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Roles of Estrogen Receptors Alpha and Beta in sexually dimorphic neuroprotection against glutamate toxicity

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Abstract

Although most agree that 17 β -estradiol is neuroprotective via a variety of mechanisms, less is known about the role that biological sex plays in receptor-mediated estradiol neuroprotection. To address this issue we isolated primary cortical neurons from rat pups sorted by sex and assessed the ability of estradiol to protect the neurons from death induced by glutamate. Five minute pretreatment with 10 to 50nM 17 β -estradiol protected female but not male neurons from glutamate toxicity 24 hours later. Both estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) are expressed in these cultures. Experiments using an ER α selective agonist or antagonist indicate that this receptor is important for neuroprotection in female cortical neurons. The ER β selective agonist conveys a small degree of neuroprotection to both male and female cortical neurons. Interestingly, we found that 17 α estradiol and the novel membrane estrogen receptor (mER) agonist STX, but not bovine serum albumin conjugated estradiol or the GPR30 agonist G1 were neuroprotective in both male and female neurons. Taken together these data highlight a role for ER α in sexually dimorphic neuroprotection.

Keywords

neuroprotection; sexually dimorphic; estrogen receptor alpha; estrogen receptor beta; primary cortical neuron; glutamate

Introduction

It is well known that 17 β - estradiol has protective effects in the brain. Early work demonstrated that estradiol increased viability in neuronal and glial cell lines (Bishop and Simpkins, 1994). Estradiol neuroprotection was subsequently shown to occur via estrogen receptor (ER) independent antioxidant (Behl et al., 1995) or ER-dependent mechanisms (Singer et al., 1996; Singer et al., 1999). Work from this laboratory further demonstrated that ER-mediated neuroprotection in primary cortical neurons involves the rapid stimulation of the src-protein tyrosine kinase (PTK)-mitogen activated protein kinase (MAPK) pathway (Singer et al., 1999). More recently, it has been shown that ER α and ER β can mediate neuroprotection against beta amyloid toxicity and glutamate mediated oxidative stress

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(Fitzpatrick et al., 2002; Mize et al., 2003). In the intervening years, ER-mediated cellular responses to estradiol have been loosely grouped into two interconnected categories: genomic and rapid non-genomic signaling (Bjornstrom and Sjoberg, 2005; McEwen, 2001). Presently, it appears that both rapid non-genomic and genomic responses to estrogen are required for estrogen's trophic and protective effects.

Despite this progress in understanding the cellular mechanisms of estradiol neuroprotection (Behl et al., 1995; Cordey and Pike, 2005; Fitzpatrick et al., 2002; Green et al., 1996; Heyer et al., 2005; Marin et al., 2003; McEwen, 2001; Mize et al., 2003; Nilsen et al., 2002; Singer et al., 1999; Singer et al., 1996; Wu et al., 2005; Xia et al., 2009; Zhao and Brinton, 2007; Zhao et al., 2005; Zhao et al., 2004), one issue has been largely ignored until recent years. What role does biological sex play in estrogen neuroprotection? Accumulating *in vitro* evidence suggests that the vulnerability to neurological insult is sexually dimorphic. Male cortical neurons are reported to be more susceptible to nitrosative stress and excitotoxicity while female cortical neurons are more susceptible to agents that induce apoptosis (Du et al., 2004). Male hippocampal neurons have been reported to be more susceptible to hypoxia than female hippocampal neurons (Heyer et al., 2005). Male organotypic hippocampal cultures are more susceptible to NMDA excitotoxicity or oxygen glucose deprivation than female counterparts (Li et al., 2005). Finally, intact female rats sustain less ischemic injury than ovariectomized female or male counterparts (Alkayed et al., 1998). In light of these data it is reasonable to hypothesize that the protective effects of estradiol are sexually dimorphic.

