

ESTROGEN RECEPTOR ALPHA AND G-PROTEIN COUPLED RECEPTOR 30 MEDIATE THE NEUROPROTECTIVE EFFECTS OF 17 β -ESTRADIOL IN NOVEL MURINE HIPPOCAMPAL CELL MODELS

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Abstract—The hippocampus is a multifaceted, complex brain structure considered to be the learning center. The use of primary hippocampal cell cultures has uncovered important cellular mechanisms involved in overall physiological function. Yet, the use of primary culture is inherently difficult, and the lack of immortalized cell lines from the murine hippocampus for mechanistic studies at the molecular level is evident. We have immortalized cell lines from embryonic (E18) and adult-derived hippocampal primary cell culture using retroviral infection of SV40 T-antigen. Four clonal embryonic lines, mHippoE-2, mHippoE-5, mHippoE-14, mHippoE-18, and one mixed adult line, mHippoA-mix, exhibited neuronal morphologies with neurite extensions and expression of neuronal markers, with unique gene expression profiles. We used these cell models to study the neuroprotective effects of 17 β -estradiol (E2) on glutamate-induced neurotoxicity. The cell lines express a relevant array of genes and receptors suggested to play a role in neuroprotection, including estrogen receptors ER α , ER β , and GPR30. We find that pretreat-

ment with E2 (10 or 100 nM) for 24 h significantly reduced cell death induced by glutamate mHippoE-14 and mHippoE-18 cells, but not the mHippoA-mix. Using 24 h pretreatment with the specific estrogen receptor (ER) agonists, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and diarylpropionitrile, 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), we linked the E2-mediated neuroprotection to ER α , but only in the mHippoE-18 cells. Since E2 activated both PI3K/Akt and STAT3 signaling pathways, we also tested whether the membrane-bound E2 receptor GPR30 was involved in its neuroprotective action. Pretreatment with the GPR30 agonist G-1 (10 and 100 nM) for 1 h, but not 24 h, significantly attenuated cell death in both mHippoE-14 and mHippoE-18 cells. The use of specific ER antagonist ICI 182780 and GPR30 antagonist G-15 linked these effects to both ER and GPR30 receptors. This is the first evidence that GPR30 may play a role in the protective effects of estrogen in hippocampal neurons. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: glutamate, immortalization, cell biology, signal transduction, hippocampus, membrane estrogen receptor.

The hippocampus is a complex structure most commonly known for its role in learning and memory. The hippocampal formation consists of CA1, CA3, and the dentate gyrus regions, and is comprised of distinct neuronal cell types including pyramidal neurons, interneurons, and granule cells. The hippocampus has been extensively targeted *in vivo* to study spatial and non-spatial memory, long-term potentiation, adult neurogenesis, neurodegeneration, and neurodegenerative diseases, such as Alzheimer's. In many studies, primary hippocampal E18 cultures have been used as a cell model for analysis of hippocampal cell function, however non-transformed primary cultures are difficult to maintain, have a short life-span and represent a heterogeneous neuronal and glial cell population, often with an inconsistent number of viable neurons. Immortalized hippocampal cell lines are scarce, and are primarily derived from embryonic cultures. A number of rat hippocampal cell lines were generated previously (Eves et al., 1992), including the H19-7 neuronal line. Further a rat adult neuronal progenitor cell line HC2S2 has been used to study neuronal differentiation (Hoshimaru et al., 1996). The HT4 neuroblastoma cell line was derived from the hippocampal neurons of mice (Lendahl and McKay, 1990), and a number of subclones from this parental line, including HT22, have been used for further studies (Morimoto and Koshland, 1990), among many others. However, these few lines do not represent the extensive population of heterogeneous cell types from the hippocampus and it is clear that new models are required to fully understand hippocampal cell biology.

