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INTERACTION BETWEEN ESTROGEN RECEPTOR AND METABOTROBIC GLUTAMATE RECEPTOR 1 MEDIATES THE DUAL EFFECT OF ESTROGEN IN NEUROPROTECTION AND NEURODEGENERATION

Doctorate thesis

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Abstract

Estrogen receptors (ER) are known to exert important action in the central nervous system, including neuroprotection. These effects are due to interaction of estrogen with membrane localized receptors that signal through rapid transduction pathways. How membrane ERs initiate rapid signal transduction has not been clarified, but they have been reported to interact with other membrane receptors, including metabotropic glutamate receptors (mGluR).

The role of estrogen in neuroprotection is not always defined and above all, neuroprotective data provided by preclinical studies have not been confirmed by the use of estrogen in humans: estrogen treatment can in fact be neuroprotective but also responsible for increased neurodegeneration. The dual role ascribed to estrogen, can be observed also with mGluR1 agonists. 3,5-Dihydroxyphenylglycine (DHPG) behaves both as a neuroprotective and neurodegenerative factor. The aim of this study has been to point out whether the similar behaviour of the estrogen and mGlu1R agonists depends on the interaction between their receptors.

Neuroprotective activity of both drugs was demonstrated in an *in vitro* model of cortical cultures exposed to beta amyloid (Aβ) toxicity. Pre-treatment with either 17β-estradiol (E2) and DHPG reduced Aβ-induced neuronal death. The neuroprotective effect was due to strict interaction between mGluR1 and ERα, as treatment with the mGluR1 antagonist, JNJ 16259685, or ER antagonist, ICI 182,780, prevented the effect induced by the respective as well as by the reciprocal agonist. Moreover, E2 and DHPG shared a common signalling pathway, as they stimulated to a similar extent phosphoinositide hydrolysis and induced enhanced phosphorylation of AKT. Both these effects were not additive, when the two agonists were

added together and they were prevented by the reciprocal antagonists. In addition, a similar effect was reproduced when each receptor was expressed in recombinant cells.

The interaction between the two receptors seems to be involved also in the neurotoxic effect of E2 and DHPG. Both compounds exacerbated NMDA induced toxicity when added after the neurotoxic challenge, while being still neuroprotective when administered before. The effect of E2 was not modified by co-treatment with DHPG and was sensitive to ICI 182,780, but also to JNJ16259685. Moreover, this potentiating effect is abolished by pre-treatment with a calpain III inhibitor. The ability of E2 to phosphorylate AKT and ERK is reduced after NMDA pulse, but this effect is partially reverted by pre-treatment with the calpain inhibitor.

Altogether the present data demonstrate that membrane ERs and mGluR1 interact to induce both neuroprotection and neurodegeneration. The different outcome may depend on the cellular conditions, thus pointing out the importance of the choice of the timing of intervention.

General introduction

Estrogen: physiological role and signaling

Estrogens belong to the class of steroid hormones representing the primary female's sex hormones. 17-beta Estradiol (E2) is the most potent compound, although there are two different metabolites, estrone (E1) and estriol (E3), whose activity has tissue-specific roles (Gruber DM et al., 1999). They are produced primarily in the ovaries and placenta, through the synthesis of androstenedione from cholesterol that is then converted to estrone or estradiol, either immediately or through testosterone. The conversion of testosterone to estradiol and of androstenedione to estrone, is catalyzed by the enzyme aromatase. Estrogen can also be produced in small amounts in the liver, adrenal glands and breast, and astrocytes have been found to express aromatase, justifying brain derived-estrogen (Gulinello M et al., 2006, Saldanha CJ et al.2005).

The role of estrogen is not only related to the development of sexual and reproductive function; its activity is essential, in both males and females, in cardiovascular, skeletal, immune functions as well as in the central nervous systems (CNS) (Gustafsson JA. et al., 2003).

In the late 50s (Jensen EV et al., 1962) the nature of an estrogen binding protein, estrogen receptor α, (ERα), was characterised, and after several years the nature of a second estrogen receptor, ERβ, was pointed out (Kuiper GG et al., 1996).

Using the models of recombinant mice, either ERα-/- , ERβ-/- or the double knockout mice, it has been demonstrated that life is possible without either or both receptors, while the reproductive system is severely impaired (Couse JF et al., 1999).

Studies on the role of the two receptors in the CNS, in the skeletal, cardiovascular and immune systems pointed out that the two receptors have distinct and not overlapping roles (Gustafsson JA. Et al., 2003, Harris H et al., 2007). It has been suggested that the two receptors are involved in opposite actions, so the effect of E2 could be the result of the balance between ERα and ERβ signaling (Liu MM et al., 2002; Heldring N et al., 2007).

Estrogen Receptors

Nuclear receptors

ERα and ERβ belong to the class of nuclear receptors. They are the products of two different genes located on different chromosomes (Enmark E et al.,1997; Menasce LP et al.,1993) and several splice variants have been described for both receptors. Similarly to other nuclear receptors, they contain evolutionary conserved domains. The NH2 terminus is not conserved and it represents the area with most variables both in length and sequence. Except for the N-terminal area, ERα and ERβ share high sequence homology, have the same affinity for E2 and bind the same DNA Estrogen Response Elements (ERE) (Nilsson S et al., 2001). When the receptor is not coupled to estrogen it binds to heat shock proteins, in a transcriptionally inactive state (Hall JM et al., 2001). Upon binding to estrogen the receptor undergoes conformational changes, homodimerization and nuclear translocation, where the complex E2/ER directly binds the ERE sequence, or interacts with other transcriptor factors, such as Fos/Jun or SP-1(Kushner PJ et al., 2000; Saville B et al., 2000). The complex ligand/receptor alters chromatin structure and allows the activity of RNA polymerase II transcriptional machinery.

Membrane receptors

In addition to the classical genomic pathway, evidence exists to suggest that estrogen is responsible for rapid responses occurring within few seconds after addition of E2. These effects include activation of kinases, phosphatases and ion fluxes across membranes (Simoncini T et al., 2006; Song RX et al., 2005; Warner M et al., 2006; Wong CW et al., 2002).

The first line of evidence of membrane-initiated signaling by estrogen were reported back in 1967, when Szego and Davis described increased cyclic andenosine mono-phosphate (cAMP) formation in uterus following exposure to E2 for 15 seconds (Szego CM et al., 1967). These rapid effects are initiated at the membrane surface, as demonstrated by the use of membrane impermeable estrogen analogs, estrogen conjugated either to serum bovine albumine (E-BSA) or to horse radish peroxidase (E-HRP) (Zheng J et al., 1996).

In the CNS, membrane-initiated effects of estrogen and activation of classical intracellular signaling cascades are involved in the control of reproductive functions, neuronal excitability, neuroprotection, neurotrophism (reviewed in Vasudevan D et al., 2008); furthermore E2 is involved in the modulation of putative nociceptive signaling in dorsal root ganglion (DRG) neurons (review in Mermelstein P et al., 2008).

The effects of estrogen stimulation are not only related to the activation of ERα or ERβ; removal of classical receptors, using double knockout mice, does not prevent all estradiol binding (Shughrue P.J et al., 2002), and estrogen maintains its activity (Dominguez-Salazar E. et a., 2006; Fugger H.N. et al., 2001). Accordingly, it has been suggested that other receptors are able to bind E2 and mediate its activity. (Kelly M.J. et al.,2008; Li L et al., 2003; Qiu J et al., 2003; Razandi M et al.,1999; Thomas P et al., 2005;

Toran-Allerand C.D et al.,2002). A new receptor, ER-X, has been described in uterus, lung and neocortex, localised at the plasma membrane and associated with caveolin proteins (Toran-Allerand C.D et al.,2005). ER-X binds 17α estradiol, and its activity is not modified by the non selective ER antagonist ICI 182,780 (both stereospecific activation by 17β estradiol and antagonism by ICI 182,780 are classical hallmarks of ERα and ERβ); the receptor is functionally coupled to Mitogen Activated Protein Kinase (MAPK) signaling (Toran-Allerand et al., 2002). In the cortex, its expression peaks in the post natal period (days 7-10), but drops off within a month; in contrast it appears to be upregulated after ischemic injury or in animal models of Alzheimer disease (reviewed in Micevych P et al., 2009).

More recently, the activity of G coupled membrane receptors, likely activated by estradiol has been described (Filardo E.J. et al., 2002; Evinger A.J. III et al., 2005). Among others, GPR30, an integral membrane protein, is the best characterised. Although initially thought to be localised at plasmatic membrane level, recently its localization has been restricted to Golgi apparatus and endoplasmatic reticulum (Matsuda K et al., 2008; Otto C. et al., 2008).

Estrogen activation of GPR30 increases activity of the cAMP pathway (Revankar C.M. et al., 2005; Thomas P. et al., 2005). The ability of ICI 182,780 to modify this effect has not been clarified as contrasting data are reported (Thomas P. et al., 2005). However there are still controversies on the possible role of GPR30 as an estrogen receptor (reviewed in Langer G et al., 2010).

Kelly and co-authors (2003) analyzed the activity of another membrane protein that is activated by a diphenylacrylamide compound, STX (Kelly MJ et al., 2003; Qiu J et al., 2006). This STX binding protein is stereospecifically activated by E2 and blocked by ICI 182,780. When

activated by STX, the receptor affects calcium oscillations and modulates gonadotropin releasing hormone (GnRH) release from hypothalamic neurons in mice and primates, with effects that are similar to those caused by estrogen treatment. To date, the molecular structure of the STX-sensitive receptor remains to be identified (Kenealy BP et al., 2011), but it has been hypothesized that the STX-activated protein is a GPCR, since downstream effects are sensitive to G protein modulation.

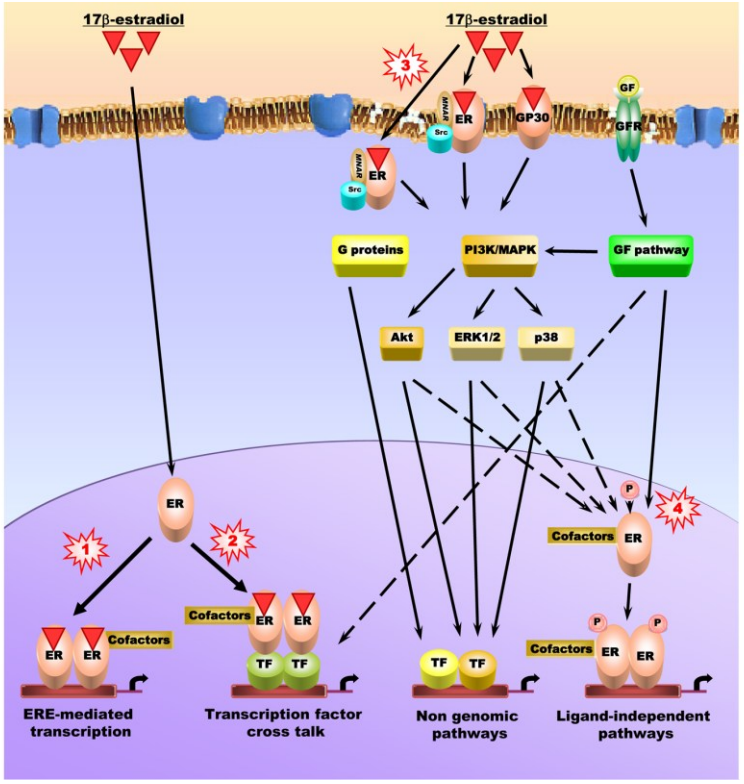
Finally, as mentioned, despite their classical nuclear localization, ERα and ERβ can also be associated with the plasma membrane and can be responsible for the rapid non genomic effects of estrogen (Razandi M et al.,1999). It is not fully understood how the nuclear receptors are trafficked to the membrane, and if they undergo post-transcriptional modification that allow their insertion into the membrane (Acconcia F et al., 2005; Milner T.A et al., 2001).

Membrane ERα and ERβ can transactivate different classes of tyrosine kinase receptors, including epidermal growth factor receptors (EGF) (Song et al., 2010) and type-I insulin-like growth factor receptors (IGF-I) (Marin et al., 2009; Varea et al., 2010).