In the present study we used primary cortical neurons sorted by biological sex to determine if estradiol neuroprotection against glutamate toxicity is sexually dimorphic. Our data indicate that estradiol protects neurons isolated from female but not male rat pups from glutamate toxicity. We provide evidence that the ER α selective ligand PPT provides dose dependent neuroprotection in female but not male cortical neurons. In contrast, the ER β selective agonist DPN conveys a small degree of neuroprotection in females and male neurons. Similarly, 17 α - estradiol and the estrogenic agent STX are neuroprotective in both male and female neurons. Taken together these data highlight a role for ER α in sexually dimorphic neuroprotection.

Methods

Primary Neuron Culture

The experiments described in this report conformed to guidelines established by the National Institutes of Health and Oregon Health & Science University for the humane treatment of animals. Primary cultures of cortical neurons were prepared from Sprague Dawley rat pups on embryonic day 19 (E19) as described in Brewer et al. (Brewer and Price, 1996; Brewer et al., 1993). Briefly, brains were sorted by biological sex (presence of testes) and dissected in Hibernate-E media (Brain Bits Inc, Springfield, Ill) containing B27 supplement. Hippocampi and olfactory bulbs were removed from cortices and discarded. Cortices were then incubated in Papain (20units/ml) in Hibernate-E minus calcium for 30 minutes at room temperature. Neurons were subsequently dissociated by manual trituration in Hibernate/B27. Neurons were counted and plated (approximately 330,000-360,000 per well) on poly-d-lysine coated glass cover slips in 12 well plates in "complete" Neurobasal media containing 2% B27, 1% Glutamax and penicillin-streptomycin (Invitrogen, Carlsbad California). Cultures were maintained in a 5% CO₂ atmosphere for 10 days *in vitro* (DIV) in a humidified incubator. Unless otherwise indicated, experiments were performed on 10-11 DIV.

Western Blot

Cultured neurons were scraped from 150 mm culture dishes (Corning) into ice cold Immunoprecipitation Buffer containing 1% triton and protease inhibitors (Bryant et al., 2005). Protein concentration was determined for each whole cell lysate using a commercially available BCA Assay (Pierce). After adding sample loading buffer, lysates were resolved using SDS-Page. Membranes were immunoblotted as described (Bryant et al., 2005) using antibodies against ER α (6F11, Novacastra), ER β (PA1-310B, Thermo) and beta actin (Sigma). Densitometry was performed using software on a UVP EpiChemi Darkroom machine as described in (Bryant et al., 2005).

Chemicals

17 β - Estradiol and 17 α - estradiol were purchased from Steraloids. ICI 182,780(ICI), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenyl]-1H-pyrazole dihydrochloride (MPP dihydrochloride or MPP), Propylpyrazole-triol (PPT), Diarylpropionitrile (DPN), R, R-Tetrahydrochrysen (R, R-THC) and ($\pm$)-1-[(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl) - 3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]- ethanone (G1) were purchased from Tocris Bioscience (Ellisville, Mo). Bovine serum albumin (BSA) conjugated estradiol was purchased from Steraloids (Newport RI). STX was kindly provided by Drs. Tom Scanlan and Martin J. Kelly, Department of Physiology and Pharmacology, Oregon Health & Science University.