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Abbreviations: Akt, homologous enzyme sequence of viral oncogene v-akt isolated from AKR mouse spontaneous thymoma cell line and also called protein kinase B; AR, androgen receptor; BDNF, brain derived neurotrophic factor; CREB, cAMP response element-binding protein; DPN, diarylpropionitrile, 2,3-bis(4-Hydroxyphenyl)-propionitrile; E2, 17 β -estradiol; E18, embryonic day 18; ER, estrogen receptor; ER α , estrogen receptor α ; ER β , estrogen receptor β ; ERK1/2, extracellular related kinase; FBS, fetal bovine serum; G-1, (\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; G-15, (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline; GFAP, glial fibrillary acidic protein; GHSR, ghrelin/growth hormone secretagogue receptor; GPR30, G protein-coupled receptor; ICI 182780, 7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; IR, insulin receptor; MAPK, mitogen activated protein kinase; MTT, methylthiazolyl-diphenyl-tetrazolium bromide; NPY, neuropeptide Y; NSE, neuron specific enolase; ObRb, leptin receptor; PI3-K, phosphatidylinositol 3-kinase; PPT, 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; RT-PCR, reverse transcriptase PCR; SSTY1, spermiogenesis specific transcript on the Y; STAT3, signal transducer and activator of transcription 3; TrkA, neurotrophic tyrosine kinase, receptor, type 1; TrkB, neurotrophic tyrosine kinase, receptor, type 2.

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Over the past 30 years researchers have extensively studied the pathogenesis of neuronal cell death and have aimed at identifying protective strategies to minimize neurodegeneration. Estrogen has been shown to provide neuroprotection against *in vivo* models of ischemia (Simpkins et al., 1997; Dubal et al., 1998; Lebesgue et al., 2010), and *in vitro* models of amyloid β toxicity (Goodman et al., 1996; Nilsen et al., 2006; Yao et al., 2007), glutamate excitotoxicity (Goodman et al., 1996; Singer et al., 1999), and oxygen/glucose deprivation (Harms et al., 2001; Cimarosti et al., 2005). Estrogen's neuroprotective actions have been suggested to be mediated by several means including; preventing down regulation of pro-survival members and attenuating pro-apoptotic members (Zhao et al., 2004; Won et al., 2005; Jover-Mengual et al., 2007; Yao et al., 2007), inhibiting caspase-3 activation (Jover-Mengual et al., 2007; Sribnick et al., 2009), attenuating activation of immediate early genes (Rau et al., 2003), enhancing neurogenesis (Suzuki et al., 2007), and interacting with growth factors (Azcoitia et al., 1999; Aguirre and Baudry, 2009). A study using knock-out mice (Dubal et al., 2001) suggested that estrogen receptor α (ER α) is likely responsible for mediating the neuroprotective effects of estrogen; although studies using the selective estrogen receptor agonists, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and diarylpropionitrile, 2,3-bis(4-hydroxyphenyl)propionitrile (DPN; Carswell et al., 2004; Zhao et al., 2004; Zhao and Brinton, 2007) dispute whether ER α and/or estrogen receptor β (ER β) is responsible. Little is known about the molecular events mediating the neuroprotective effects of estrogen, although mitogen activated protein kinase (MAPK) (Singer et al., 1999; Mize et al., 2003; Jover-Mengual et al., 2007; Zhao and Brinton, 2007), signal transducer and activator of transcription-3 (STAT3) (Dziennis et al., 2007), phosphoinositide 3-kinase (PI3K/Akt) (Honda et al., 2000; Harms et al., 2001; Cimarosti et al., 2005), and cAMP-response element binding protein (CREB) (Jover-Mengual et al., 2007) signaling have been suggested to play a role. Because the models available to study neuroprotection include *in vivo* models, primary culture, explants cultures and neuroblastoma cell lines, studying the cellular mechanisms governing neuroprotection within specific neuronal phenotypes of the hippocampus is limited. As a means to overcome this challenge, we have generated an array of immortalized clonal cell lines from the embryonic and adult mouse hippocampus using the retroviral transfer of SV40 T-antigen into primary neurons, a technique previously exploited by our laboratory to generate clonal mouse hypothalamic cell lines (Belsham et al., 2004, 2009). The clonal hippocampal cell lines have been extensively characterized and each line displays a unique gene expression profile with expression of neuronal markers. Furthermore, the clones mHippoE-14, mHippoE-18, and mHippoA-mix were used to investigate the neuroprotective effects of estrogen against glutamate neurotoxicity and define the role of ER α , ER β , and the newest estrogen receptor (ER), GPR30, in the neuroprotective effect of estrogen. These findings provide evidence that these novel immortalized cell lines are functional models to study

neuroprotection of the hippocampus, as well as other avenues of hippocampal function and physiology.