E2 is able to modulate ionic movements through the membrane, particularly potassium and calcium, due to activation of cAMP and Protein Kinase A (PKA), suggesting the occurrence of mechanisms mediated by a Gs coupled protein (Aronica S.M. et al., 1994, Gu Q., et al.., 1996, Nabekura J. et al., 1986). It can also activate phospholipase C (PLC)/PKC thus modulating a Gq protein (Vasudevan, D.W et al., 2008; Boulware M.I et al.,2005; Dewing P et al., 2007; Dewing P. et al., 2008; Kelly MJ et al., 2003), and signaling related to Gi/o protein has been also demonstrated (Navarro C.E et al.,2003; Wyckoff M.H et al., 2001).

How estrogen receptors activate G protein-coupled proteins is not fully understood; recently, it has been suggested that ERs interact with membrane receptors coupled to G proteins; the ensuing transactivation of a G protein-coupled receptor involve also metabotropic glutamate receptors (see paragraph ***Estrogen receptor and mGluR)***

Fig 1.



Blas et al., 2009

Estrogen activity in the CNS

The role of estrogen in the CNS has been largely studied both in physiological and pathological conditions. Estrogen activities in the brain are related not only to the regulation of hormonal feedback in the hypothalamic pituitary system (Kalra SP et al., 1983; MacLusky NJ et al.,

1978). Estrogen in fact can modulate motor behaviour, mood and mental state, pain perception, etc (Beatty WW et al., 1978; Fink G et al., 1996; Harlan RE et al., 1988; Harlan RE et al., 2001).

In rats, estrogen treatment increases synapse formation in the arcuate nucleus during postnatal development (Arai Y et al., 1978), while in adults it is responsible for synapse remodelling. This occurs not only in response to axonal injury (Matsumoto A et al., 1978), but also in a continuously ongoing process involved in memory and learning, as demonstrated by synapse remodelling in the arcuate nucleus and CA1 area in hippocampus, depending on hormonal fluctuation during the estrous cycle (Olmos G et al., 1989).

The effects of estrogen on synaptic morphology and number of dendritic spines, occurring both in postnatal period and adulthood, have been described in midbrain (Reisert I et al., 1987), cortex (Garcia-Segura LM et al., 1989), hippocampus (Gould E et al., 1990), spinal cord (VanderHorst VG et al., 1997), pituitary (Chun TY et al., 1998) and are related to changes in brain morphology, sexual behaviour, learning and memory. The increased transcription of neurotrophic factors is one of the mechanisms through which E2 induces these effects: in physiological conditions, in fact, E2 increases the transcription of Nerve Growth Factor (NGF) and its receptors in cholinergic neurons (Toran-Allerand CD et al., 1996) and sensory neurons (Sohrabji F et al., 1994), while it modulates the release of Transforming Growth Factor Beta 1 (TGF-β1; Ma YJ et al., 1992) and IGF-1 (Pons S et al., 1993) in hypothalamic neurons and Brain Derived Neurotrophic Factor (BDNF) in cortex (Sohrabji F et al., 1995).

Neuroprotection

In 1991 Hall and coworkers pointed out that female gerbils had less severe brain damage after occlusion of carotid artery versus male animals. These

data were confirmed using different models of brain ischemia (middle cerebral artery occlusion, MCAO) in mice and rats (Alkayed N et al., 1998; Park EM et al., 2006); all females had greater survival rates compared to males after brain injuries.

In human beings, women, at least before menopause onset, are less exposed to stroke risks, (Murphy SJ et al., 2004; Niewada M et al., 2005) while a worse outcome in women parallels the reduced estrogen levels that occurs after menopause (Hochner-Celnikier D et al., 2005).

Evidence that E2 is the neuroprotective factor responsible for the gender-related difference in the outcome of brain ischemia is provided by studies in which estrogen administration reduced infarct volume after global or focal ischemia in both ovariectomized female or male mice, as well as in rats and gerbils (Jover T et al., 2002; Miller NR et al., 2005; Alkayed NJ et al., 1999).

The effects of estrogen consist of a significant improvement in recognition, working and spatial memory, and a reduction in the sensorimotor dysfunctions (Gulinello M et al., 2006; Plamondon H et al., 2006; Li X et al., 2004).

Several studies (Jover T et al., 2002; Plamondon H et al., 2006; Dubal DB et al., 1998) demonstrated that at least a 24 hours estrogen pretreatment is needed to achieve the reduction in the volume of ischemic area. ERα seems to be more implicated in estrogen neuroprotective activity, due to the lack of protective effects in ERα-/- mice (Dubal DB et al., 2001; Sampei K et al., 2000), and the upregulation of ERα in the penumbra area following MCAO in rats (Dubal DB et al., 1999). However, the involvement of ERβ cannot be completely excluded (Miller NR et al., 2005; Sampei K et al., 2000; Carswell HV et al., 2004).

The effects of estrogen are related to its ability to increase the expression of anti-apoptotic genes, in particular *bcl-2* in the penumbra following global ischemia or MCAO*,* in *in vivo* models (Dubal DB et al., 1998). Accordingly, i*n vitro,* E2 increases the expression of *bcl-2* in neuronal hippocampal cultures (Zhao L et al., 2004; Wu TW et al., 2005) and neuronal continuous cell lines (human NT2) (Singer CA et al., 1998); furthermore, E2 reduces the expression of the pro-apoptotic protein BAD (Dubal DB et al., 1999; Zhao L et al., 2004), cytochrome c translocation (Bagetta G et al., 2004) and DNA fragmentation (Rau SW et al., 2003).

The neuroprotective activity of estrogen could be extended to chronic or acute brain injuries. It has in fact protective effects in seizure models where it reduces NMDA- and kainate-induced seizure numbers and duration (Hoffman GE et al., 2003; Velisek L et al., 2002; Kalkbrenner KA et al., 2003). Estrogen prevents cerebellar damage and behavioural decline after ethanol withdrawal (Jung ME et al., 2002) and ameliorates the outcome in models of amyotrophic lateral sclerosis (Kruman II et al., 1999).

There is also evidence suggesting a role of estrogen in different models of brain injury. Through reduction of inflammation, estrogen protects against spinal cord injury (Yune TY et al., 2008; Sribnick EA et al., 2003) and reduces edema and blood brain barrier permeability after traumatic brain injury (Sribnick EA et al., 2005).

Parkinson's disease

Differences in symptoms severity and treatment outcome between males and females have been reported in Parkinson's disease. More specifically males have higher risk in developing the pathology and generally they are affected by more severe symptoms, whereas women have a better outcome,

although they exhibit more frequently levodopa-induced dyskinesia (Rajput MI et al., 2004; Baba Y et al., 2005; Scott B et al., 2000).

Studies in animal models of Parkinson's Disease demonstrate that E2, acting on ERα, reduces toxicity induced in the striatum by 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (D'Astous M et al., 2004), and maintains motor function after 6-hydroxydopamine lesions. In nigrostriatal dopaminergic neurons the latter effect has been related to the interaction with the IGF-1 system (Quesada A, et al., 2004).

In humans, there is evidence that correlates reduced estrogen levels during life to higher risk in developing Parkinson's Disease (Currie LJ et al., 2004). In line with this, estrogen replacement therapy seems to protect against this condition (Westberg L et al., 2004), also through an increased availability of the dopamine transporter in putamen (Gardiner SA et al., 2004).

Finally estrogen treatment induces human neuronal stem cell proliferation and enhances their differentiation in dopaminergic neurons (tyrosine hydroxylase positive cells) (Kishi Y, et al., 2005).

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, characterised by severe cognitive decline. AD is a multi-factorial disease influenced by a combination of genetic and environmental factors. According to the age of onset, AD has been classified into two forms. A rare familial form of AD (FAD), accounting for about 5% of cases, characterized by early onset (45–60 years of age) and linked to causative genetic mutations (Bertram L et al., 2001; Williamson J.,2009). Sporadic AD accounts for the remaining 95% of AD cases and is characterized by late onset (>65 years). This form has not been associated with specific gene

mutations, but with genetic risk factors that seem to underlie an increased chance to develop the disease (Williamson J.,2009).

Pathological hallmarks of AD are extracellular senile plaques and intracellular neurofibrillary tangles (NFTs), results of the aberrant aggregation of misfolded proteins, respectively amyloid beta protein (Aβ) and hyper-phosphorylated tau protein (Selkoe D et al., 2001).

Tau is a microtubule-associated protein that is involved in microtubule assembly and stabilization. Hyperphosphorylation and abnormal phosphorylation are major biochemical abnormalities of the protein. They are early events in the development of the neurofibrillary lesions (Braak E et al., 1994) and, as a result, tau is unable to bind to microtubules (Bramblett GT et al., 1993; Yoshida H et al., 1993). The hyperphosphorylated, insoluble, filamentous tau is the main component of NFTs. Although associations per se cannot prove cause-effect relationships, tau inclusions are widely thought to contribute to the pathogenesis of the disease because they occur in specific brain regions whose functions are altered and NFT formation correlates with the duration and progression of AD (Giannakopoulos et al., 2003; Ihara,2001). Tau inclusions also appear to modulate the clinical features of other neurodegenerative diseases, known as tauopathies (Iqbal K et al., 2005).

The classical and widely accepted mechanism proposed for AD pathogenesis is the amyloid cascade theory (De Strooper d et al., 2010; Pimplikar SW et al., 2009).

Aβ is a short peptide (39-42 amino acids) derived from the proteolytic cleavage of the transmembrane amyloid precursor protein (APP). APP is cleaved at different sites by transmembrane proteolytic complexes known as secretases. Cleavage of the N-terminal ectodomain of APP could be alternatively generated by either α- or β-secretases (β-site APP-cleaving

enzyme, BACE). The activity of α-secretase produces a non-amyloidogenic soluble fragment (sAPPα), and prevents the formation of amyloidogenic fragments due to the activity of BACE. γ- Secretase, a complex of at least 5 different proteins (presenilin 1 and 2, nicastrin, APH1 and PEN2), operates a transmembrane C-terminal cut at alternative sites to generate, when coupled to BACE, Aβ species of 39–42 amino acids. The 40 and 42 aminoacids isoforms have an increased propensity to self aggregate. According to the amyloid theory, an imbalance leading to an over-production of the highly aggregation-prone Aβ 42 species triggers its accumulation and aggregation first into low-molecular-weight oligomers, then into fibrils and finally into plaques, in specific brain regions.

Plaques contain Aβ and degenerating neurites, and evoke strong inflammatory response; activated astrocytes and microglia are attracted to the area of the plaques and release cytokines, chemokines and complement components. The significance of the resulting inflammatory state is not fully understood, since it could represent a protective mechanism against neurodegeneration or rather it is responsible for increased neuronal damage in the areas surrounding the plaques (Schlachetzki JC et al., 2009; Zilka N et al.,2006). Recently it has been shown that oligomers may be highly synapto-toxic species able to cause neuronal synaptic dysfunction and degeneration (De Strooper D et al., 2010, Walsh DM et al., 2009, Sakono m et al., 2010).

The use of estrogen in AD is justified by the effects that E2 exerts in memory functions, through the modulation of synaptic morphology and density (Gould E et al., 1990; Woolley, C et al., 1990), and the regulation of neurotransmission, including catecholaminergic, GABAergic, cholinergic and serotoninergic systems (McEwen, B et al., 2002). Furthermore E2 modulates the expression of apoptotic proteins (Pike, C. J et al., 1999) and exerts effects as antioxidant agent (Greene R et al., 2000).

The effects of estrogen therapy on AD have been widely studied in different preclinical and clinical settings.

Preclinical evidence of estrogen effects in AD

Several *in vitro* studies demonstrate that E2 treatment is able to reduce Aβ toxicity in neuronal cultures (Chen S et al., 2006; Goodman Y et al., 1996; Green SG et al., 1996; Cordey M et al., 2005; Sortino MA et al., 2004), and to reduce the toxic effects caused by oxidative stress (Sawada H et al., 1998; Behl C et al., 1998) and excitotoxicity (Goodman Y et al., 1996; Singer CA et al., 1999; Singer CA et al., 1991; Weaver CE et al., 1997), both events related to neuronal damage in AD.

