{(25-35)}$ was solubilized in water at an initial concentration of 2.5 mM. All stock solutions were diluted in culture media as appropriate before use. [^{3}H]-Myo-inositol (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 Nunc (Rochester, NY). All drugs were used at concentrations reported in 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 carried out 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 HBSS (pH 7.4). Cells were plated onto 75 mm^2 flasks and maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), at 5% CO_2 and 37°C for 14 DIV.

Confluent cultures were shaken for 7 hours 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 1-2 x 10^5 cells/cm^2 and used when appropriate confluency was reached.

Cultures of pure cortical neurons were obtained from rats at embryonic day 15 (Harlan), prepared according to a procedure previously described (Sortino et al., 2004). Briefly, cortices were dissected in Ca^{2+}/Mg^{2+}-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 (50 U/ml), streptomycin, (50 μg/ml), albumin from bovine serum (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), progesterone (20 nM) (all from Sigma-Aldrich). Arabinoside cytoside, (Ara-C, 5 μM), was added 18 hours after plating to reduce non-neuronal element proliferation and maintained for 72 hours. Subsequent partial medium replacements were carried out every 2 days. After 7 DIV, cultures were treated for the experiments. These conditions yield a pure neuronal culture as shown by 99% immunostaining to the specific neuronal marker MAP2 as previously 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 MEM supplemented with penicillin (50 U/ml), streptomycin, (50 μg/ml), glucose (6 mg/ml), 10 % FCS, 10% horse serum, glutamine (2 mM) (all from Sigma-Aldrich). At 5 DIV FCS was removed from the medium and cells were supplemented with 5 μM Ara-C for 72

hours. Subsequent partial medium replacements were carried out every 2 days. The cultures were used for experiments at 14 DIV. Mature cultures contained about 40% neurons.

Assessment of neuronal death in mixed cortical cultures

$A\beta_{(1-42)}$ and $A\beta_{(25-35)}$ peptides were applied to serum-deprived mature mixed cortical cultures at 14 DIV. After 24 hours 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 eighty and three hundreds dead neurons per field were counted. All experiments were carried out in the presence of the glutamate receptor antagonists MK801 (10 μM) and DNQX (30 μM) to avoid endogenous glutamate toxicity.

Immunoblot analysis

Astrocytes and neurons were harvested in RIPA 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-lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 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-PAGE and transferred to nitrocellulose membranes using a Transblot semidry transfer cell. After blocking in 1% non-fat dry milk, membranes were incubated with primary rabbit antibody anti-ERα (1:500 Millipore, Billerica, MA), rabbit anti-mGluR1 (1:750 Millipore), rabbit anti-pAkt (1:750 ; Cell Signaling Technology, Beverly, MA), followed by incubation with anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein loading was determined using anti-Akt (1:1000; Cell Signaling Technology). In selected experiments, the same membranes were then reblotted with anti-β-actin (Sigma; not shown).

Specific bands were detected by enhanced chemiluminescence using the Immobilon detection system (Millipore). Full-range rainbow markers (GE Healthcare) were used to assess the size of the band. Densitometric analysis of band intensity was carried out with the aid of the 'Image J' software, developed by NIH and in public domain.

Coimmunoprecipitation

Neurons were harvested in RIPA buffer and 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 hour at 4 °C, in a rotating stirrer with 25 µl of rabbit serum to reduce non-specific binding. Twenty µl of protein G PLUS-Agarose (Santa-Cruz Biotechnology) were then 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, Millipore) or rabbit anti-mGluR1 (1:100, Millipore) were added to supernatants and placed in a rotating stirrer at 4 °C for 7 hours. The antibody-protein complex was adsorbed with 20 µl of protein G-Plus Agarose (Santa Cruz Biotechnology) in a rotating stirrer at 4°C for 10 hours and then washed 5 times with a solution containing PBS and 1% Tween-20 (Sigma-Aldrich). Samples were run on SDS-PAGE, using 4-15% gradient gels (Bio-Rad Laboratories, Milan, Italy) and transferred to nitrocellulose membranes. After blocking in PBS solution containing 2% non-fat milk and 0.1% Tween-20, membranes were incubated with primary rabbit anti-mGluR1 antibody (1:750 Millipore) or rabbit anti-ERα (1:100, Millipore), followed by incubation with HRP-conjugated anti-rabbit secondary antibody. Detection of specific bands was carried out with the Immobilon detection system (Millipore).

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) overnight at 4 °C; mouse

anti-glial fibrillary acidic protein (GFAP; 1:300, Cell Signaling) and mouse anti-microtubule associated protein (MAP2; 1:120, Millipore) for 2 hours at room temperature. For fluorescent immunodetection the following fluorochrome-conjugated antibodies were used: Alexa-Fluor 488 anti-mouse (1:300, Invitrogen) and anti-rabbit Texas Red (1:75, Santa Cruz Biotechnology).

Studies in heterologous expression systems

Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). Cells were transfected in 10-mm dishes using 10 µl of LipofectAMINE2000 in OptiMEM medium and 18 µg 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 carried out for 4 h and then cells were plated in culture medium in 6 well plates, previously coated with 0.01% poly(L-lysine). Using this procedure, about 80-85% of HEK293 cells are immunopositive to cotransfected green fluorescent protein. Experiments were performed 72 h after transfection, following a serum starvation of 16-18 h.

Measurement of polyphosphoinositide hydrolysis in cultured neurons.