EXPERIMENTAL PROCEDURES

Generation of mouse hippocampal cell lines and subcloning

The generation of the mouse hippocampal cell lines was carried out using the same methodology to that of the generation of mouse hypothalamic cell lines as previously described in (Belsham et al., 2004, 2009). All procedures were conducted in accordance with the regulations of the Canadian Council on Animal Care and approved by the University Animal Care Committee. Briefly, male and female Swiss Webster mice (Charles River; embryonic cell lines) were bred and on embryonic day 18 (E18) the pregnant mice were deeply anesthetized with isofluorane (Sigma, Oakville, ON, Canada) and the fetal hippocampi were dissected, pooled together, triturated into a single cell suspension, and finally plated on a 60 mm² culture dish coated with poly-L-lysine in primary culture medium Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated defined fetal bovine serum (FBS), 10% heat-inactivated horse serum, 1% penicillin-streptomycin, and 20 mM D-glucose (all from Life Technologies/Life Technologies, Inc., Rockville, MD, USA). The suspension was plated on a 60 mm² cell culture dish coated with poly-L-lysine, then placed in the incubator (5% CO₂) for 7 days allowing cells to adhere. For the adult-derived cultures, wild type C57Bl/6 male mice (Charles River Canada, Montreal) were used. Animals 8–12 weeks old were euthanized by CO₂ and immediately decapitated. Brains were explanted, the individual hippocampus dissected and stored on ice in Hank Balanced Salt Solution (HBSS; GIBCO Invitrogen Corporation, USA). Tissues were transferred to cell culture dishes, washed in 0.1 M Phosphate Buffered Saline (PBS, pH 7.4), incubated for 5 min in a 37 °C water bath in 0.25% trypsin (GIBCO Invitrogen Corporation, USA), and washed again with PBS. Each hippocampus was transferred into 1 mL of adult neuronal growth media consisting of Neurobasal-A medium (GIBCO Invitrogen Corporation, USA), B-27 Supplement (1:50; GIBCO Invitrogen Corporation, USA), 0.5 mM L-glutamine (GIBCO Invitrogen Corporation, USA), 10% fetal bovine serum (Hyclone, USA), 5% normal horse serum (heat inactivated, GIBCO Invitrogen Corporation, USA), and 1% Penicillin-Streptomycin (GIBCO Invitrogen Corporation, USA). Each tissue piece was gently triturated starting with a 1000 μ L pipette followed by a 200 μ L pipette, until uniform cellular dissociation was achieved, incubated on ice for 3 min eliminating larger tissue debris, and the supernatant was further diluted 1:16 in adult neuronal growth medium. The cultures were treated with 10 ng/ml recombinant rat CNTF in PBS for 5–7 consecutive days.

Primary cultures were immortalized by transfection with recombinant murine retrovirus harboring simian virus (SV40) T-antigen and the neomycin resistance gene from the pZIPNeo SV(X) 1 vector, as previously described (Belsham et al., 2004, 2009). After 48 h incubation in retrovirus cells were treated with 400 μ g/ml geneticin (G418) every 3 days for 2 weeks, followed by 250 μ g/ml for an addition 2–3 weeks. Resistant colonies appearing 2–3 weeks later were selected using cloning cylinders and further expanded. New clones were further selected and expanded if they demonstrated predominant neuronal morphology. Mixed populations of embryonic-derived hippocampal cells were further subcloned through successive dilutions of the trypsinized cells into 96-well tissue culture plates coated with poly-L-lysine. The optimal dilution allowed only one or two cells per well. The cells were incubated in a 1:1 ratio of conditioned medium from the mixed cultures and fresh DMEM containing 15% FBS (Life Technologies). The adult-derived cell lines were maintained as a mixed cell population, and were not further sub-cloned. Cell colonies were allowed to grow and then successively split into

24-well plates, and finally into 60-mm plates for RNA analysis and cryopreservation.