Furthermore it has been demonstrated that estrogens are involved in the modulation of APP metabolism: E2 increases the activity of the non amyloidogenic α-secretase (Jaffe AB et al., 1994; Manthey D et al., 2001) and stimulates the activity of enzymes responsible of Aβ clearance. Enzymes involved in the degradation of Aβ are matrix metalloproteases (MMPs) and the synaptic zinc metallo-endopeptidase neprilysin (NEP). NEP is considered the dominant Aβ-degrading enzyme in the brain and it recognizes 5 different Aβ cleavage sites (Howell S et al., 1995). NEP has been shown to fully degrade Aβ(1-42) peptide injected into rat hippocampus (Iwata N et al., 2000). Accordingly, infusion with NEP inhibitors produces a significant increase of Aβ levels, leading to formation of extracellular deposits similar to plaques (Takaki Y et al., 2000; Iwata N et al., 2000; Marr RA et al., 2004). In different AD mouse models transgenic NEP was overexpressed using viral vectors: increased NEP levels inhibit Aβ deposition by significantly increasing its degradation, and such event totally prevents neurodegeneration and plaque deposition in these animals (Leissring MA et al., 2003, Marr RA et al., 2004, El Amouri SS et al.,

2008). Convincing evidence finally comes from several studies on brains from AD patient, all showing a significant decrease of NEP mRNA and protein levels in high plaque load areas (Yasojima K et al., 2001; Reilly CE 2001). There are several reports indicating that estrogen regulates the expression and activity of NEP; the NEP gene promoter contains more than one steroid response element, and estrogen is able to up-regulate the transcription of NEP gene through both ERα and ERβ (Xiao ZM et al., 2009). In particular, ERα has proven to be more efficient than ERβ in activating gene transcription in this system. Finally estrogen has also been shown to directly affect enzymatic activity of NEP in different brain areas (hippocampus, cerebellum and caudatum; Huang J et al., 2004).

The matrix metalloproteinases (MMPs) belong to a large family of zinc-dependent endopeptidases and are also involved in the metabolism of Aβ. MMP2 and -9 are the major type found in the CNS. MMP2 is prevalently expressed by astrocytes whereas MMP9 is the prevalent type expressed by neurons (Roher AE et al., 1994; Backstrom JR et al., 1996). Both MMPs have been shown, in vitro, to hydrolyze Aβ(1-42) peptides, purified from AD patients, at several specific sites (Roher AE et al, 1994; Backstrom JR , et al., 1996) and MMP9 has been shown to degrade both soluble and fibrillar Aβ in APP/PS1 transgenic mice (Yan P et al., 2006).

Although estrogen action has been related with modulation of MMPs in areas other than the CNS, only recent data have highlighted the involvement of MMPs in the Aβ-degrading activity of estrogen (Merlo and Sortino, unpublished). Furthermore, a study on healthy post-menopausal women subjected to estrogen replacement therapy with conjugated equine estrogens showed increased plasma levels of both MMP2 and MMP9 compared to untreated women (Lewandowski KC et al., 2006).

E2 induces dephosphorylation of tau protein and prevents its phosphorylation in neurons (Alvarez de la Rosa M et al 2005); it also increases phosphorylation of AKT (Znamensky V et al., 2003; Zhang L et al., 2001), while reducing the activity of BAD and GSK3β (Singer CA et al., 1991; Goodenough S et al., 2005). *In vivo* studies on different AD transgenic mice showed the protective effect of estrogen in reducing Aβ levels and its aggregation in plaques (Amtul Z et al., 2007; Yue X et al., 2005; Carroll JC et al., 2007), as well as tau hyperphoshorylation (Carroll JC et al., 2007).

Clinical evidence on estrogen effects in AD

The effects of estrogen treatments in cross-sectional, longitudinal studies and randomised, control trials have been reviewed by Sherwin and collaborators (Sherwin B et al., 2009).

Evidence has emerged from cross sectional studies showing that elderly women (average age 75) treated with an estrogen substitutive therapy started just after surgically induced menopause (mean 45 years) (Verghese, J et al., 2000) or exposed to an *early estrogen initiation* treatment (before they were 56 years old in the case of natural menopause, or within 5 years after oophorectomy) (MacLennan, A. H et al., 2006), had a better outcome in cognitive functions, compared to women that did not undergo any estrogen treatment or were subjected to a *late initiation* therapy.

Improved performances in short and long term verbal memory, verbal fluency and abstract formation were also confirmed by longitudinal studies comparing elderly women that had initiated estrogen therapy at the time of

menopause *(ever users)* to women who had not received estrogen treatment (*never users*) (Jacobs, D. M et al., 1998).

All these studies suggest that estrogen treatment can reduce the cognitive decline occurring 20-25 years later, especially when an early initiation therapy is established. This has been also demonstrated by another longitudinal study, the *study of osteoporotic fractures* (Matthews K et al., 1999), that pointed out that *past users* (women who started therapy at 46) performed better on Mini Mental state examination and mental flexibility tests compared to *current users* (that started the therapy at 52) and *never user*s.

However, in contrast with these encouraging results, the WHIMS (Women Health's Initiative Memory Study), the largest randomised controlled trial, failed to demonstrate the beneficial effects of estrogen treatment and pointed out that the effects of the therapy could also cause cognitive decline and dementia in women who at the time of therapy initiation were older than 65 (Espeland, M. A et al., 2004; Shumaker, S. A et al., 2004).

The WHI involved the use of estrogen plus a progestin (conjugated equine estrogen [CEE] 0.625 mg daily plus medroxyprogesterone acetate [MPA] 2.5 mg daily) or placebo in women in menopause, or CEE or placebo in hysterectomized women. The combined estrogen and progestin treatment group exhibited a higher risk in developing all-cause dementia compared with the placebo group (Espeland, M. A et al., 2004). In the estrogen-only group, in contrast, no significant differences were found in the incidence of probable dementia or mild cognitive impairment compared with the placebo group, although a non significant increase was observed in the risk of probable all-cause dementia (Shumaker, S. A et al., 2004).

Several criticisms have been moved on the design of the study, in particular it is now known that progestin, and MPA in particular, might counteract the

protective effect of estrogen treatment (Gould, e et al., 1990). Moreover CEE is predominantly comprised of estrone and at least 10 other hormones (Rocca, W et al., 2007). Estrone is biologically less active than estradiol and its affinity for ER is less than 50%, so its activity is not comparable to estradiol (Kuiper, G et al., 1997). Finally the use of Mini Mental State examination, used to test the cognitive function in women in treatment, could not be the most appropriate exam, due to the fact that it fails to examine some specific neuropsychological tasks that are improved by estrogen treatment (verbal and working memory) (LeBlanc et al., 2001).

However the major criticism that can be moved on the study is the involvement of women older than 65. This study in association with others that examined the different impact of estrogen therapy depending on treatment timing (MacLennan, A. H et al., 2006; Matthews, K et al., 1999) suggested the *'critical period hypothesis'*: according to this theory, estrogen therapy effectively decreases the cognitive decline associated with normal aging if the treatment is initiated at the time of menopause or very early in the postmenopausal period and is continued for several years. In contrast, estrogen therapy has no effect, or might even be harmful, when treatment is initiated decades after the menopause begins (Sherwin, B et al., 2009).

This hypothesis has found support in *in vitro* models, that have generated the so called *"healthy cell bias of estrogen benefit"* (Zhao L & Brinton RD 2004): if estrogen treatment is started while neurons are healthy, their response to estrogen is beneficial for their function and survival, but if neurons are exposed to estrogen when their health is already compromised, the hormone exacerbates neuronal death. To support this hypothesis, hippocampal neuronal cultures were exposed to different treatment paradigms (acute, continuous or intermittent E2 exposure before Aβ), or to acute E2 treatment either before or after Aβ exposure. When cultures were

pretreated with E2 in an acute, continuous or intermittent protocol, the neuronal death induced by Aβ exposure was reduced, while E2 treatment after Aβ insult was ineffective to reverse Aβ-induced neurodegeneration, and even exacerbated Aβ-induced cell damage (Chen S et al., 2006).

Mechanisms of action of estrogen

As already described the neuroprotective activity of estrogen could be due to induction of gene transcription that can, for example, increase the expression of neurotrophic factors (NGF, TGF-β, BDNF, IGF-1), and modulate the expression of genes related to apoptotic process (increases of bcl-2 and reduction of BAD expression).

In addition to these genomic effects, estrogen is also able to exert non genomic effects: activation of extracellular signal-regulated kinases (ERK) and phosphoinositol-3-kinase (PI3K)-Akt pathways by E2 have been reported (Singer CA et al., 1999, Singh M et al., 2001). The Akt pathway could in turn phosphorylate and inactivate BAD and GSK3β (Goodenough S et al., 2006; Datta SR et al., 1997; Cross, D et al., 1995).

The effect of estrogen is not confined only to neurons, but it can involve also astrocytes and microglia.

Astrocytes *in vitro* express both ERα and ERβ and E2 induces increased release of TGF-β, from 6 to 36 hours after estrogen treatment (Zhu Y et al., 2002). The increased production of TGF-β at astrocytic level protects cortical and hippocampal neuronal cultures exposed to Aβ toxicity or serum deprivation (Sortino MA et al., 2004; Dhandapani KM et al., 2003a). Astrocyte-derived TGF-β induces also protection after brain ischemia (Ruocco A et al., 1999; Dhandapani KM et al 2003b). Furthermore, estrogen increases the activity of glutamine synthetase, an astrocyte specific enzyme necessary for producing glutamine, that neurons reuptake to increase the

formation of glutamate (Mong JA et al., 2006). Estrogen enhances also the expression of GLAST (glutamate aspartate transporter) and GLT1 (glutamate transporter 1), reducing the levels of extracellular glutamate, potentially harmful (Pawlak J et al., 2005). As astrocytes express aromatase they likely increase the brain derived-estrogen (Gulinello M et al., 2006, Saldanha CJ et al., 2005).

Estrogen has important activity also on microglia, cells that are activated after neuronal insults in different neurodegenerative diseases. Estrogen treatment reduces microglial release of IK-β, NFK-β, PGE2, COX2 and iNOS after brain ischemia (Wen Y et al., 2004; Bruce-Keller AJ et al., 2000; Morale MC et al., 2006; Lewis DK et al., 2008); furthermore estrogen pretreatment reduces the release of superoxide anion, phagocytic activity and expression of inflammatory markers in microglia primary cultures (Bruce-Keller AJ et al., 2000).

E2 seems to be able to reduce also oxidative stress, a condition that affects specifically neurons, and that can lead to both necrotic and apoptotic death. Estrogen reduces brain mitochondrial ROS production, inhibiting mitochondrial superoxide production (Razmara A et al., 2007). It stabilises ATP production increasing mitochondrial efficiency (Simpkins JW et al., 2004), and at physiological concentration modulates antioxidant enzyme activity, including superoxide dismutase, catalase, and glutathione sintetase (Azevedo RB et al., 2001).

Metabotropic glutamate receptor

Metabotropic glutamate receptors (mGluRs) are a class of membrane receptors belonging to class C G Protein Coupled Receptor (GPCRs).

Genes encoding for 8 different mGluR subtypes have been identified and they are widely expressed in different cell types in the CNS. The receptors are subdivided into three different groups, based on sequence and pharmacological homologies (Table 1).

Class I receptors, coupled to a Gq protein, include mGluR1 and mGluR5; class II, coupled to a Gi/o protein, includes mGluR2 and mGluR3, whereas class III, coupled to a Gi/o protein, includes mGluR4, mGluR6, mGluR7 and mGluR8 (reviewed by Niswender and Conn 2010).

mGluRs modulate also other signaling pathways: class I receptors activate phospholipase D, Jun kinase, components of the MAPK/ERK pathway, and the mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway (Li XM et al., 2007; Saugstad, JA et al., 2008, Page G et al., 2006; Hou Let al., 2004). Group II and III receptors also activate MAPK pathway and PI3 kinase pathways (Iacovelli L et al., 2002).