Cortical neuronal cultures were incubated overnight with myo[^{3}H] 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 chloroform and 600 µl water, samples were centrifuged at low speed to facilitate phase separation and the upper aqueous phase was loaded into Dowex 1-X-8 columns for separation of [3H]inositol phosphate (InsP).

Statistical analysis

Data shown are always mean ± SEM of 3 to 6 independent experiments each run in triplicates. Data were analyzed by one-way Anova followed by 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. mGlu1a 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 mGlu1a receptor expression was confirmed by immunocytochemical analysis carried out 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. 1c,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. Co-immunoprecipitation was increased in cultures treated with 10 nM 17βE2 for 30 min (Fig. 1e). Similarly, a 30 min exposure to DHPG increased co-immunoprecipitation 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 $A\beta_{1-42}$ for 24 h. Under these conditions, neuronal death, assessed by cell counting after labeling with the cell dye trypan blue, increased by 2-3 fold. Pretreatment with 10 nM 17βE2 for 30 min reduced $A\beta_{1-42}$-induced neuronal death by about 30% (Fig. 2a). Identical results were obtained when cultures were challenged with 25 μM of a shorter fragment of β-amyloid, $A\beta_{25-35}$, which rapidly forms toxic aggregates in cultures (Fig. 2b).

17βE2 was equally effective as neuroprotectant when added 24 hours prior to the addition of $A\beta_{25-35}$ (Fig. 2b). Thus, 17βE2 was routinely applied 30 min prior to $A\beta_{25-35}$ in all further experiments. A BSA-conjugated form of 17βE2 (100 nM), which is not cell permeable, protected cortical neurons against $A\beta_{25-35}$ toxicity to the same extent as free 17βE2 (Fig. 2c). This 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 agonist, DHPG. A 30-min pretreatment with DHPG (100 μM), produced a neuroprotective effect comparable to that observed with 10 nM 17βE2 or 100 nM E2-BSA against $A\beta_{25-35}$ 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, ICI182,780 (1 μM), or the selective mGlu1 receptor antagonist, JNJ16259685 (100 nM). Both drugs were applied 5 min prior to 17βE2 or DHPG. As expected, treatment with ICI182,780 abolished the protective activity of 17βE2 against $A\beta_{25–35}$ neurotoxicity, whereas treatment with JNJ16259685 abolished the neuroprotective activity of DHPG. It was unexpected, however, that ER receptor blockade with ICI182,780 abolished neuroprotection by DHPG, and mGlu1 receptor blockade with JNJ16259685 abolished neuroprotection by 17βE2 (Fig. 5a,b). It was ERα that 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, JNJ16259685, whereas the ERβ selective agonist, DPN (1 nM), was only slightly neuroprotective and its action was insensitive to JNJ16259685 (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 classically linked to mechanisms of neuroprotection. Accordingly, 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 $A\beta_{25-35}$ (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 avoids the confounding effect produced by the stimulation of glial ERα 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. Accordingly, the ERα antagonist, ICI182,780 abolished the activation of the PtdIns-3-K/Akt pathway produced by DHPG, whereas the mGlu1 receptor antagonist, JNJ16259685, abrogated the action of 17βE2 (Fig. 7b). Both ICI182,780 and JNJ16259685 were on their own devoid of any effect (not shown). The study was extended to HEK293 cells expressing both ERα and mGlu1 receptors. Cells were co-expressing 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. 7c,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 co-administration of 17βE2 and quisqualate was abrogated by pre-treatment with ICI182,780 and/or JNJ16259685 (Fig. 7c). JNJ16259685 inhibited Akt phosphorylation induced by 17βE2 and ICI187,680 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 (Kuo et al., 2009; Micevych and Sinchak, 2008a). DHPG (100 nM) substantially increased [^{3}H]InsP formation (an indicator of PI hydrolysis) in cultured cortical neurons whereas17βE2 (10 nM) produced a slight stimulation of [^{3}H]InsP accumulation without modifying the stimulation of PI hydrolysis by DHPG (Table 1) Both ICI182,780 (1μM) and JNJ16259685 (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 to the neuroprotective effect of estrogen against Aβ toxicity. Although several signalling 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 behaviour 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 co-localized in cultured cortical neurons, in agreement with previous studies showing a co-localization 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 JNJ16259865, which blocks mGlu1 receptors, or with MPEP, which blocks mGlu5 receptors. Neuroprotection was abolished by JNJ16259865 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, reviewed by (Bruno et al., 2001b; Nicoletti et al., 1999). Baudry and his associates 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 Ca^{2+} influx mediated by NMDA receptor activation (Xu et al., 2007). 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 Methods section). We were surprised to observe a full interdependence between ERα and mGlu1 receptors in causing neuroprotection. Accordingly, 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 (NAM), JNJ16259865, and neuroprotection by DHPG was blocked by the ER antagonist, ICI182,780. Remarkably, 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 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 (Carbonaro et al., 2009; Sortino et al., 2004). We favour the hypothesis that ERα and mGlu1 receptors directly interact in cortical neurons (where they co-localize), and their combined activation is required to signal neuroprotection. This interaction involves a Gq-mediated signalling as demonstrated by increased InsP formation following