Reverse transcriptase PCR (RT-PCR) cell line screening

Each clonal hippocampal cell line was analyzed for the expression of specific markers by reverse transcriptase PCR (RT-PCR). cDNA was made using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Foster City, CA, USA). PCR products separated by electrophoresis in a 2% agarose gels and stained in an Ethidium Bromide solution (10 mg/ml in Tris–acetate–EDTA). Gels were visualized under UV light and relative gene expression levels were quantified by densitometry on an imaging station (Kodak Image Station 2000R) and analyzed using Kodak 1D Image analysis 3.6 software (Eastman Kodak, Rochester, NY, USA). All primers were designed using mouse mRNA sequences. See Suppl. Table 1 for primer sequences.

Real time RT-PCR

RNA was harvested from mHippoE14 and mHippoA-mix cell lines using the guanidium isothiocyanate phenol chloroform extraction method. cDNA was made using the Applied Biosystems High Capacity cDNA reverse transcriptase Kit (Foster City, CA, USA). Real-time RT-PCR reactions were performed with 100 ng of cDNA template using SYBR Green PCR master mix and run on the Applied Biosystems Prism 7000 realtime PCR machine. The primer sequences used were: cFOS sense 5' CAA CGA GCC CTC CTC CGA CT-3', antisense 5'-TGC CTT CTC TGA CTG CTC ACA-3'; and Histone 3A sense, 5'-CGC TTC CAG AGT GCA GCT ATT-3', antisense 5'-ATC TTC AAA AAG GCC AAC CAG AT-3'. Whereas the receptor SYBR primers are as follows: GPR30 sense 5'-GTG GCC AAG CCT CAA CAC TCA C-3', antisense 5'-GGT GGA CAG GGT GTC TGA TGT CTG-3'; ER α sense 5'-CTC CCG CCT TCT ACA GGT CTA A-3', antisense 5'-TCG TTA CTG CTG GAC AGT CTC TCT-3'; ER β sense 5'-AGA CAA GAA CCG GCG TAA AA-3', antisense 5'-TCG GTA CCC ACA CCT TTC TC-3'.

Pharmacological agents

L-glutamic acid (glutamate), methylthiazolylidiphenyl-tetrazolium bromide (MTT), 17 β -estradiol (E2), and brain derived neurotrophic factor (BDNF) were purchased from Sigma (Oakville, ON, Canada). DPN; ER β agonist; 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; ER α agonist); (\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 (G-1; GPR30 agonist); 7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182780; ER antagonist) and (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline (G-15; GPR30 antagonist)? were purchased from Tocris Bioscience (Ellisville, MO, USA). MTT, 5 mg/ml in PBS was filter sterilized through a 0.2 μ m syringe filter prior to use. Stock solutions of glutamate (200 mM in PBS), BDNF (50 μ g/mL in water) and finally E2, PPT, DPN, PPT, G-1, ICI, and G-15 (1–10 mM in 95% ethanol) were prepared and further diluted to final working concentrations in phenol-red free DMEM immediately prior to use.

MTT cell viability assay

mHippoE-14, mHippoE-18, and mHippoA-mix cells were plated into 96-well plated in DMEM, containing 5% FBS and 1% penicillin/streptomycin. Once a confluency of 80% was reached, media was replaced with phenol-red free DMEM containing 1% charcoal stripped FBS and 1% penicillin/streptomycin. After 16 h cells were pre-treated with potential neuroprotective agents for 24 or 1 h followed by 24-h treatment with glutamate. MTT was added to the

medium 22 h post glutamate treatment at a final concentration of 1 mM. Media was aspirated off the cells and 200 μ L of DMSO was added to the wells to lyse the cells and dissolve the formazin product that accumulated inside the living cells. Absorbance of each well was assessed at 570 nm using a spectrophotometer.