Live Dead Assay

All stock solutions (except glutamate and STX, G1 and BSA conjugated estradiol) were made in 95 % ethanol. STX and G1 were dissolved in DMSO. BSA conjugated estradiol was dissolved in 50mM Tris pH 8.5. Free estradiol was washed away using a Microcon 3kd centrifugal filter device (Millipore, Bedford MA) as described in (Stavis et al., 1999). Stock solutions were diluted in buffer "G" containing 2 mM KCl, 1mM MgSO₄, 2.5mM CaCl₂, 1mM NaH₂PO₄, 4.2 mM NaHCO₃, 12.5mM Hepes, 10mM glucose, 0.1 M NaCl (Singer et al., 1996) prior to treatment. L-Glutamate was dissolved in buffer G and diluted further in the same buffer. All treatments were performed on at 10DIV. In experiments using ER ligands (17 β -estradiol, 17 α - estradiol, DPN, PPT, R, R-THC, STX G1, BSA-estradiol), neurons were exposed to vehicle or ER ligand for 5 minutes followed by a 5 min exposure to glutamate. After a total of 10 minutes, neurons received one wash of complete neurobasal media. Fresh media was placed on neurons and they were returned to the incubator. In experiments using ER "antagonists" (ICI or MPP), neurons were pretreated for 15 minutes with the antagonist or vehicle followed by a 5 minute ER ligand or vehicle exposure followed by a 5 minute glutamate exposure. Neurons were thus exposed to the antagonist for a total of 25 minutes. The neurons received one media wash and then were returned to the incubator in fresh complete media. Twenty four hours after treatment (11DIV), neurons were stained using the commercially available Live/Dead (Invitrogen) assay using manufacturer's instructions. Live cells were stained fluorescent green by Calcein AM, which stains esterases in living cells. Dead cells are stained fluorescent red by the ethidium bromide homodimer. Cover slips were placed on microscope slides and examined using FITC and TRITC filters on a Zeiss Axioskop FS microscope. Three images (TIFFs) were captured from each cover slip. Three cover slips were sampled for each experimental condition. A minimum of three platings (biological replicates) was used for each experiment. Percent survival (death) was calculated by dividing the number of live (dead) cells by the total number of cells. All data was normalized to the mean of vehicle (or glutamate, in the case of percent death) treated cells. A two way (Sex by Treatment) ANOVA was calculated followed by post-hoc Bonferroni t-tests for each experiment. ANOVAs were always statistically significant due to the glutamate mediated cell death.

Unless otherwise indicated, we only report post-hoc t-tests comparing glutamate to ER ligand + glutamate (i.e. neuroprotection).

Results

Five minute pre-exposure to estradiol (50nM) protected female but not male neurons from glutamate (5mM) toxicity (***)Bonferroni post hoc $t_{(female\ glutamate\ vs.\ female\ estradiol+ glutamate)}=3.911, p<0.001$ Figure 1). Since susceptibility to glutamate/glycine toxicity has been reported to be sexually dimorphic (Du et al., 2004), we wanted to determine if there was a sex difference in neuronal survival 24 hours after a 5 minute glutamate exposure. We did not observe sexually dimorphic susceptibility to glutamate toxicity in the absence (Figure 2) or presence (data not shown) of glycine in our experimental paradigm. In order to determine whether the observed sexually dimorphic neuroprotection involved ER activation, male and female neurons were pre-exposed to vehicle (ethanol) or ICI 182,780 (1uM), for 15 minutes, followed by a 5 minute exposure to estradiol (10nM) and a subsequent 5 minute glutamate (5mM) exposure. We observed neuroprotection in estradiol treated female neurons but not male neurons (Figure 3a) (***)Bonferroni post hoc $t_{(glutamate\ vs.\ estradiol+ glutamate)}=4.977, p<0.001$ in vehicle pretreated neurons. Pre-exposure to ICI, blocked neuroprotection in female neurons and had no effect on male neuron survival (Figure 3b). Although ICI can have SERM-like effects, it has historically been used as an ER antagonist that implicates an ER α / β mediated response. Immunoblots of whole cell lysates (Figure 4) demonstrated that at 10DIV, ER α content of female neurons was greater than that male neurons (* $t=4.773, p=0.0412$). In contrast to ER α , there was no sex difference in ER β expression.