activation of both receptors and prevention of this effect in the presence of antagonists for ERα or mGlu1 receptor. Although Gαiβγ mediates ERα-induced neuroprotective effect (Dominguez et al., 2009), a Gq-mediated signalling has also been linked to membrane ERα 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 (Meitzen and Mermelstein, 2011; Micevych et al., 2009). MAP kinase signalling is known to participate to the neuroprotective effect of estrogen. However, in our conditions, increased phosphorylation of extracellular signal regulated kinase (ERK) by 17βE2 was not affected by pretreatment with JNJ16259865 (not shown), suggesting that this signalling pathway is not primarily involved following coupling of the two receptors. Activation of ERα or mGlu1 receptors is also known to induce neuroprotection *via* the PtdIns-3-K pathway (Ferraguti et al., 2008; Harms et al., 2001; Honda et al., 2000). Here, ERα and mGlu1 receptors were interdependent in activating the PtdIns-3-K pathway in pure neuronal cultures, and the PtdIns-3-K blocker, 10-DEBC, prevented neuroprotection caused 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 carried out 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 ICI182,780 or JNJ16259865; 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 ICI182,780 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 signalling 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 behaviour of native *vs.* 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). Interestingly, 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: Sortino, Nicoletti, Battaglia, Bruno

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

Performed data analysis: Spampinato, Merlo

Wrote or contributed to the writing of the manuscript: Sortino, Nicoletti

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Footnotes

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Simona Federica Spampinato: PhD program in Neuropharmacology

Figure legends

Fig. 1. **Expression of ERα and mGlu1 receptors in cultured cortical neurons and astrocytes.** Immunoblot of ERα (a) and mGlu1 receptor (b) reveals two bands of approximately 66 and 142 kDa, respectively. Co-localization of ERα and the neuronal marker MAP2 and the astrocyte marker GFAP (c). Neurons, immunopositive to MAP2 (d), but not astrocytes, immunopositive to GFAP, express mGlu1 receptor (d). Scale bar = 15 μm. In the insets a 3 fold magnification of single cells is shown. Immunoprecipitation of ERα and mGlu1 receptor in neurons is increased following treatment for 30 min with both 10 nM 17βE2 (e) and 100 μM DHPG (f).

Fig. 2. **Protective effect of 17βE2 against Aβ peptide toxicity.** Cortical neurons were exposed to 17βE2 (10 nM) for 30 min or 24 h (pre), BSA-conjugated 17βE2 (100 nM; 30 min) prior to treatment with $A\beta_{1-42}$ (100 nM; a) or $A\beta_{25-35}$ (25 μM; b,c) for 24 h. Data are expressed as % of $A\beta_{1-42}$- and $A\beta_{25-35}$-induced neuronal death evaluated as the number of trypan blue including neurons. Data are mean ± SEM of 3 to 4 experiments each run in triplicates. Five to 8 different fields per well were counted. *$p<0.05$ vs untreated control; §$p<0.05$ vs respective Aβ treatment.

Fig. 3. **Protective effect of DHPG against $A\beta_{25-35}$–induced toxicity.** Cortical neurons were exposed to 100 μM DHPG, 10 nM 17βE2, 100 nM BSA-conjugated 17βE2 alone or in combination with DHPG, for 30 min prior to treatment with $A\beta_{25-35}$ (25 μM) for 24 h. Data are expressed as % of $A\beta_{1-42}$- and $A\beta_{25-35}$-induced neuronal death evaluated as the number of trypan blue including neurons. Data are mean ± SEM of 3 experiments run in triplicates. *$p<0.01$ vs untreated control and §$p<0.01$ vs $A\beta_{25-35}$ alone.

Fig. 4. **Neuroprotection induced by DHPG is mediated by mGlu1 receptor.** Cortical neurons were treated with the mGlu5 receptor antagonist MPEP (1μM) and 30 min later 17βE2 (10 nM) and DHPG (100 μM) or both drugs together were added for additional 30 min. Neurons were then exposed to $A\beta_{25-35}$ for 24 h and neuronal death was evaluated by counting trypan blue positive cells.

Data are mean ± SEM of three independent experiments run in triplicates in which 4 to 6 fields per well were counted. *p<0.01 vs untreated control and §p<0.05 vs $A\beta_{25-35}$.

Fig. 5. Effect of ERα and mGlu1 receptor antagonists on 17βE2 and DHPG neuroprotective effect. Neurons were treated with 1μM ICI 182,780 (ICI; a) or 100 nM JNJ16259685 (JNJ; b) for 30 min. 17βE2 (10 nM), DHPG (100 μM) or both were added for 30 min prior to treatment with 25 μM $A\beta_{25-35}$ for additional 24 h. Neuronal death was then evaluated by cell counting of trypan blue stained cultures. Data are mean + SEM of 3 independent experiments run in triplicates. *p<0.05 vs $A\beta_{25-35}$ alone; §p<0.05 vs respective treatment in the absence of antagonist.

Fig. 6. Specific interaction of ERα, not ERβ, with mGlu1 receptor. Neurons were treated with the selective ERα (PPT, 100 nM) and ERβ (DPN, 100 nM) agonists for 30 min prior to exposure to 25 μM $A\beta_{25-35}$ for 24 h. When used, the mGlu1 receptor antagonist JNJ16259685 (JNJ; 100 nM) was added 30 min before ER agonists. Data are mean ± SEM of 9 determinations obtained in 3 independent experiments. *p<0.05 vs untreated control; §p<0.05 vs $A\beta_{25-35}$; °p<0.05 vs PPT alone.