Table 1

Group	Receptor/ Splice variants	CNS Expression	Synaptic localization	Signaling pathway
Group I	mGluR1 a, b, c, d, e, f	Widespread in neurons	Predominantly post synaptic	Phospholipase C stimulation, Stimulation of adenylyl cyclase (selected systems), MAP kinase
	mGluR5 a, b	Widespread in neurons, astrocytes		
Group II	mGluR2	Widespread in neurons	Presynaptic and Postsynaptic	Inhibition of adenylyl cyclase Activation of K^+ channels Inhibition of Ca^{++} channels
	mGluR3	Widespread in neurons, astrocytes		
	mGluR4	Widespread in neurons High in cerebellum	Predominantly pre synaptic	Inhibition of adenylyl cyclase Activation of K^+ channels

GroupIII	mGluR6 a, b, c	Retina	Postsynaptic in ON-bipolar retinal cell	Inhibition of Ca^{++} channels Stimulation of cGMP phosphodiesterase (mGluR6)
	mGluR7 a, b, c, d, e	Widespread in neurons	Active zone of presynaptic terminals	
	mGluR8 a, b, c	Lower and more restricted expression than mGluR4/7	Predominantly pre synaptic	

Mod from Nicoletti et al., 2010

Several proteins can interact with the C-terminal tails of mGluR, regulating their signaling. In particular Homer proteins interact with the last several aminoacids of mGluR1-5 (Tu JC et al., 2008); through the interaction with Homer, class I mGluRs are associated with the long isoform of protein PI3 kinase enhancer (PIKE-L), preventing neuronal apoptosis (Rong R et al., 2003).

mGluRs are diffused in the CNS, where they can be localised at synaptic and extrasynaptic levels in both neurons and glia. They mainly modulate neuronal excitability and synaptic transmission, through their control on ion fluxes and regulation of signalling proteins.

Group I mGluRs are generally localised postsynaptically and their activation leads to cell depolarization and synaptic excitability. Group II and III are localised at presynpatic levels where they reduce the release of neurotrasmitters in both excitatory (glutamate), inhibitory (GABA) and neuromodulatory (Ach) terminals (reviewed in Niswender and Conn 2010).

Preclinical studies suggested a potential role for drugs targeting mGluRs in multiple CNS disorders including depression (Pilc A et al., 2008), anxiety (Swanson CJ, et al., 2005), schizophrenia (Conn PJ et al., 2008; Moghaddam B et al., 2004), pain syndromes (Bleakman D et al., 2006), epilepsy (Alexander GM et al., 2006), Alzheimer's disease (Lee HG et al.,

2004), and Parkinson's disease (Conn PJ et al., 2005). These data are now beginning to be validated also in clinical studies.

mGluR II agonists have been proved, both in preclinical and clinical trials, to have good effects in the treatment of schizophrenia and anxiety disorders, (Conn PJ et al., 2008; Swanson CJ, et al., 2005,174, 175 ,176) as well as in pain, additive disorders, depression and epilepsy (reviewed in Brady, AE et al., 2008). In contrast, mGluR3 seems to be selectively implicated in neuroprotection, through the release of neurotrophic factors by astrocytes (Corti C et al., 2007).

mGluR4 agonists are promising drugs for the treatment of PD (Conn PJ et al., 2005), and evidence exists to suggest a role for class III mGluRs in depression, anxiety (Pilc A et al., 2008), neuroblastoma treatment (Iacovelli L et al., 2006) and neuronal differentiation (Saxe JP et al., 2007).

mGluR5 antagonists are useful tools in anxiety disorders (Swanson CJ, et al., 2005), chronic pain, addiction, depression, some neurodegenerative disorders (Slassi A, et al., 2005), migraine (Keywood C et al., 2008a) and gastroesofageal reflux (Keywood C et al., 2008b). mGluR5 is also a target in the treatment of X fragile syndrome (Bear MF et al., 2004; Yan QJ et al., 2005).

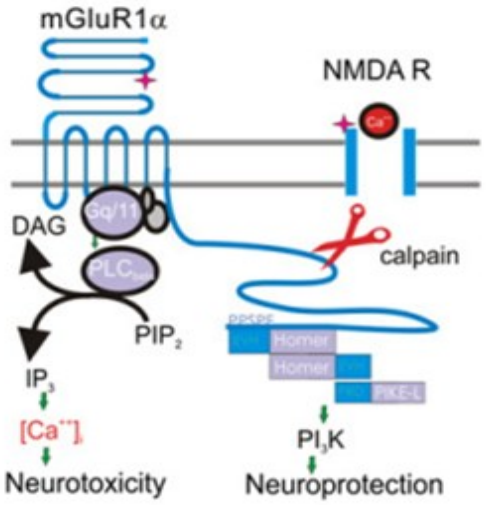
The role of mGluR1 in neurodegeneration is ambiguous. Due to the fact that mGluR1 activity produces excitatory effects in neurons, its activation has been related to induction and progression of excitotoxic neuronal death. Accordingly, mGluR1 agonists have been shown to have neurotoxic effects both in *in vivo* and *in vitro* models (Camon L et al., 1998; Bruno V et al., 1995). However, evidence also suggests that mGluR1 agonists induce neuroprotection (Pizzi M et al., 2000; Battaglia et al., 2001; Emery et al., 2010; Pshenichkin et al., 2008; Scartabelli et al., 2008; Zhou et al., 2009).

The role of mGluR1 in neurodegeneration or neuroprotection has been largely examined (Nicoletti F et al., 1999, Bruno V et al., 2001b) and it can be concluded that the dual activity observed depends on the cellular context and the experimental paradigm applied.

In an attempt to explain the dual role of mGluR1, Baudry and coworkers have proposed a model in which mGluR1 conformation can be modified as a consequence of high intracellular calcium concentration (Xu et al.,2007). As already described, mGluR1a signaling involves activation of a Gq protein, responsible for the stimulation of phospholipase C (PLC), which hydrolyzes membrane phosphoinositides and leads to inositol trisphosphate (IP3)-mediated Ca^{2+} release from intracellular stores (Masu et al., 1991). The neuroprotective effect of mGluR1 seems instead to be mediated by activation of PI3 K-Akt pathway through the formation of a mGluRI-Homer-PIKE-L signaling complex (Rong et al., 2003).

According to Baudry' s group (2007) the increased calcium influx mediated by NMDA receptor activation, leads to calpain-mediated cleavage of the C-terminal tail of mGluR1, where the Homer binding motif is located, preventing the activation of the neuroprotective signaling. When activated after the cleavage, mGluR1 can only activate the PLC pathway, leading neurons to death (Fig 2).

Fig 2



Xu W et al., 2007 Neuron

Estrogen receptor and mGluR

As previously reported membrane ERs can interact with other receptors. In 2005 for the first time evidence emerged that ERs could transactivate mGluRs (Boulware MI et al., 2005).

In cultured hippocampal neurons, membrane ERα interacts with mGluR1 to increase, within few seconds, the phosphorylation of CREB. E2 also decreases L-type calcium channel-dependent CREB phosphorylation, through the interaction between either membrane ERα or ERβ and mGluR2/3 (Boulware MI et al., 2005). These effects were the result of treatment at physiological estrogen concentrations and were mimicked using E2 conjugated to BSA, highlighting the involvement of a membrane receptor, and inhibited by the ER antagonist, ICI 182,780. Moreover agonists/antagonists of mGluRs mimicked or prevented the effect of estrogen, respectively (Kuo, J., et al.,2009).

The physical interaction between the receptors was demonstrated using co-immunoprecipitation studies (Dewing M.I et al., 2007). Specifically, in the

hippocampus ERα couples with mGluR1 whereas both ERα and ERβ co-immunoprecipitate with mGluR2/3. In contrast, in the striatum ERα couples to mGluR5 and both ERα and ERβ are linked to mGluR3 (Grove-Strawser D et al., 2010) suggesting that ERs can be coupled to different mGluRs (Table 2).

Table 2

Striatum	ERα	CAV1	mGluR5	Gq
	ERβ	CAV3	mGluR3	Gi/o
Hippocampus	ERα	CAV1	mGluR1a	Gq
	ERβ	CAV3	mGluR2	Gi/o
Arcuate Nucleus	ERα	CAV1?	mGluR1a	Gq
Hypothalamic Astrocytes	ERα	CAV1?	mGluR1a	Gq
DRG Neurons	ERα	CAV3?	mGluR2/3?	Gi/o

Mermelstein 2010

Interactions between receptors are favoured by caveolins (CAV). These are membrane proteins that organise the signal transduction machinery (Patel HH et al., 2008), and that are known to interact with mGluRs (Patel HH et al., 2008; Murray F et al., 2008) and ERs (Luoma JI, et al., 2008).
Mermelstein and co demonstrated that CAV1 is necessary for the interaction between ERα and mGluR1, while CAV3 is needed to allow the connection between mGluR2/3 and ERα and ERβ (Boulware MI et al., 2007; Grove-Strawser D et al., 2010). The interaction between these receptors has been demonstrated to occur in physiological conditions.

Interaction between mGluR1 and ERα in the arcuate nucleus seems to be important for lordosis behaviour (Dewing MI et al., 2007) while at the hypothalamus it mediates the production of neuroprogesterone, necessary for the LH surge (Sinchak K et al., 2003; Micevych P and Sinchak K, 2008; Micevych P et al., 2010).

Interaction between group II mGluR and ERs occurs also at the level of DRG, where estrogen stimulation inhibits L-type calcium channels (Chaban VV et al., 2003; Chaban VV and Micevych P, 2005). mGluRs signaling is also necessary for the estrogen-induced masculization of adult rat sex behavior and increases in dendritic spine density in the medial preoptic area (Wright and McCarthy, 2009)

Chapter I

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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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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 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 preliminar 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-lisine-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-GFAP (1:300 Cell Signaling) and mouse anti-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 (PI) 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 phosphoERK 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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[1]PhD program in Neuropharmacology

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 each the respective treatment in the absence of the antagonist

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. 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.

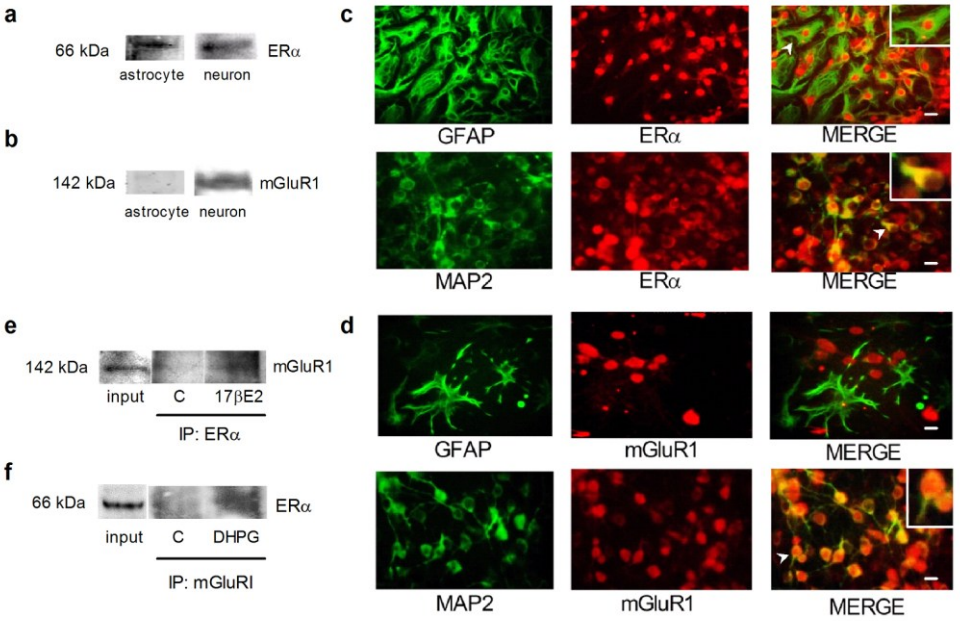
Fig. 3. **Protective effect of DHPG against $A\beta_{25-35}$–induced toxicity.** Cortical neurons were exposed to 100 μM DHPG, 10 nM 17βE2, 100 nM BSA-conjugated 17βE2 alone or in combination with DHPG, for 30 min prior to treatment with $A\beta_{25-35}$ (25 μM) for 24 h. Data are expressed as % of $A\beta_{1-42}$- 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.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astrocyte
neuron
b
142 kDa
mGluR1
astrocyte
neuron
c
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MERGE
MAP2
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MERGE
e
142 kDa
mGluR1
input
C
17βE2
IP: ERα
d
GFAP
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MAP2
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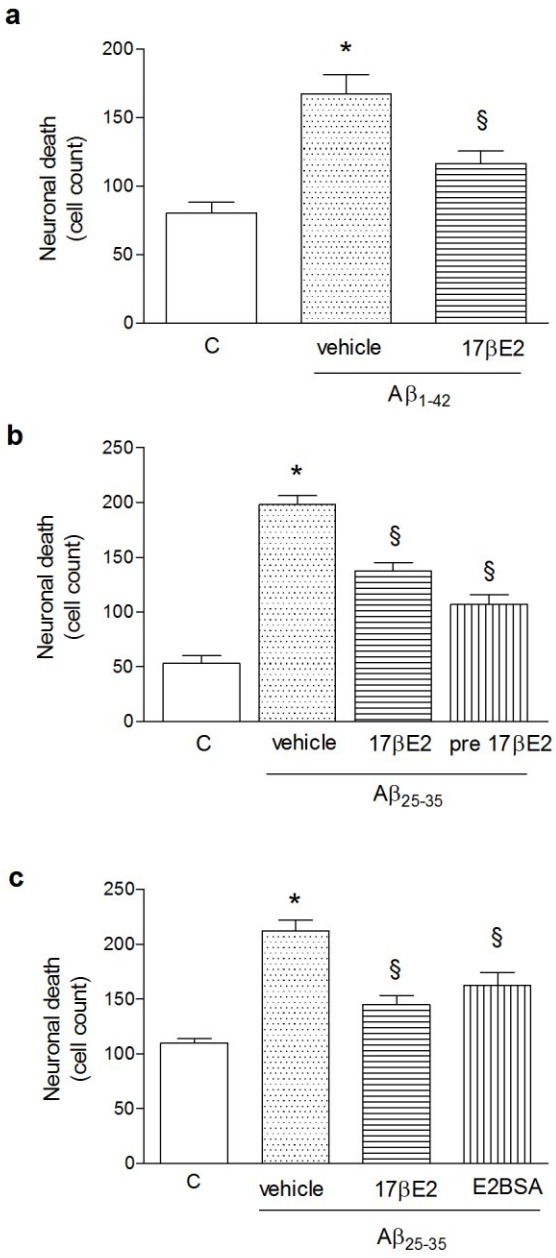
figure 1