Given the presence of both ERs in our cultures, pharmacological probes were used to assess the role of each receptor in neuroprotection. Neurons were pre-exposed to the ER α agonist propylpyrazole-triol (PPT) or the ER β agonist diarylpropionitrile (DPN) for 5 minutes prior to a 5 minute glutamate exposure. The initial doses of PPT (10nM) and DPN (1nM) were chosen based on demonstrated transcriptional activation of estrogen response elements (EREs) via the target receptor but not the counterpart (Harrington et al., 2003). Female neurons exposed to PPT exhibited statistically significant neuroprotection ($t=2.610, p<0.05$ Figure 5a). PPT had no effect on male survival. In order to further evaluate the role of PPT in sexually dimorphic neuroprotection, we tested a range of doses, including 10nM, 1nM and .1nM. A two way (Sex X Treatment) analysis of variance indicated that there was an effect of Sex on PPT neuroprotection ($F=6.11, p=0.02$, Figure 5b). Bonferroni post-hoc tests indicate that PPT was neuroprotective at 10nM ($t=2.42, p<0.05$) and 1nM ($t=6.96, p<0.05$) but not 0.01nM in female neurons. At a 1nM concentration, DPN did not protect female or male neurons (Figure 5c). However, a follow up experiment examining a range of doses from 1nM to 0.01nM indicated that there was a modest overall effect of treatment in DPN exposed neurons ($F=4.02, p=0.01$). Post-hoc comparisons using the Bonferroni post test were not statistically significant. In order to further investigate the role of ER α in neuroprotection in female neurons we pretreated male and female neurons with the ER α antagonist MPP dihydrochloride (MPP, 1 uM) for 15 minutes prior to exposure to E2 and glutamate. MPP blocked E2 neuroprotection in female neurons (Figure 6). Thus, although our data suggests that the sex difference in estrogen neuroprotection is primarily ER α dependent, ER β has a role in neuroprotection.

Since other ER candidates have been postulated in recent years, we assayed neuroprotection using ligands reported to be selective for these ER candidates. Neurons were exposed to STX, a novel diphenylacrylamide compound that has 100,000 fold reduced affinity for ER α and ER β (Tobias et al., 2006). Five minute STX (10nM) pretreatment protected both male (***)Bonferroni post hoc $t_{(glutamate\ vs.\ STX+ glutamate)}=2.557, p<0.05$) and female (*Bonferroni

post hoc $t_{(\text{glutamate vs. STX+ glutamate})} = 3.076$, $p < 0.05$) neurons from glutamate toxicity (Figure 7a). Since GPR30 has been reported to be a membrane estrogen receptor (Filardo et al., 2000; Revankar et al., 2005; Thomas et al., 2005), we evaluated the neuroprotective effect of the selective non-steroidal GPR30 agonist G1 on neuroprotection in female and male neurons. G1 was not protective in male or female neurons (Figure 7b). We also evaluated the neuroprotective effects of 17α -estradiol and bovine serum conjugated estradiol. 17α -estradiol was neuroprotective in both male (post hoc $t_{\text{glutamate vs } 17\alpha\text{-estradiol + glutamate}} = 3.507$, $p < 0.01$) and female (post hoc $t_{\text{glutamate+ } 17\alpha\text{-estradiol + glutamate}} = 3.18$, $p < 0.05$) neurons (Figure 7c). Finally, a 5 minute pre-exposure to bsa conjugated estradiol did not protect against glutamate toxicity (Figure 7d).

Discussion

ER α activation is important for neuroprotection in female cortical neurons

Taken together, the data from this study indicate that in E19 isolated primary cortical neurons, estradiol neuroprotection against glutamate toxicity is sexually dimorphic. Furthermore, this sexual dimorphism is mostly mediated by ER α in rat female cortical neurons. The ER β selective agonist, DPN provides some degree of neuroprotection in both male and female neurons. Similarly, 17α -estradiol and STX are also neuroprotective in both sexes. Sex segregation of primary cortical neuron cultures has allowed us to dissociate the neuroprotective effectiveness of PPT and DPN. The implication of these data is that there is a “sex by receptor” interaction underlying ER mediated neuroprotection in cortical neurons. Our findings are consistent with the literature, which highlight a role for ER α in estradiol neuroprotection in female mice and rats. Dubal et al., (Dubal et al., 2001) demonstrated that ER α is required for neuroprotection against delayed cell death after permanent middle cerebral artery occlusion (MCAO). In their studies, estradiol protected ER β KO mice but not ER α KO mice 24 hours after the onset of MCAO. They also observed an induction of ER α mRNA and protein in the cortex of MCAO treated estradiol replaced female rats (Dubal et al., 2006). We also observe more ER α expression in whole cell lysates from rat female neurons than male neurons at 10DIV. Estradiol is also cardioprotective in female rats against myocardial ischemia via an ER α -mediated mechanism (Jeanes et al., 2008). The ER α agonist ERA-45 was cardioprotective while the ER β antagonist failed to block estrogen cardioprotection, further highlighting a role for ER α in estradiol protection against insult. In contrast to these findings, data from other studies did not support the idea that ER α is protective against transient MCAO (Sampei et al., 2000). This may be due to methodological differences such as permanent (Dubal et al., 2006; Dubal et al., 2001) versus transient MCAO (Sampei et al., 2000) and the use of intact animals in the later study compared to ovariectomized and/or estradiol replaced animals in the prior studies.