Fig. 7. Involvement of PtdIns-3-K/Akt pathway in the neuroprotective effect of 17βE2 and DHPG. In a, mixed cortical cultures were treated with 10 μM of the Akt/PKB inhibitor 10-DEBC, 30 min before treatment with 10 nM 17βE2, 100 μM DHPG or a combination of the two drugs. $A\beta_{25-35}$ was then added for additional 24 h and neuronal death evaluated by cell counting after trypan blue staining. Data are mean + SEM of 3 independent experiments each run in triplicates. *p<0.05 vs untreated control; §<0.05 vs. Aβ alone; °p<0.05 vs. respective treatment in the absence of 10-DEBC. In b, western blot analysis of Akt phosphorylation induced in pure cortical neurons by a 10 min exposure to 10 nM 17βE2, 100 μM DHPG or both. When the antagonists ICI182,780 (1 μM) and JNJ16259685 (100 nM) were used they were added 5 min before the agonists. A representative blot is shown and bars are mean ± SEM of at least three determinations. *p<0.05 vs untreated control; °<0.05 vs. each agonist alone; °p<0.05 vs. either agonist in the absence of antagonists. Panels c and d report representative western blot analysis of Akt phosphorylation in

HEK293 cells transiently transfected with ERα, mGlu1 receptor and EAAC1 and exposed to 10 nM 17βE2, 200 μM quisqualate or both for 10 min. ICI182,780 (1 μM) and JNJ16259685 (100 nM) were added 5 min before the agonists. Bars are mean ± SEM of three experiments. *$p<0.01$ vs untreated control; °$p<0.05$ vs either agonist alone or in combination; § $p<0.05$ vs. quisqualate or 17βE2 alone.

Table 1 Effect of 17βE2, DHPG in the presence and absence of antagonists on [^{3}H]InsP formation in cultured cortical neurons.

Treatment	[^{3}H]InsP formation (% of control)
Control	100.0 ± 9.4
DHPG (100 μM)	186.1 ± 20.7*
17βE2 (10 nM)	138.0 ± 8.2*
DHPG + 17βE2	175.0 ± 9.0*
DHPG + JNJ	131.0 ± 9.9**
DHPG + ICI	117.5 ± 4.6*
17βE2 + JNJ	93.9 ± 8.0**
17βE2 + ICI	104.9 ± 11.7**
JNJ16259685 (100 nM)	116.7 ± 3.7
ICI 182,780 (1 μM)	99.4 ± 2.9

Data are mean + SEM of 3 to 8 independent experiments. *$p<0.05$ vs control; **$p<0.05$ vs respective treatment in the absence of the antagonist.

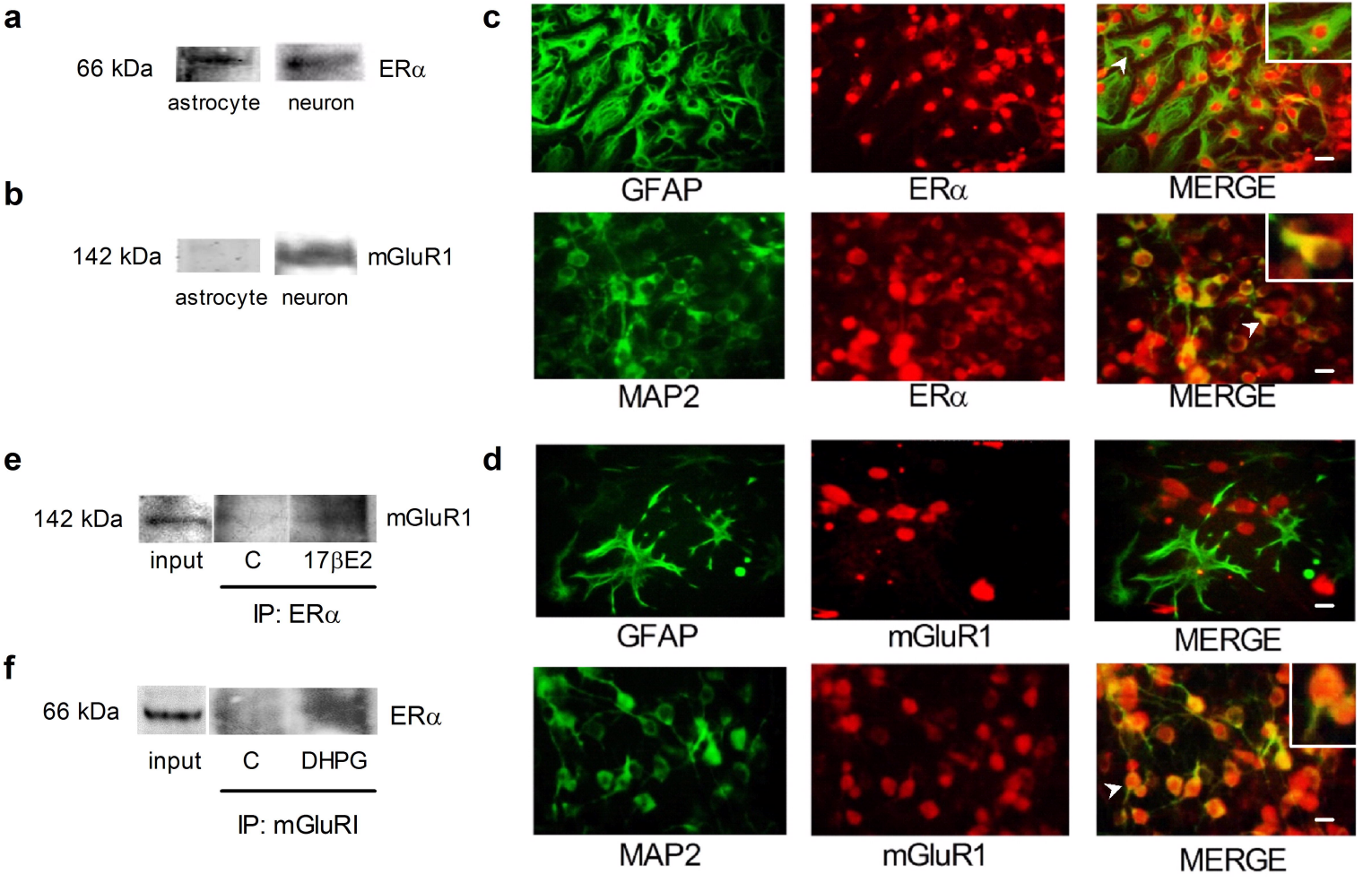
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Figure 1