Western blot analysis

mHippo-E14 and mHippoE-18 cells were grown to 80–90% confluency and serum starved for 16 h followed by treatment with E2 (10 nM) or vehicle (ETOH) for 5, 15, 30 and 60 min. Cells were washed with ice cold PBS and protein was harvested by adding ice cold cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma). Protein was quantified using the biocinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, IL, USA). Protein (30 μ g) was resolved on an 8% SDS–PAGE gel and transferred onto immuno-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% bovine serum albumin (Sigma) in Tris-buffered saline containing 0.2% Tween 20 (TBS–T) for 1 h followed by incubation in primary antibodies; phospho–AKT (Ser473, 1:1000 Cell Signaling Technology), phospho–CREB (Ser133, 1:1000: Cell Signaling Technology), phospho–STAT3 (Tyr705, 1:1000, Cell Signaling Technology), and G protein beta, G β (1:5000: Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C. Membranes were then incubated in secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Immunocomplexes were visualized using enhanced chemiluminescence (ECL kit, GE Healthcare, UK) and levels of phosphorylated protein expression were determined by calculating the ratio of phosphoprotein density to G β density for each treatment group using an imaging station (Kodak Image Station 2000R) and Kodak 1D Image analysis 3.6 software (Eastman Kodak, Rochester, NY, USA).

Statistical analysis

Data were analyzed by Student's *t*-test or one-way ANOVA followed by a post hoc Student–Newman–Keuls multiple comparison test using Sigma Stat. Mean values were obtained from a minimum of three independent experiments and data were considered statistically significant when $P < 0.05$.

RESULTS

Generation and characterization of hippocampal cell models

We have previously developed methods to mass immortalize all dividing cell types present in primary embryonic hypothalamic cultures (Belsham et al., 2004) and CNTF-induced dividing cells of primary adult-derived hypothalamic cultures (Belsham et al., 2009). We have now expanded these techniques into immortalization of primary embryonic and adult hippocampal cultures. The immortalized mixed embryonic cell populations were serially diluted and subcultured as clonal cell populations. Four representative clonal cell lines; mHippoE-2, -5, -14, and -18, were isolated from the initial generation of the heterogeneous cell populations and have been further characterized, while the adult cell line, mHippoA-mix, is the original immortalized mixed adult cell population. Each cell line has a distinct phenotype, indicating that they represent unique cell types and demonstrating the potential diversity from the heterogeneous cells, although all the cells have some common characteristics, such as overall neuronal morphology and the appearance of neurites (Fig. 1A). We