ER β conveys some degree of neuroprotection in male and female neurons

Although our data indicate that ER α has a prominent role in sexually dimorphic neuroprotection, it appears that ER β is modestly neuroprotective in both sexes. Our findings contrast with Xia et al., (Xia et al., 2009) who did not observe neuroprotection in mixed primary cortical neurons exposed to 0.1 nM PPT or 0.1nM DPN (co-administration or 24 hour pretreatment) 18 hours after glutamate exposure. The lack of protection may be due to ligand-dependent ER down-regulation. For example, ER α expression is down-regulated in human aortic smooth muscle cells after 48 hour exposure to Estradiol, PPT, and DPN (Barchiesi et al., 2004). This data, along with our own highlight the interaction of ligand exposure time and ER expression levels on ER mediated responses. Consistent with our findings, ER β has been shown to be protective in males under other experimental conditions. Estradiol and the ER β selective agonist DPN were neuroprotective against transient global ischemia resultant from cardiac arrest/cardiopulmonary resuscitation (CA/CPR) in the

hippocampal CA1, caudate putamen of male mice (Noppens et al., 2009). Conversely, the selective ER α agonist, PPT was not protective in the caudate putamen or the CA1 field of the hippocampus. Taken together these data indicate that ER mediated sexually dimorphic neuroprotection can vary from one brain structure to the next.

Despite differences between our experimental protocol and that of published reports, consistent themes do emerge. Cordey and Pike (Cordey and Pike, 2005) used mixed (male and female) cortical neurons to determine which ER was important for neuroprotection against beta amyloid (A β) toxicity. Neurons were exposed to ER ligands for 1 hour prior to a 24 hour A β exposure. They observed ICI sensitive neuroprotection with estradiol, 17 α -estradiol, PPT and DPN. Furthermore, PKC activation was required for neuroprotection via all of these ligands except DPN (Cordey and Pike, 2005). Our data is consistent with these findings in that 17 α -estradiol is neuroprotective and the response to DPN is not identical to that of via estradiol or PPT. Our data is somewhat consistent with that of Zhao et al., (Zhao et al., 2004), who demonstrated that ER α and ER β activation is important for neuroprotection against glutamate toxicity in mixed primary hippocampal neurons. Neuroprotection was most robust in the picomolar range which minimally activates several transcription factors including ERE, sodium – hydrogen exchanger regulatory factor ezrin-radixin-moesin binding protein 50 (NHE-RF/EBP50), and TGF β after 24 hours (Harrington et al., 2003). Although our current findings are generally consistent with (Zhao et al., 2004) and prior work in this laboratory (Fitzpatrick et al., 2002; Mize et al., 2003), we find that sexually dimorphic neuroprotection appears to be due solely to ER α .