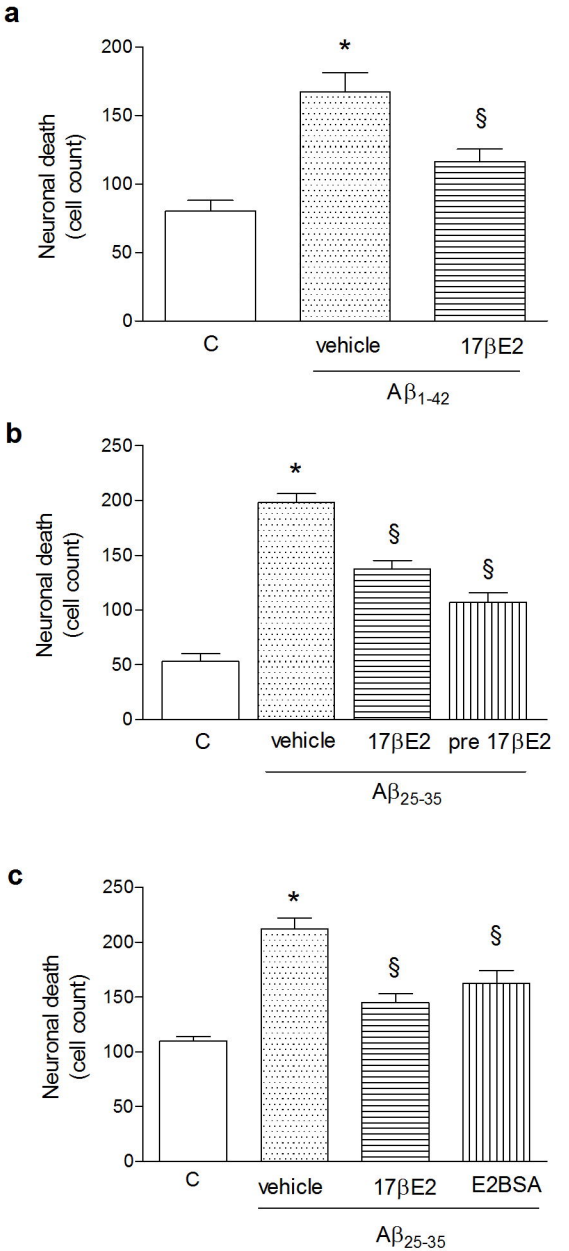
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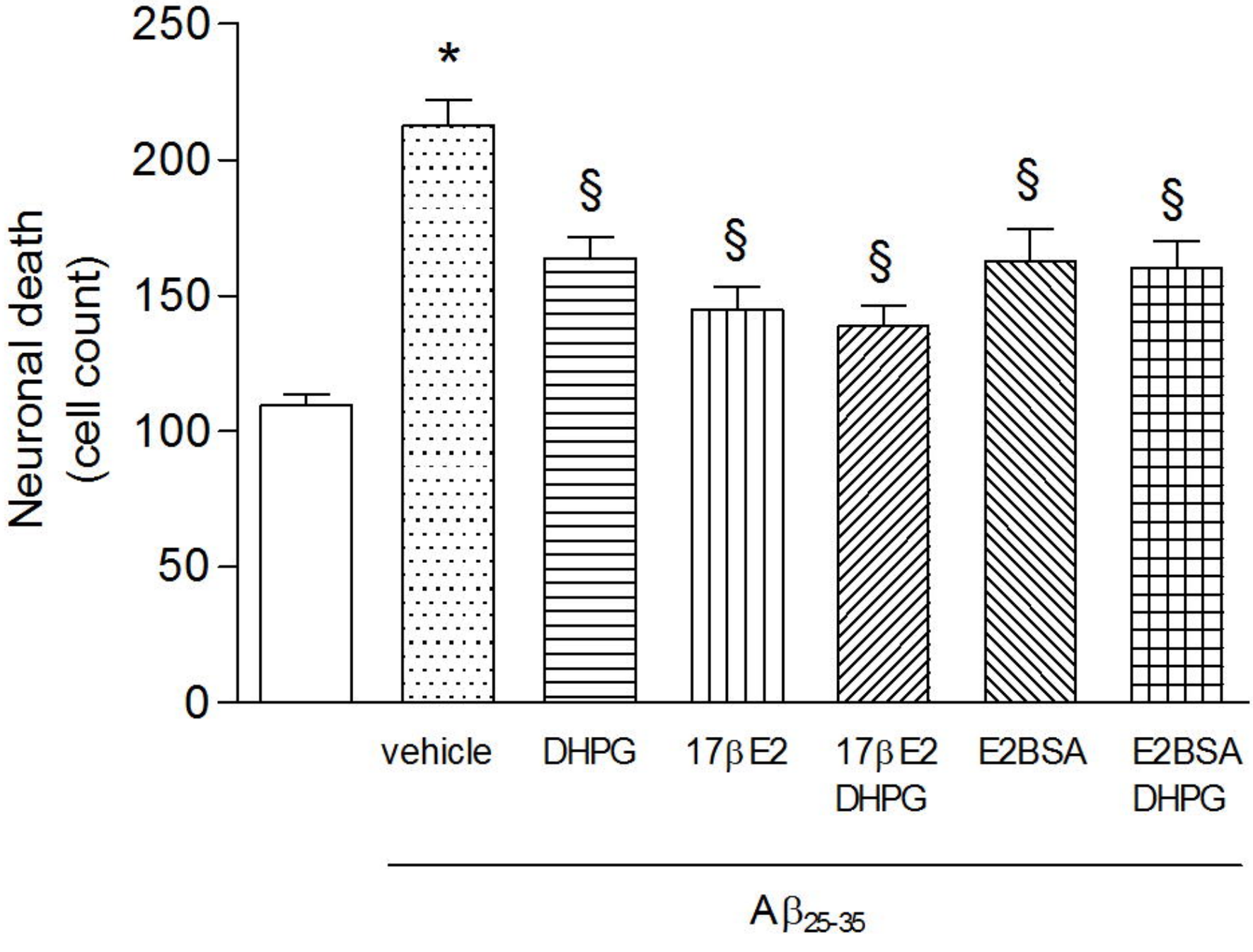