a
Neuronal death (cell count)
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vehicle
17βE2
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b
Neuronal death (cell count)
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Neuronal death (cell count)
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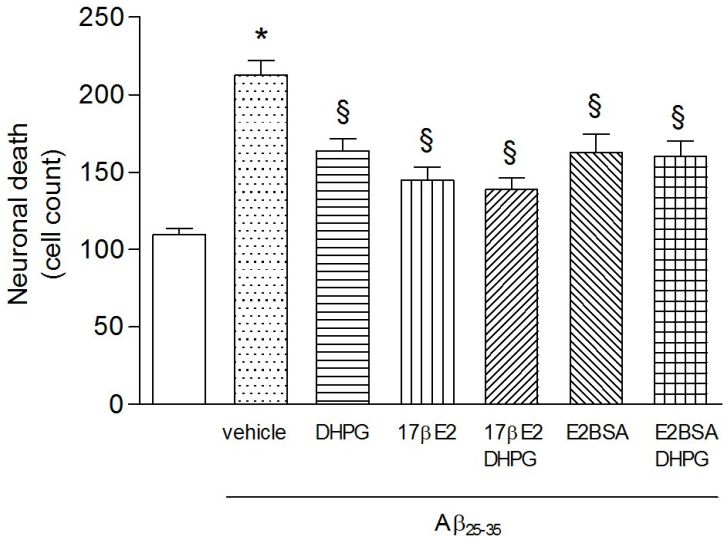
Figure 2



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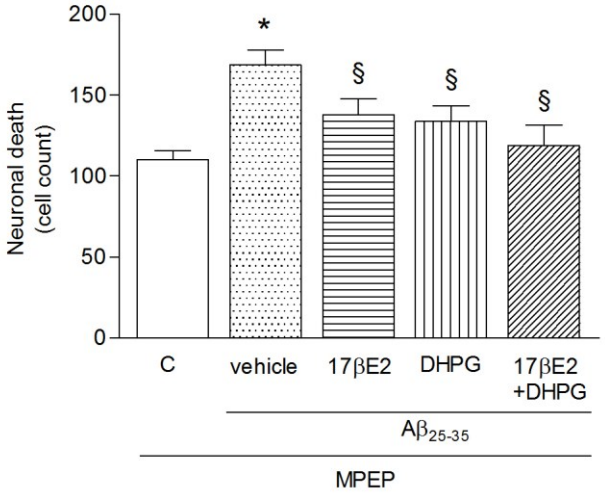
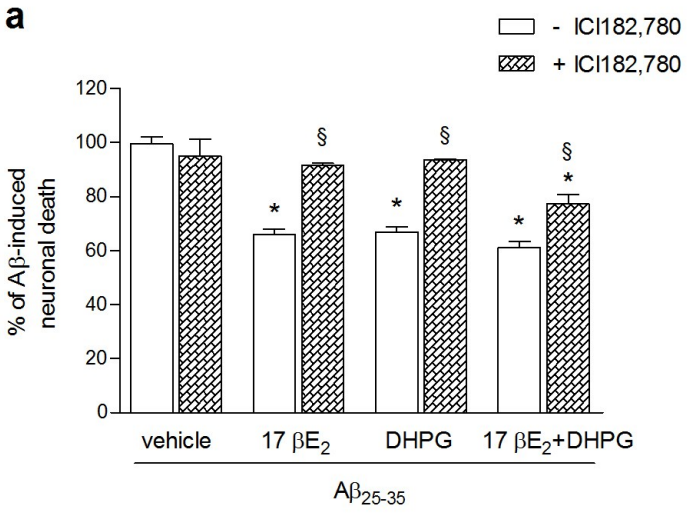


Figure 4


a
- ICI182,780
+ ICI182,780
% of Aβ-induced neuronal death
120
100
80
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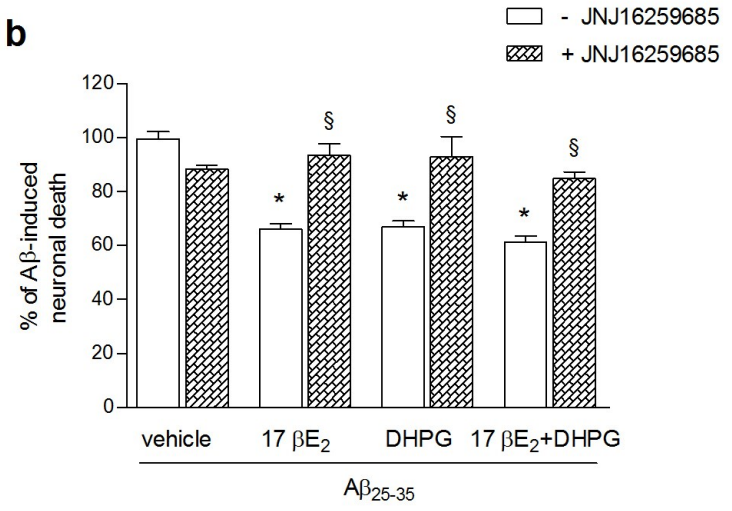
vehicle
17 βE2
DHPG
17 βE2+DHPG
Aβ25-35


b
- JNJ16259685
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120
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Figure 5

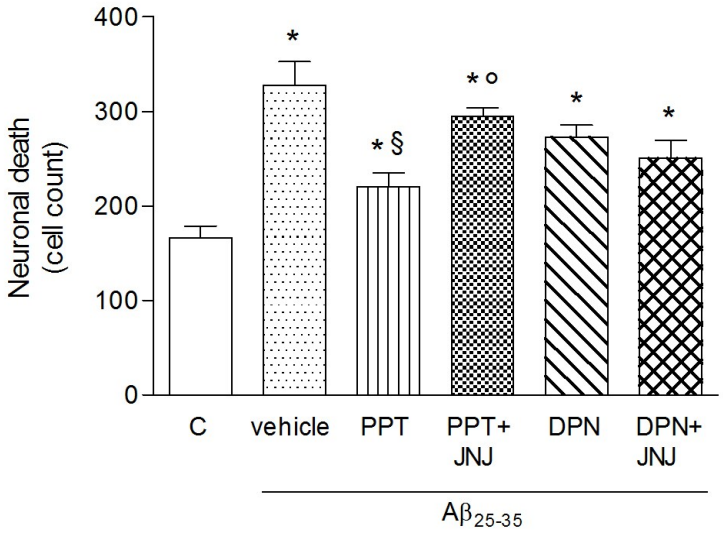
Neuronal death
(cell count)
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Figure 6

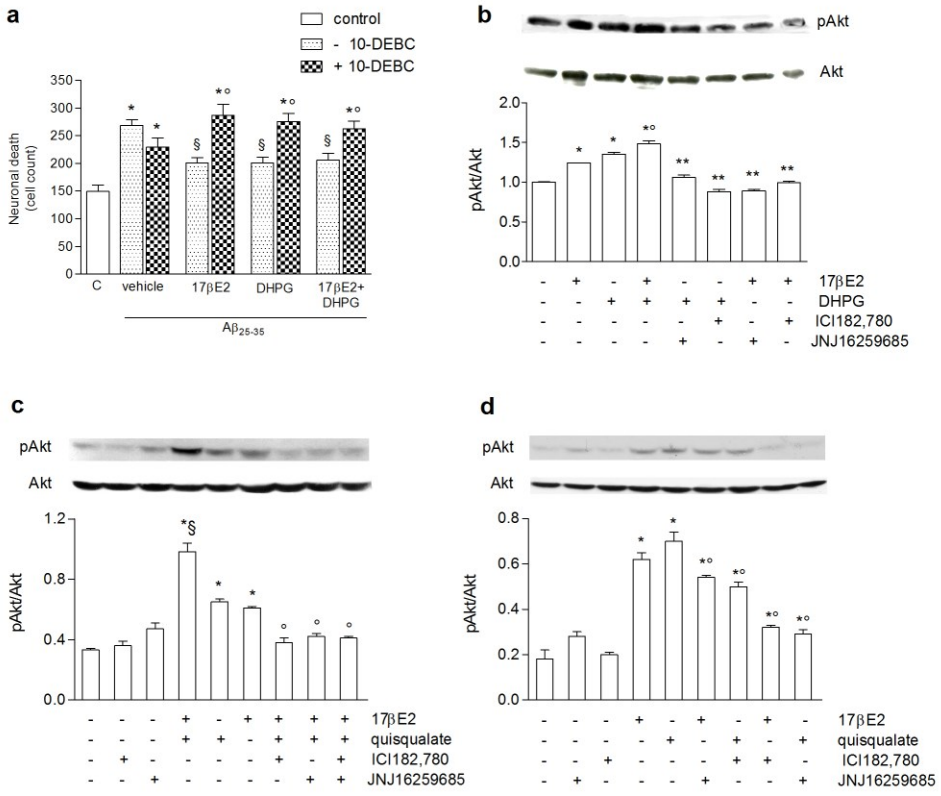
a
control
- 10-DEBC
+ 10-DEBC
Neuronal death (cell count)
C
vehicle
17βE2
DHPG
17βE2+ DHPG
Aβ25-35
b
pAkt
Akt
pAkt/Akt
17βE2
DHPG
ICI182,780
JNJ16259685
c
pAkt
Akt
pAkt/Akt
17βE2
quisqualate
ICI182,780
JNJ16259685
d
pAkt
Akt
pAkt/Akt
17βE2
quisqualate
ICI182,780
JNJ16259685

Figure 7

Chapter II

Estrogen receptor and metabotropic glutamate receptor 1 share a common pathway in potentiating NMDA-induced toxicity

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Abstract

In mixed cortical cultures, treatment with 10 nM 17β-estradiol (17βE2) and 100 μM of the metabotropic glutamate receptor 1 (mGluR1) agonist, DHPG, before a 10 min pulse with NMDA produces a similar protective effect, as assessed by lactate dehydrogenase (LDH) release and cell counting of trypan blue including cells. Neuroprotection by 17βE2 was prevented by treatment with the estrogen receptor antagonist ICI182,780 and the mGluR1 antagonist JNJ6259685. An opposite effect, i.e. an exacerbation of NMDA toxicity, was observed when 17βE2 and DHPG were added following a low concentration NMDA pulse (45 μM). This effect was sensitive to blockade of either estrogen receptor or mGluR1 by selective antagonists. 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 pre-treatment with 17βE2. Treatment with calpain III inhibitor (10 μM), 2 h before NMDA, prevented the increased damage induced by the two agonists. NMDA per se induced a reduction of the full length (140 kDa) mGluR1 expression, an effect partially reversed by calpain inhibitor. In the presence of NMDA, the ability of 17βE2 to stimulate phosphorylation of AKT and ERK was impaired, an effect partially restored by pre-treatment with the calpain inhibitor. The present data confirm the dual role of estrogen in neurotoxicity/neuroprotection and highlight the role of timing of exposure to estrogen.