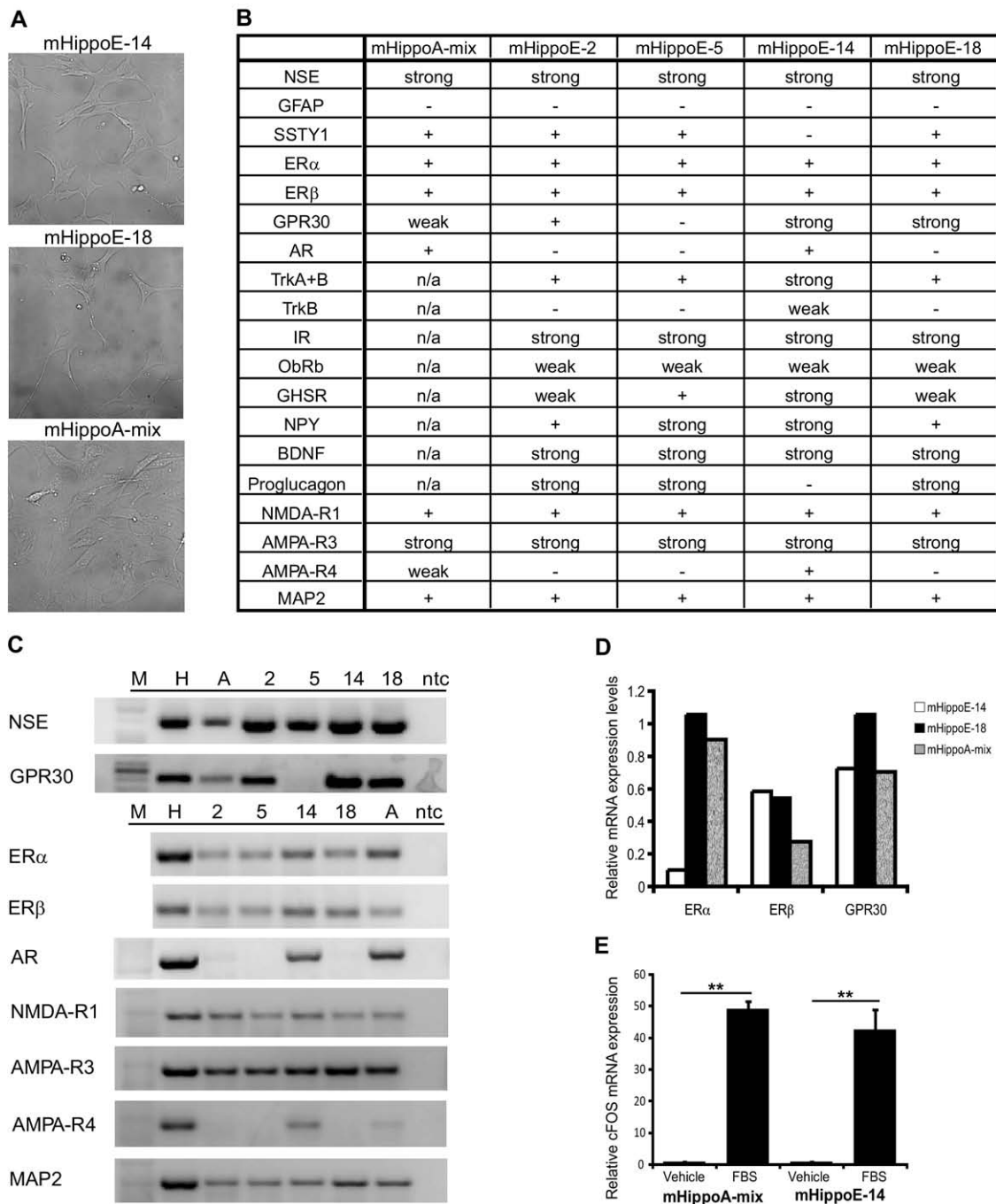


Fig. 1. Characterization of an array of genes expressed in immortalized, hippocampal cell lines exhibiting neuronal morphology and function. (A) Representative phase contrast micrographs of cell lines, mHippoE-14, and mHippoE18 and mHippoA-mix. (B) RT-PCR of hippocampal cell lines mHippoE-xx or mHippoA mix. Mouse hypothalamic RNA was used as a positive control, whereas reactions without template were also included, as indicated (ntc). NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; SSTY1, spermiogenesis specific transcript on the Y 1; ER α , estrogen receptor α ; ER β , estrogen receptor β ; GPR30, g-protein coupled receptor 30; AR, androgen receptor; TrkA, neurotrophic tyrosine kinase, receptor, type 1; TrkB, neurotrophic tyrosine kinase, receptor, type 2; IR, insulin receptor; ObRb, leptin receptor; GHSR, ghelin/growth hormone secretagogue receptor; NPY, neuropeptide Y; BDNF, brain derived neurotrophic factor; proglucagon; NMDA-R1, *N*-methyl *D*-aspartate-receptor 1; AMPA-R3, AMPA-receptor 3; AMPA-R4; MAP2, microtubule-associated protein 2. Expression levels were analyzed by semi-quantitative RT-PCR and range from strong, moderate (+), weak, negative (-). n/a=not available. (C) Representative images of RT-PCR products of NSE and the steroid hormone receptors (GPR30, ER α , ER β , and AR) in mHippoE-xx and mHippoA-mix cell lines. (D) Quantitative levels of ER α , ER β and GPR30 in the mHippoE-14, mHippoE-18 and adult hippocampal cells as measured by real-time RT-PCR. Relative mRNA levels were normalized to the internal control Histone3A. (E) cFOS mRNA expression in mHippoA-mix and mHippoE14 cell lines following a 30 min treatment with 20% FBS or vehicle quantified by real-time RT-PCR. Relative cFOS mRNA levels were normalized to the internal control Histone3A. Results shown are relative to corresponding control cFOS levels and are expressed as mean \pm SEM ($n=3$ independent experiments). ** $P<0.001$.