Figure 2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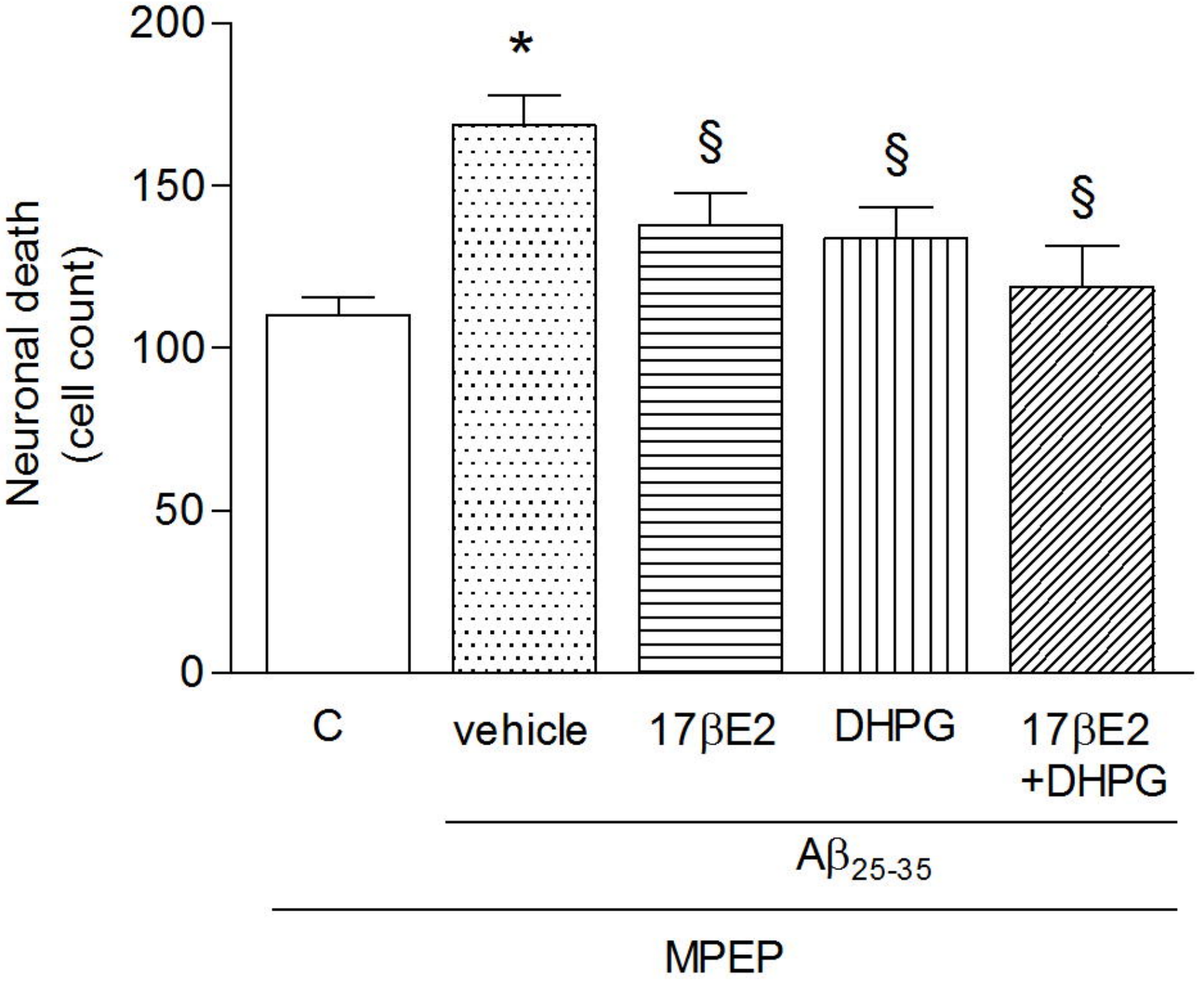
E2BSA
DHPG
Aβ25-35