Introduction

The effects of estrogen in the central nervous system (CNS) go far beyond the sole regulation of reproduction and sexual behaviour; estrogen in fact modulates motor behaviour, mood and mental state, pain perception and has important roles in neuroprotection (McEwen, 2002). A large body of preclinical data support a role for estrogen in reducing neuronal death in different experimental models in vitro and in vivo, including global or focal ischemia (Dubal et al., 1998; Lebesgue et al., 2009), excitotoxicity (Cimarosti et al., 2005; Singer et al., 1999), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced neurodegeneration (Bourque et al., 2009), beta amyloid (Aβ) toxicity (Chae et al., 2001; Cordey and Pike, 2005; Goodman, 1996; Marin et al., 2003; Sortino et al., 2004). The latter observations led to predict a promising use of estrogen in neurodegenerative diseases. Accordingly, clinical trials have demonstrated reduced cognitive impairment in women treated with estrogen replacement therapy (ERT) at the onset of menopause (Jacobs et al., 1998; MacLennan et al., 2006; Matthews et al., 1999; Verghese et al., 2000). However, the Women Health's Initiative (WHI), the largest randomized controlled trial on ERT outcomes, pointed out that estrogen therapy caused cognitive decline and dementia in women who at the time of initiation of ERT were older than 65 (Espeland et al., 2004; Shumaker et al., 2004). This controversy represents the basis for the development of the *'critical period hypothesis'* (Sherwin, 2009), according to which estrogen therapy is helpful in reducing cognitive decline associated with normal aging, if started at the time of menopause or very early in the postmenopausal period, while it has no effect, or is even harmful if initiated decades after menopause begins.

The effects of estrogen in the CNS involve classical intracellular as well as membrane estrogen receptors (ER). Membrane ERs have been shown to interact with other membrane receptors (Marin et al., 2009; Song et al., 2010; Varea et al., 2010), including G protein-coupled receptors and recently the interaction between ERs and metabotropic glutamate receptors (mGluRs) has been described in different brain areas (Dominguez and Micevych, 2010; Mermelstein, 2009; Micevych and Mermelstein, 2008). We have recently shown that in cortical neurons in culture, estrogen reduces Aβ toxicity by transactivating mGluR1 (Spampinato et al., 2011). Interestingly, mGluR1 mediates neuroprotection or neurodegeneration, depending on the cellular context and experimental paradigm examined (Bruno et al., 2001; Nicoletti et al., 1999). The dual role of both ER and mGluR1 led us to investigate whether these receptors interact also in mediating potentiation of neurotoxicity. We here report that estrogen through mGluR1 exacerbates N-methyl-D-aspartate (NMDA)-induced neuronal death, an effect that can be prevented by inhibition of calpain activity secondary to NMDA activation.

Materials and Methods

Reagents

17-β-estradiol (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 (DMSO, Sigma); 2-methyl-6-(phenylethynyl)pyridine (MPEP; Tocris) and N-Methyl-D-Aspartic acid,

(NMDA, Sigma) were dissolved in water. All 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 days of gestation, as described previously (Bruno et al., 2001). In brief, dissociated cortical cells were plated in 24 or 48 multiwell vessels (Falcon) or 35-mm dishes (Nunc, Rochester, NY), precoated with 0.1 mg/ml poly-D-lysine (Sigma). Cultures were maintained in MEM-Eagles salts supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM), and glucose (21 mM). After 3-5 days in vitro, cultures were exposed to 10 μM cytosine arabinofuranoside for 1-3 days and then were shifted to a maintenance medium identical to the plating medium but lacking fetal bovine serum. Subsequent partial medium replacements were carried out twice a week. Neuronal death was studied in cultures at 13-14 days in vitro (DIV).

Assessment of NMDA toxicity in culture

For the induction of excitotoxic neuronal death, cultures were exposed for 10 min to 45-100 μM NMDA at room temperature in a solution containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 20 mM HEPES, 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 in the 2 h or 30 minutes 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 (0.4% for 5 min), 20 hours after 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 minutes, 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 (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 3% no fat milk blocking solution, membranes were incubated with primary rabbit anti-mGluR1(1:700 Millipore, Billerica, MA), rabbit anti-pAkt (1:700; Cell Signaling Technology, Beverly, MA), rabbit anti-pERK (1:700, Cell Signaling Technology) followed by incubation with anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein loading was determined using anti-Akt and anti-ERK (1:1000; Cell Signaling Technology) and anti-actin (1:1000 , Sigma). Specific bands were detected by enhanced chemiluminescence using the Immobilon detection

system (Millipore). Full-range rainbow markers (GE Healthcare, Milan, Italy) 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.

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

Mixed cortical cultures at 14 DIV were exposed to 10 nM 17βE2 for 30 min prior to a brief pulse with NMDA (60 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 further corroborated by cell counting of trypan blue including cells (Fig. 1b). Stimulation of mGlu1 receptor by the group 1 mixed agonist DHPG (100 μM), in the presence of the mGlu5 receptor selective antagonist MPEP (1 μM), produced a similar protective effect (Fig. 1a). Inhibition of NMDA-induced neuronal death by 17βE2 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 JNJ16259685 (100 nM) (Fig.1b). Such effect of DHPG was not present any more when the drug was added to neuronal cultures after or in

combination with the NMDA pulse (60 or 90 μM for 10 min) (Fig. 2b,c). However, when 100 μM DHPG was added after (or at the same time of and only during) a pulse with a low concentration of NMDA (45 μM for 10 min), that per se induced only a mild neuronal damage, an exacerbation of the excitotoxic effect was observed (Fig. 2a). 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), and when the two drugs were added together, no additivity was observed (fig. 3 a,b). The potentiating effect of 17βE2 was prevented by the ER antagonist ICI 182,780 (1 μM), but also by the mGlu1R antagonist JNJ16259685 (100 nM). 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 during the NMDA pulse and throughout the following 24 h of neuronal damage development) (Fig. 4a). Similarly, increased neuronal death observed when DHPG was added after the NMDA pulse was prevented by pre-treatment with 10 nM 17βE2, 30 min before NMDA exposure (Fig. 4b). As mGluR1 is known to undergo calpain-mediated changes in its conformation following NMDA activation, 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 neurons were pulsed with NMDA. This concentration was chosen as 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 mGlu1R, its expression and signaling were tested. As expected, a 10 min-pulse with 45

μM NMDA reduced the expression of the full length mGlu1R (approximately 140 kDa; Fig. 5b), already 2 h after the pulse, an effect likely due to receptor cleavage at the C-terminal tail. This reduction was in fact partially prevented by pre-treatment with 10 μM calpain III inhibitor, added 2 h before the NMDA pulse. Signaling pathways initiated by mGlu1R activation were then tested. For this purpose, neurons were exposed to the NMDA pulse, left to recover for 2 h, and then challenged with 10 nM 17βE2 for 10 min. When calpain III inhibitor was used, it was added to the culture 2 h before the NMDA pulse. As shown in fig. 5c, 17βE2 induced enhanced phosphorylation of ERK and AKT, but the effect was reduced when neurons were subjected to the NMDA pulse. Pre-treatment with calpain III inhibitor rescued the ability of 17βE2 to phosphorylate ERK and slightly modified the reduced AKT phosphorylation (Fig. 5c).

Discussion

The present data demonstrate that 17βE2 acts through mGluR1 receptor to exacerbate NMDA-induced neurotoxicity. Such effect is present only when estrogen treatment follows the excitotoxic insult and is likely mediated by calpain-induced cleavage of mGlu1R. These data are consistent with our recent observation that 17βE2, through ERα, transactivates mGluR1 to protect cortical neurons against Aβ-induced neuronal death (Spampinato et al., 2011). At the same time, however, our data appear in contrast with our previous report demonstrating an involvement of both receptors in neuroprotection. Indeed, such a dual role for estrogen has been largely reported. In fact, low concentrations of estrogen, administered before Aβ insult, are protective, whereas they potentiate neuronal damage, if given after the neurotoxic challenge (Chen et al., 2006). Interestingly, such an observation well supports clinical data showing that early estrogen therapy

at the time of menopause, is beneficial to aging-related cognitive decline, but the opposite may occur if a delayed regimen treatment is applied (Sherwin, 2009). Interestingly, the dual role of estrogen in neurodegeneration/neuroprotection somehow recapitulates what observed with mGlu1R, whose stimulation results in either enhancement or attenuation of excitotoxic neuronal death, depending on the experimental conditions (Bruno et al., 2001).

In the mixed cell culture we have used, stimulation of ERα and mGluR1 before addition of a medium/high concentration of NMDA caused reduction of neuronal death. Neuroprotection observed following activation of both receptors was of similar entity and, more intriguingly, it was not modified by concomitant treatment with agonists of the two receptors, 17βE2 and DHPG. In addition, the neuroprotective effect of 17βE2 was prevented by blockade of mGluR1 with JNJ16259685. These results are consistent with our recent report demonstrating that mGluR1 is involved in the neuroprotective effect of 17βE2 against Aβ toxicity (Spampinato et al., 2011) and find support in the demonstrated interaction between the two receptors, as shown by co-immunoprecipitation studies (Spampinato et al., 2011). However, a similar receptor cooperation seems to occur also when ERs and mGluR1 are stimulated following NMDA toxic insult, a condition that is characterized by 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 blockade of mGluR1. Stimulation of each receptor before the NMDA pulse impedes exacerbation of neuronal damage induced by treatment with the other agonist after NMDA. In our hands, this observation strongly supports the close interaction of ER and mGluR1 and 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, exploits the signalling pathway of mGluR1, with a variable response depending on the cellular condition at the time of receptor activation. The interaction between ER and mGluR1 is not exclusively linked to neuroprotection, but has been demonstrated to play a role also in physiological functions such as the control of sexual behaviour in female rats (Dewing et al., 2007) and the regulation of progesterone synthesis by glia (Kuo et al., 2010). mGluR1, that belongs to class I mGluR, stimulates phospholipase C (PLC) via coupling to Gq/11 with ensuing enhanced phosphoinositide hydrolysis and intracellular Ca^{2+} release (De Blasi et al., 2001). Increased phosphorylation of ERK (Pabst and Redecker, 1999; Pace and Del Negro, 2008) and PI3K (Pace and Del Negro, 2008; Pacey et al., 2011) following mGluR1 activation have also been described.

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 activate the neuroprotective PI3K/AKT pathway (Xu et al., 2007). Consistent with this finding, we here report that treatment with calpain III inhibitor reduces the exacerbation of NMDA toxicity induced by either 17βE2 or DHPG. Moreover, the stimulation of AKT phosphorylation by 17βE2 is reduced following NMDA, 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 specific culture conditions that may be affected by the presence of glia, also sensitive to 17βE2 stimulation. In parallel, also the ability of 17βE2 to stimulate ERK phosphorylation was impaired after NMDA treatment, but addition of calpain inhibitor rescued this response. Coupling of mGluR1 stimulation to ERK phosphorylation occurs independently of PI3K/AKT activation (Thandi et al., 2002), but equally

involves the adaptor protein homer (Ronesi and Huber, 2008; Rong et al., 2003). Thus, it is plausible that cleavage of mGlu1 by calpain modifies the receptor function so that it cannot signal anymore through PI3K/AKT but also through ERK pathway. In this respect, the correlation between mGluR1 and ERK signalling in neuroprotection has been demoinstrated (Emery et al., 2010).