Sexually dimorphic neuroprotection does not involve novel ER candidates

Our data suggest that sexually dimorphic neuroprotection against glutamate toxicity is a consequence of ER α and not other ER candidates. Several candidate estrogen receptors have been postulated, including the STX-responsive membrane estrogen receptor (Qiu et al., 2003; Qiu et al., 2006), ER"X" (Toran-Allerand et al., 2002) and GPR30 (Filardo et al., 2000; Revankar et al., 2005; Thomas et al., 2005). We find that STX is neuroprotective in both male and female neurons. Our data is consistent with previously reported STX neuroprotection, *in vivo*. It protects neurons in the CA1 field of the female rat hippocampus from transient global ischemia (Lebesgue et al., 2010). STX is a diphenylacrylamide compound (Tobias et al., 2006) that is known to stimulate rapid, estradiol-sensitive, non-genomic responses such as, phospholipase C and protein kinase A and protein kinase C, MAPK and PI3K (Qiu et al., 2003; Qiu et al., 2006; Lin et al., 2009). STX has a 100,000-fold reduced affinity for ER α and ER β compared to estradiol (Tobias et al., 2006) and alters gene expression (Roepke et al., 2008) but it is not uterotrophic *in vivo* (Qiu et al., 2003). Furthermore, STX does not stimulate or block ERE transcription via ER α or ER β (Lin et al., 2009). Although direct STX binding to GPR30 has not been demonstrated, recently published data suggests that GPR30 could be involved in STX stimulated non-genomic and genomic signaling. STX stimulates the phosphorylation of the estrogen steroidogenic factor 1(SF-1) via MAPK and PI3K. (Lin et al., 2009). SiRNA knock down of GPR30 blocks estradiol and STX stimulated aromatase (*Cyp19a1*) transcription (Lin et al., 2009). The idea that GPR30 is a *bona fide* estrogen receptor is controversial as the evidence for estradiol binding for GPR30 has been called into question (Langer et al., 2009). Finally, our data are consistent with the idea that GPR30 and the STX responsive membrane estrogen receptor are not the same vis-à-vis neuroprotection given the lack of G1 neuroprotection in this experimental paradigm.

Although 17 α - estradiol has historically been considered an inactive isomer, there is evidence that it is neuroprotective in HT22 cells (Behl et al., 1997) and SK-N-SH cells (Green et al 1997). At the time it was postulated that 17 α - estradiol worked via an estrogen receptor independent mechanism. In contrast to this early data, 17 α -estradiol has more

recently been postulated as a selective ligand for the ER candidate ERX. Evidence has been published supporting the idea that 17 α -estradiol activates MAPK signaling via developmentally regulated, caveolae localized ERX (Toran-Allerand et al., 2002). Our data are consistent with early work which indicates that 17 α -estradiol is a neuroprotective steroid. Our data also imply that this ligand might operate via a mechanism that is independent of ER α , at least in male neurons. Finally, we did not observe neuroprotection in neurons exposed to bovine serum albumin conjugated estradiol. This data implies that sexually dimorphic neuroprotection involves primarily an ER α mediated transcriptional response.

We did not observe sexually dimorphic susceptibility to glutamate toxicity in the absence of presence (data not shown) of glycine in our experimental paradigm. Our findings contrast somewhat with Du et al, (Du et al., 2004), who reported that male neurons were more susceptible to glutamate/glycine excitotoxicity than female neurons. In their excitotoxicity experiments, neurons were exposed to glutamate/glycine for 24 hours prior to examining cell viability. However, our findings are consistent with Du et al (Du et al., 2004) in that we observe estradiol neuroprotection in female neurons but not male neurons using a 10 or 50nM dose..

Conclusions

Taken together, our data indicate that estradiol neuroprotection is sexually dimorphic in 10-11DIV neurons. This sex difference appears to involve ER α and not ER β and is correlated with increased ER α expression in female neurons. Ligands selective for ER β , the STX sensitive membrane receptor and ERX are also neuroprotective in both sexes, suggesting that (non) genomic responses downstream of these receptor candidates are not identical to that of ER α . Ongoing work is aimed at examining sexually dimorphic downstream signaling responses to estradiol.

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Figure 1.

50nM estradiol protects female but not male neurons from glutamate toxicity. At 10DIV, male and female primary cortical neurons were exposed to 0.005% ethanol or 50nM estradiol for 5 minutes prior to a five minute exposure to 5mM glutamate. After 1 wash in complete Neurobasal media, cells received fresh media and were returned to the incubator for 24 hours. **A.** Representative images from the Live/Dead assay (Invitrogen). Living neurons are stained green by Calcein AM. Dead cells are stained red by the ethidium bromide homodimer. **B.** 5 minute pre-exposure to 50nM estradiol protects female neurons (***) Bonferroni $t_{(\text{glutamate vs. estradiol+ glutamate})} = 3.911$, $p < 0.001$) but not male neurons from glutamate toxicity ($n=5$).

Figure 2.