Figure 3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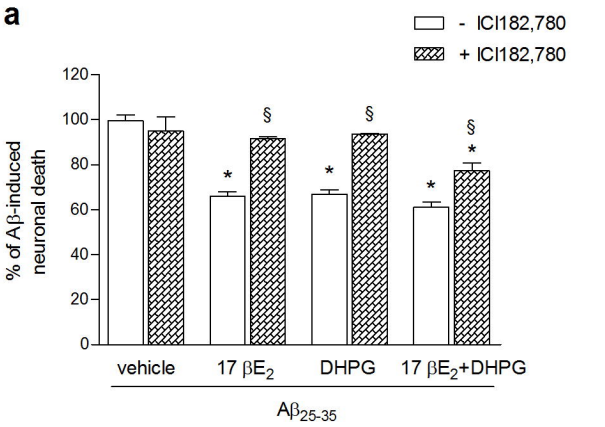
Aβ25-35
MPEP

Figure 4


a
- ICI182,780
+ ICI182,780
% of Aβ-induced neuronal death
120
100
80
60
40
20
0
*
§
vehicle
17 βE2
DHPG
17 βE2+DHPG
Aβ25-35

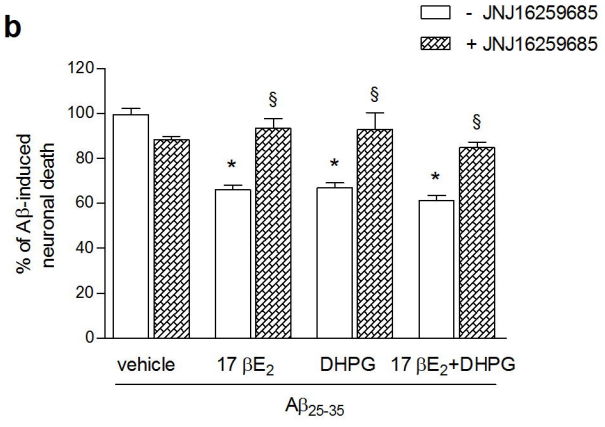

b
- JNJ16259685
+ JNJ16259685
% of Aβ-induced neuronal death
120
100
80
60
40
20
0
*
§
vehicle
17 βE2
DHPG
17 βE2+DHPG
Aβ25-35