In conclusion, estrogen exhibits the dual ability to protect and to exacerbate NMDA-induced toxicity depending on the time of hormone treatment. Both these effects rely on the ability of ER to couple to mGluR1 and use its signalling pathway. Under basal conditions, pretreatment with 17βE2 results in neuroprotection, whereas following an excitotoxic insult, exposure to 17βE2 causes potentiation of neurotoxicity. The latter effect, as for the mGluR1 agonist DHPG, may be secondary to calpain-mediated cleavage of mGluR1 that is not able anymore to couple to PI3K/AKT and ERK pathway and signal only through the phospholipase C-induced Ca^{2+} increase, responsible for the neurotoxic effect. The results reported here provide an experimental correlate to the unresolved issue of the use of estrogen as a neuroprotective agent and further underline the importance of timing in the correct use of the hormone.

Figure legends

Fig. 1. **Effect of pre-treatment with 17βE2 and DHPG on NMDA-induced toxicity.** Mixed cortical cultures were treated with 10 nM 17βE2 or 100 μM DHPG for 30 min prior to a 10 min pulse with 60 μM NMDA. Neuronal death was assessed 20 h later by measurement of LDH release (a) and counting of cells that included trypan blue (b). When used, the ER antagonist ICI 182,780 (ICI; 1 μM) and the mGluR1 antagonist JNJ16259685 (JNJ, 100nM) were added 30 min before 17βE2.
Data are mean ± SEM of at least three independent experiments each run in triplicates. For cell counting 5 to 8 different fields per well were counted. *$p<0.05$ vs respective control

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 (a), 60 (b) or 90 (c) 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 3-4 separate experiments. *p<0.05 vs untreated control; §<0.05 vs NMDA alone.

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 then treated with 17βE2 (E2; 10nM), DHPG (100 μM) or both compounds for 20 additional h. When antagonists were used, either ICI 182,780 (ICI, 1 μM) or JNJ16259685 (JNJ, 100 nM), they were added 30 min before agonists. The release of LDH was used as indicator of neuronal death (a) or trypan blue including cells were counted in 5 to 8 different fields per well (b,c). Data are mean ± SEM of 3 independent determinations (a). In the case of cell counts, at least 5-8 different fields/well were counted. *p<0.05 vs NMDA alone; §p<0.05 vs E2.

Fig. 4. **Different effect of 17ßE2 and DHPG on NMDA-induced toxicity depending on the time of treatment.** Cultures were exposed to DHPG (100 μM; a) or 17βE2 (E2 10 nM; b) for 30 min prior a 10 min pulse with NMDA (45 μM) and subsequent treatment with 17βE2 (a) or DHPG (b) for additional 20 h. Data are mean ± SEM of determinations from 3-5 independent experiments. *p<0.05 vs NMDA; §p<0.05 vs treatment with DHPG after NMDA (DHPG post; a) or treatment with 17ßE2 after NMDA (E2 post; b).

Fig. 5 **Effect of calpain inhibition on 17ßE2 and DHPG effect on NMDA toxicity.** Cultures were pretreated with calpain inhibitor III (CI; 10μM), 2 h before NMDA pulse, and treated with 17βE2 (10 nM), DHPG 100 μM or

both for additional 20 hours before evaluating neuronal death by cell counting (a). *p<0.05 vs. NMDA alone; §p<0.05 vs corresponding vehicle-treated. In b, the expression of mGluR1 following treatment with NMDA and NMDA + calpain inhibitor III (CI). *p<0.05 vs control; §p<0.05 vs NMDA. In c, phosphorylation of AKT and ERK after a 10-min treatment with 17βE2 (10 nM), following a pulse with NMDA. When used, calpain inhibitor III (CI) was added 2 h before NMDA. *p<0.05 vs control; §p<0.05 vs NMDA; °p<0.05 vs NMDA + 17ßE2.

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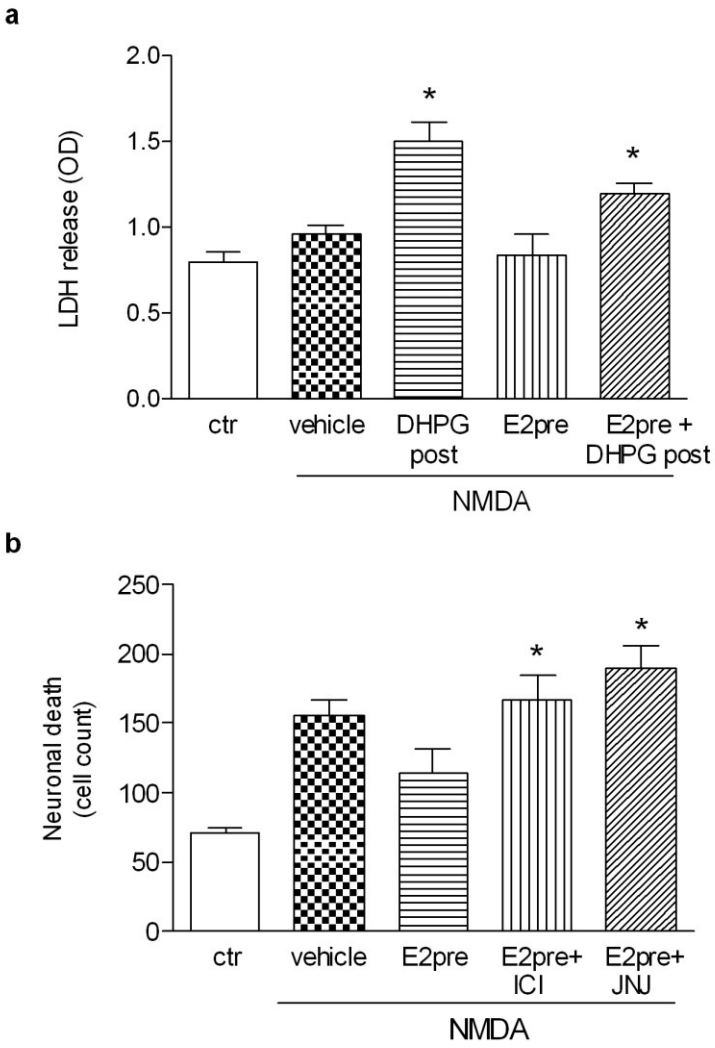
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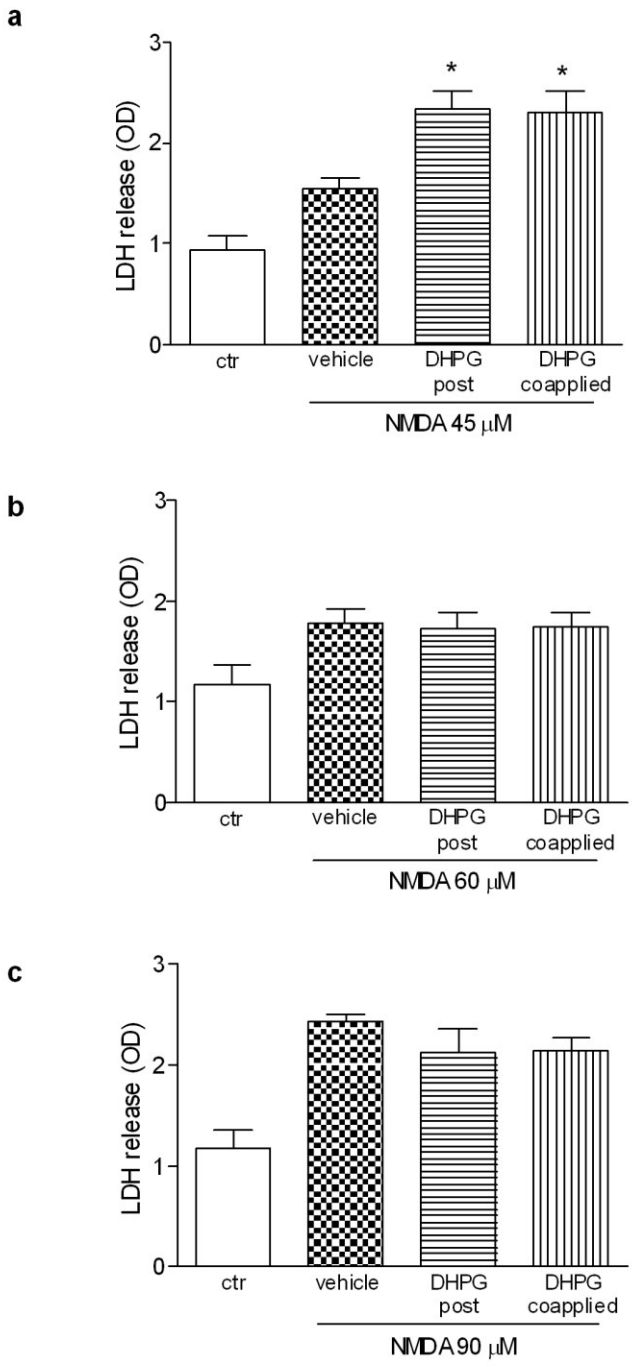
Figure 1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DHPG coapplied
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Figure 2