Glutamate toxicity is not sexually dimorphic in our experimental protocol. Male and female neurons were exposed to varying doses (.5mM, 1mM and 5mM) for 5 minutes. Neurons were handled as described in figure 1 and the methods section. There was no sex difference in percent survival (n=3).

Figure 3.

ICI 182, 780 blocks estrogen neuroprotection in female neurons. Male and female neurons were exposed to (A) 0.01% ethanol or (B) 1uM ICI for 15 minutes prior to a 5 minute exposure to 0.001% ethanol or 10nM estradiol. Neurons were subsequently exposed to 5mM glutamate for 5 minutes. (A) Estradiol (10 nM) protected female neurons but not male neurons from glutamate toxicity in ethanol pretreated neurons (***Bonferroni post hoc t (glutamate vs. estradiol+ glutamate) =4.977, $p < 0.001$, $n=4$). (B) ICI pretreatment blocked estradiol protection in female neurons ($n=4$).

Figure 4.

Female neurons contain more immunoreactive ERα than male neurons at 10DIV. Whole cell lysates from female and male neurons were immunoblotted using antibodies against ERα (6F11, Novacastra) ERβ (PA1-310B, Thermo) and beta actin (Sigma). Densitometric analysis revealed that there was a statistically significant difference in the ratio of ERα to beta actin (* $t=4.773$, $p=0.0412$, $n=2$) between female and male neurons.

Figure 5.

The ER α selective ligand PPT is neuroprotective in female neurons only. Male and female neurons treated with the ER α selective ligand PPT (**A**, **B**) or the ER β selective ligand DPN (**C**, **D**) for 5 minutes prior to a 5 minute glutamate exposure. (**A**) PPT was neuroprotective in female neurons Bonferroni t (glutamate vs. ppt+ glutamate)=2.610, $p<0.05$, $n=3$) but not male neurons. (**B**) PPT was neuroprotective in female neurons at 10nM ($t=2.42$, $p<0.05$) and 1nM ($t=6.96$, $p<0.05$) but not 0.01nM concentrations. (**C**) At a 1 nM concentration, DPN did not protect male or female neurons ($n=4$). (**D**) Neurons were exposed to three concentrations of (1nM, 0.1nM and 0.01nM) of dPN. A two way (Sex X Treatment) ANOVA indicated that there was an overall effect of treatment in neurons exposed to DPN ($F=4.02$, $p=0.01$). Post hoc tests were not statistically significant, however.

Figure 6.

The selective ER α antagonist MPP blocks estradiol neuroprotection.

(A) Male and female neurons were pretreated with 1 μ M MPP dihydrochloride for 15 minutes prior to exposure to estradiol and glutamate. Estradiol was not protective in female neurons under these conditions (n=4).

Figure 7.

(A) STX and 17 α -estradiol protect male and female neurons. Neurons were exposed to 10 nM STX for 5 minutes prior to a 5 minute exposure to 5mM glutamate. STX protected both male (*Bonferroni post hoc $t_{\text{(glutamate vs. STX+ glutamate)}}=2.557, p<0.05, n=3$) and female (*Bonferroni post hoc $t_{\text{(glutamate vs. STX+ glutamate)}}=3.076, p<0.05, n=3$) neurons from glutamate toxicity. (7b) Neurons were exposed to 10nM of the non-steroidal GPR30 agonist G1 for 5 minutes prior to exposure to glutamate. G1 was not neuroprotective ($n=3$) (7c) Neurons were exposed to 10 nM of 17 α estradiol for 5 minutes prior to a 5 minute exposure to 5mM glutamate. 17 α - estradiol is neuroprotective in male (post hoc $t_{\text{glutamate vs 17}\alpha\text{- estradiol+glutamate}}=3.507, p<0.01$) and female (post hoc $t_{\text{glutamate vs 17}\alpha\text{-estradiol+glutamate}}=3.18, p<0.05$) neurons ($n=3$). (7d) Neurons pre-exposed to 10nM bovine serum albumin conjugated estradiol for 5 minutes were not protected from glutamate toxicity ($n=3$).