Figure 5

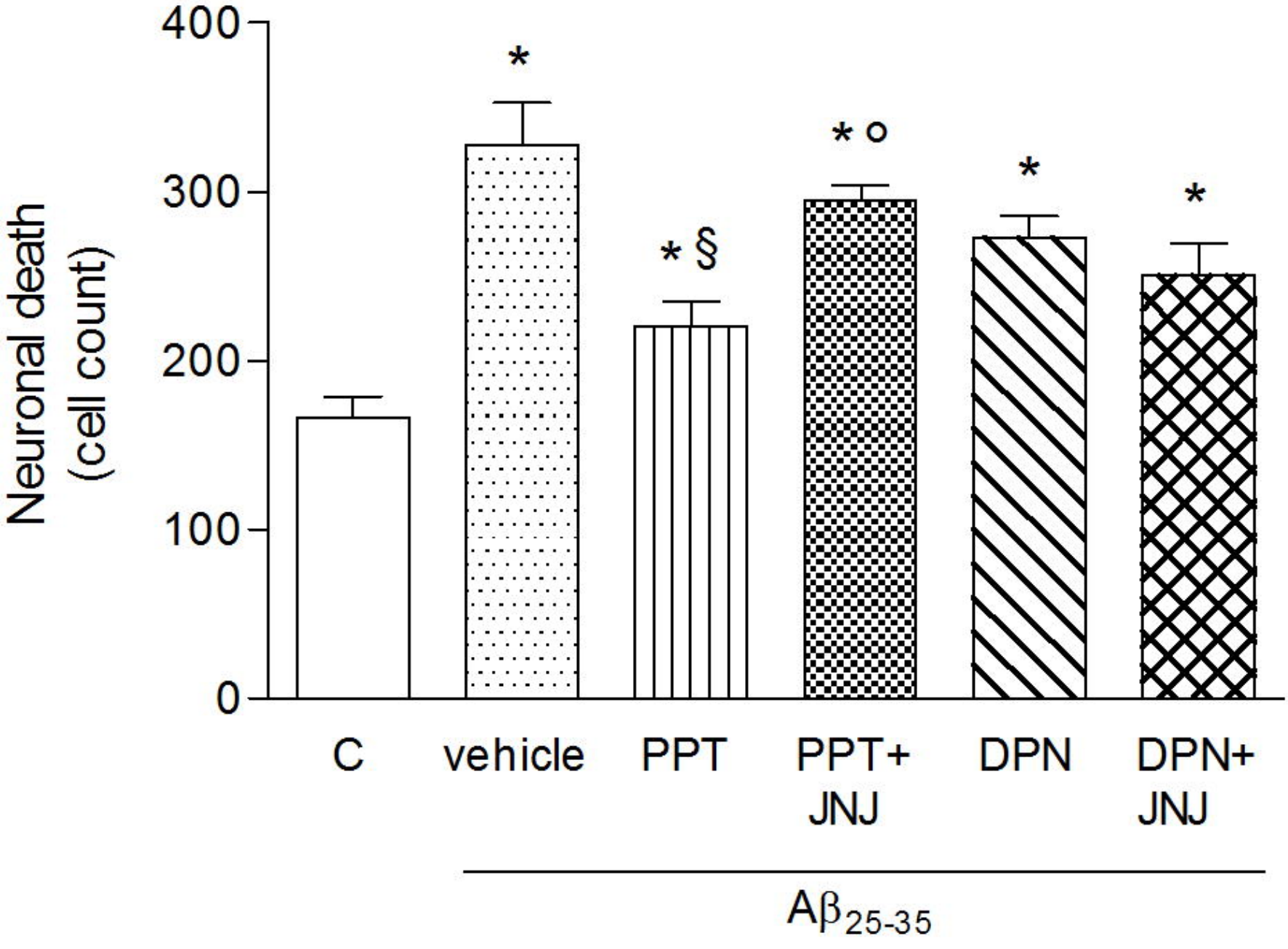

Neuronal death
(cell count)
400
300
200
100
0
*
* §
* o
*
*
C
vehicle
PPT
PPT+
JNJ
DPN
DPN+
JNJ
Aβ25-35

Figure 6

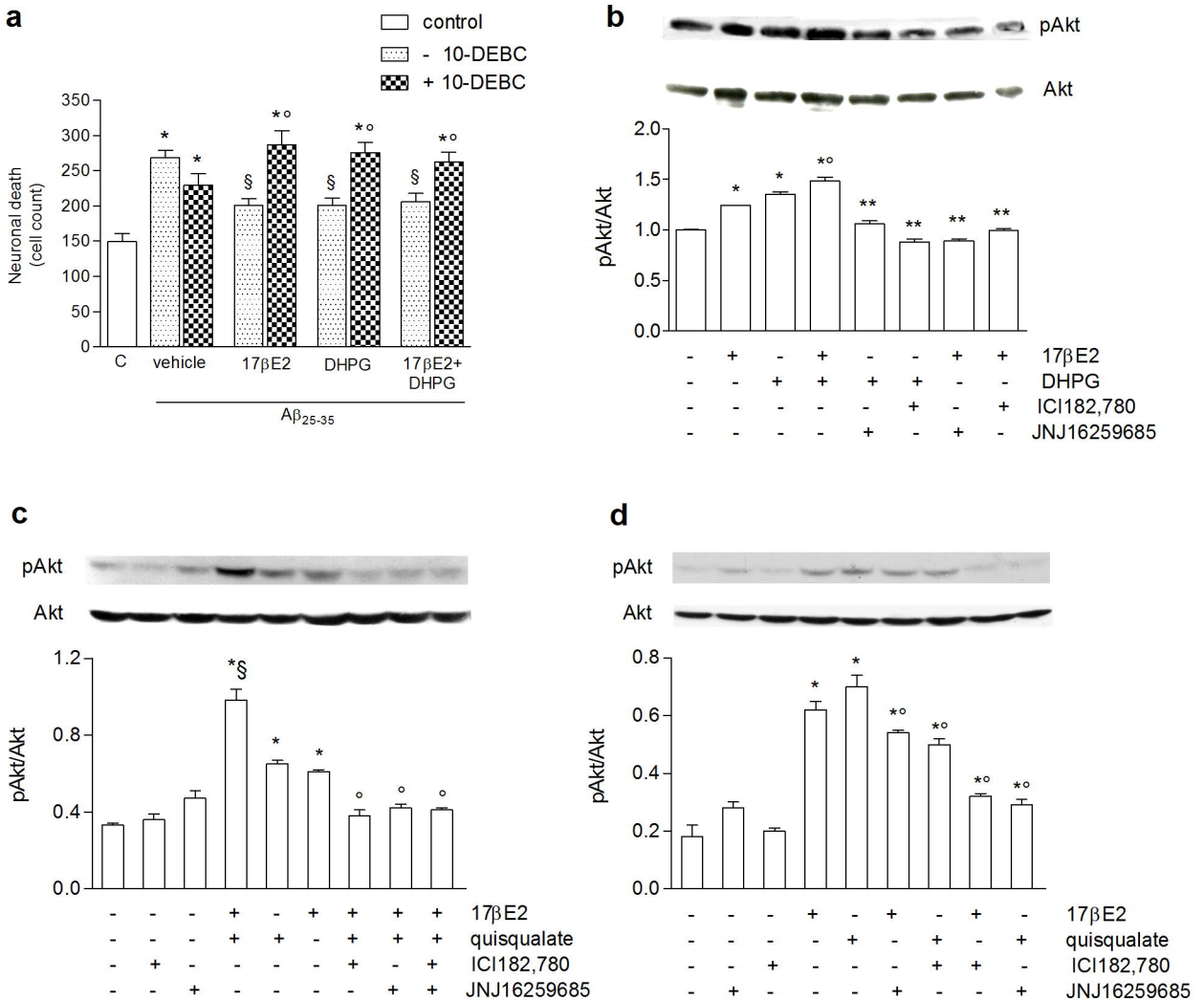

a
control
- 10-DEBC
+ 10-DEBC
Neuronal death (cell count)
C
vehicle
17βE2
DHPG
17βE2+ DHPG
Aβ25-35
b
pAkt
Akt
pAkt/Akt
17βE2
DHPG
ICI182,780
JNJ16259685
c
pAkt
Akt
pAkt/Akt
17βE2
quisqualate
ICI182,780
JNJ16259685
d
pAkt
Akt
pAkt/Akt
17βE2
quisqualate
ICI182,780
JNJ16259685

Figure 7