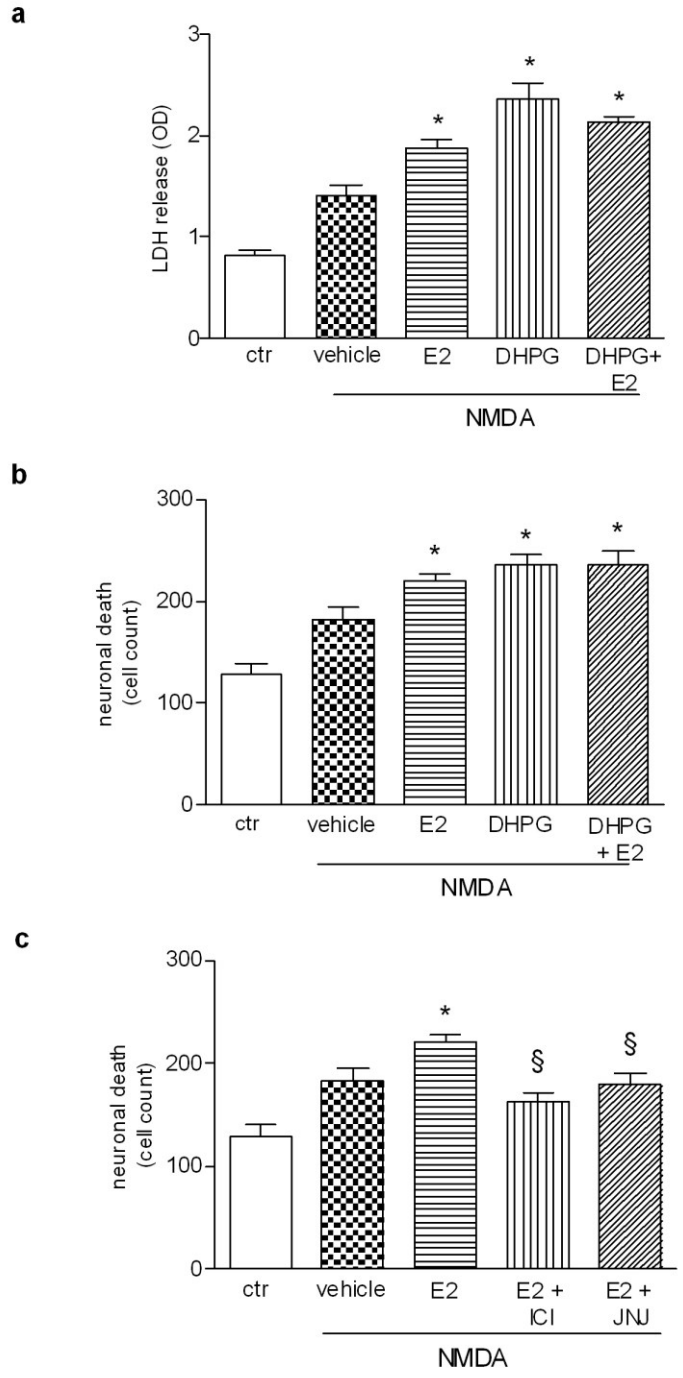
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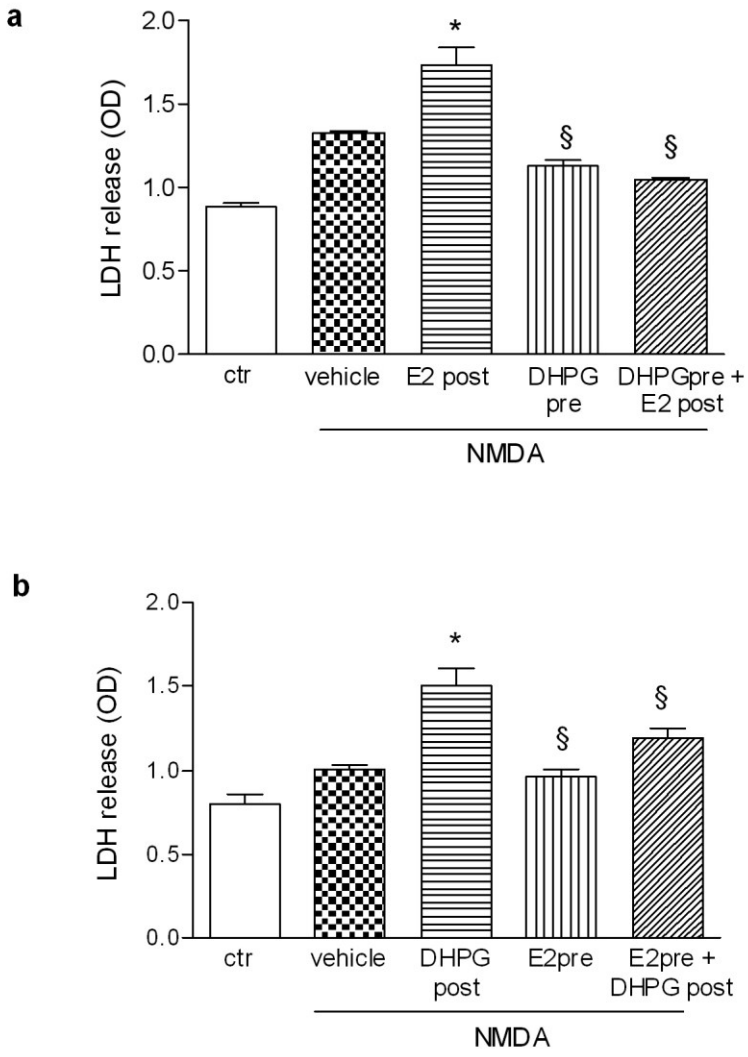
Figure 3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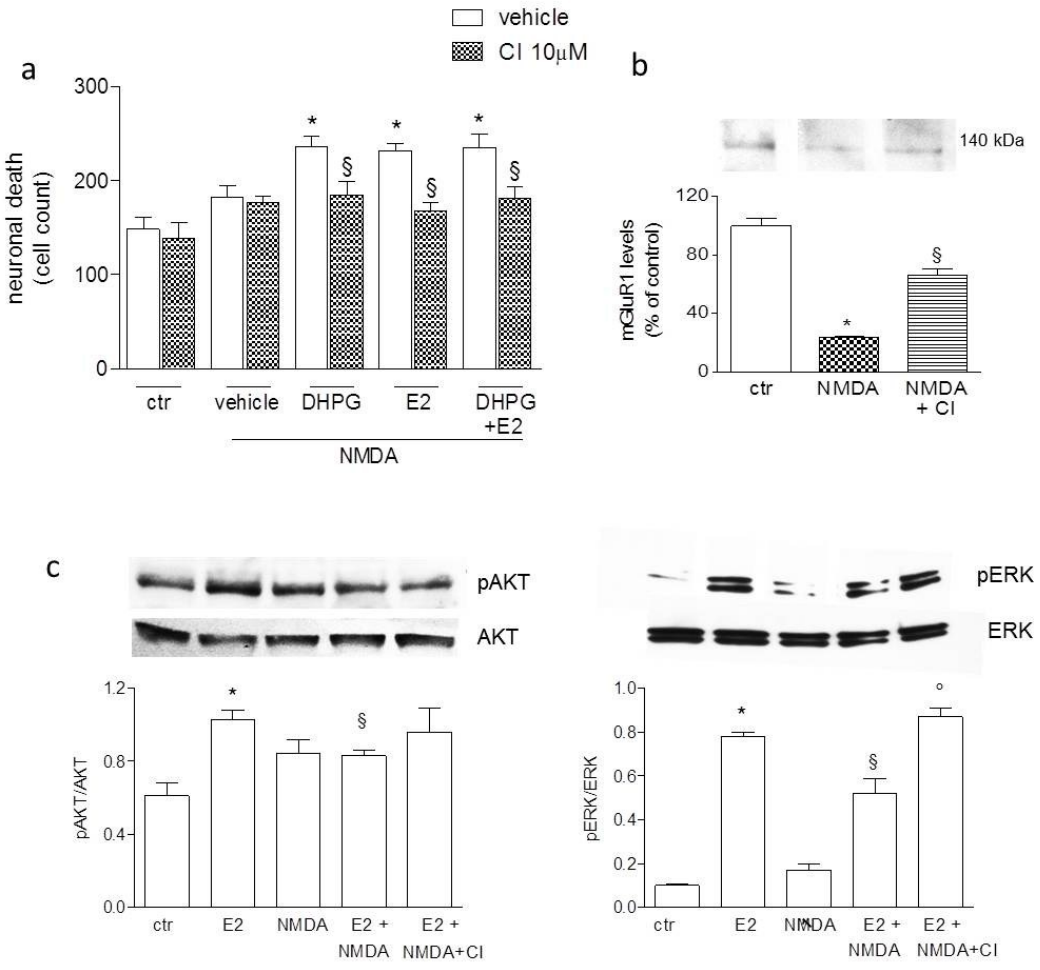
NMDA

Figure 4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NMDA
b
140 kDa
mGluR1 levels (% of control)
120
80
40
0
ctr
NMDA
NMDA + CI
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pAKT
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pERK
ERK
pAKT/AKT
1.2
0.8
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Figure 5

General discussion and conclusions

Although over the past years life expectancy is increased, this improvement has not been followed by a decrease in the rate of disability before death, especially due to the growing development of neurodegenerative diseases. Hence, the development of strategies to prevent and/or to delay their onset has become a priority in public healthcare. In this regard, attention has been paid to the effects of estrogen. Evidence demonstrates in fact the important role of estrogen in CNS in both development and neuroprotection in physiological and pathological conditions; moreover several pathologies in women start after menopause, when estrogen levels decline. These observations led to predict a promising use of estrogen in neurodegenerative diseases, supported also by the large body of preclinical in vitro and in vivo data showing a reduction in neurodegeneration and cognitive decline after estrogen treatment. However, these data were not confirmed by clinical trials. In particular the WHI study pointed out that ERT is not able to prevent neurodegenerative diseases, but it may rather worsen cognitive decline, if treatment is started after 65. Following the publication of the WHI results, the dual role of estrogen has been largely investigated, and several hypotheses to explain its role in neuroprotection/neurodegeneration have been proposed.

One of the factors that seems to be critical is the timing of estrogen therapy. If the treatment takes place early, just after menopause, it exerts neuroprotective activity, while if neuronal damage has already started, estrogen could be responsible for increased toxicity.

The dual effect of estrogen resembles the activity exerted by the stimulation of mGluR1 that is, as well, responsible for both neuroprotection and neurodegeneration. Although the real mechanism responsible for its dual

role is not fully understood, it may depend on a conformational modification of the receptor, able to activate different intracellular pathways.

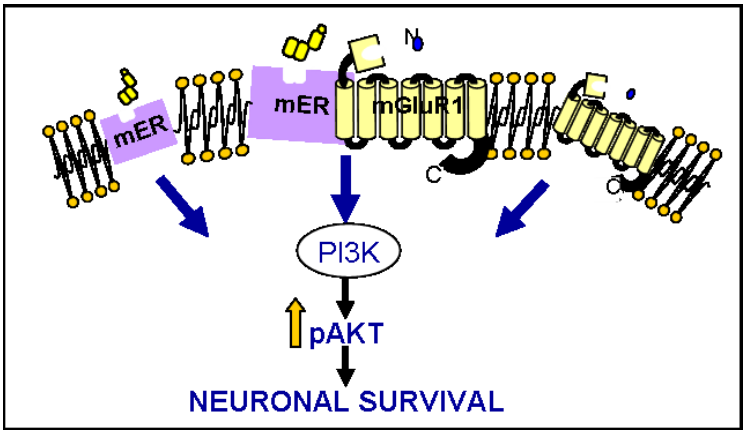
In basal conditions mGluR1, coupled to a Gq protein, activates PLC and stimulates the accumulation of inositol trisphosphate and the release of intracellular calcium. Through its C terminal tail, mGluR1 is also linked to Akt pathway, whose phosphorylation is linked to neuroprotection. The release of massive calcium concentrations in the cells, as a consequence of the activity of NMDA receptor, may be responsible for the cleavage of mGluR1's C tail, so that the receptor modifies its signaling.

As it has been demonstrated that mGluRs interact with membrane ERs in the brain, being involved in functions such as the control of sexual behaviour or mechanisms of pain perception, we wondered whether this interaction could be also responsible for the dual activity of estrogen in neuroprotection/neurodegeneration.

Two different in vitro models have been used to achieve a basal conditions of healthy neurons pretreated with estrogen and then exposed to Aβ challenge and a condition in which neurons were first exposed to a mild excitotoxic insult and then treated with estrogen. Using these two experimental paradigms we have tried to mimic an early pre-treatment with estrogen on one side and a late intervention with estrogen, when a mild neuronal damage is already present.

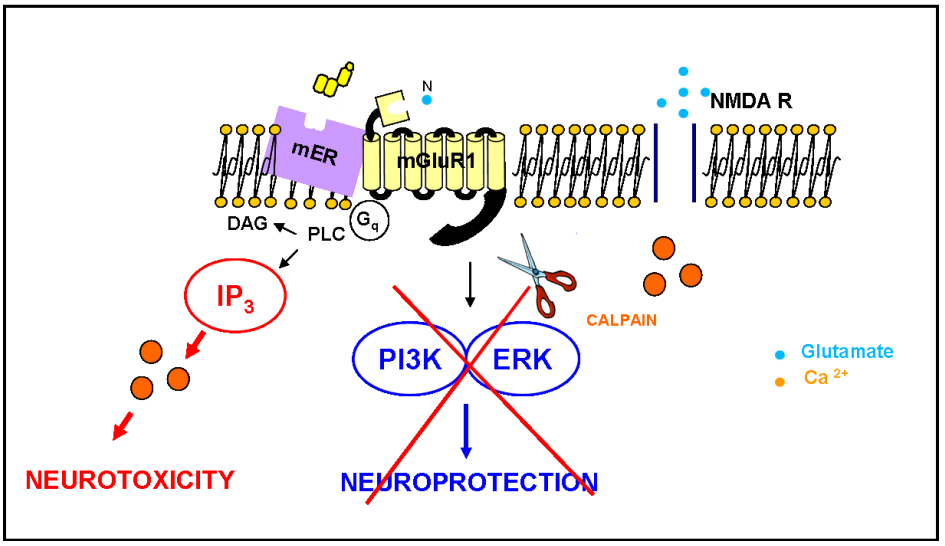
The results obtained indicate that under both conditions, membrane ERs couple to mGluR1and the signalling of this receptor brings about the final outcome. Under basal conditions, in fact, activation of mERα with estrogen causes its coupling to mGluR1 and stimulation of PLC as well as of the PI3K/AKT pathway with a resulting neuroprotective effect (Fig. 3).

Figure 3



In contrast, if estrogen stimulates its receptor when a slight neuronal damage is already present (as in the case of exposure to low concentrations of NMDA), ER couples to mGluR1, that can signal only through the PLC pathway, but is not able anymore to initiate the PI3K/AKT or ERK neuroprotective signalling (Fig. 4).

Figure 4



In conclusions, our results confirm that interaction between ER and mGluR1 is involved in the action of estrogen at the CNS and controls also mechanism of neuroprotection – neurodegeneration. Specifically, it is the status of mGluR1 and its ability to activate intracellular pathways that determines the response to estrogen.

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