have analyzed gene expression of neuron specific enolase (NSE); glial fibrillary acidic protein (GFAP); spermiogenesis specific transcript on the Y 1 (SSTY1); ER α ; ER β ; G-protein coupled receptor 30 (GPR30); androgen receptor (AR); neurotrophic tyrosine kinase, receptor, type 1 (TrkA); neurotrophic tyrosine kinase, receptor, type 2 (TrkB); insulin receptor (IR); leptin receptor (ObRb); ghelin/growth hormone secretagogue receptor (GHSR); neuropeptide Y (NPY); BDNF; progucagon; NMDA-R1, N-methyl D-aspartate-receptor 1; AMPA-receptor 3 (AMPA-R3); AMPA-R4; microtubule-associated protein 2 (MAP2). Expression levels were analyzed by semi-quantitative RT-PCR and range from strong, moderate (+), weak, or negative (–) when compared to the strong expression of each gene in hypothalamus controls (Fig. 1B). Although this is not completely quantitative, the semi-quantitative method provides a baseline for expression levels of the individual genes in the cell lines for further analysis. Representative PCR gels showing expression of selective genes in the immortalized hippocampal cell lines carried out by semi-quantitative RT-PCR is depicted (Fig. 1C). To more completely quantify the levels of the ER α , ER β , and GPR30 in the mHippoE-14, mHippoE-18, and mHippoA-mix lines, we used quantitative RT-PCR (Fig. 1D). These results indicate that all three lines express the ERs and GPR30, but the level of ER α in the mHippoE-14 is substantially lower than the other two lines. As for functionality, we were initially interested in determining if these newly developed cell lines were responsive to a known inducer of cellular activity, fetal bovine serum. To detect activation, mHippoE-14 and mHippoA-mix were treated with 20% FBS for 30 min and cFOS mRNA expression was measured using real-time RT-PCR. Indeed, FBS induced a massive increase in cFOS mRNA expression in the mHippoE-14 and mHippoA-mix cells (49-fold and 43-fold increase, respectively, compared to vehicle treated control), an indicative activation response to serum treatment (Fig. 1D). Based on the analysis of gene expression results, we further investigated the neuroprotective effects of estrogen on glutamate-induced excitotoxicity on the mHippoE-14, mHippoE-18, and mHippoA-mix cell lines, as these cell lines express a relevant array of genes and receptors suggested to play a role in neuroprotection including; BDNF, TrkA, ER α , ER β , and GPR30.

Estrogen activates PI3K/Akt and STAT3 signaling pathways

A variety of kinase signaling pathways have been shown to be activated by estrogen, including MAPK (Jover-Mengual et al., 2007; Zhao and Brinton, 2007), PI3K/Akt (Cimarosti et al., 2005), STAT3 (Dziennis et al., 2007) and CREB (Jover-Mengual et al., 2007). In order to determine whether estrogen acutely activates kinase signaling pathways in the immortalized clonal hippocampal cell lines; mHippoE-14 and mHippoE-18, we exposed these cells to an E2 (10 nM) time course of 5, 15, 30 and 60 min and measured levels of phosphorylated Akt, STAT3 and CREB. Western blot analysis demonstrated that in the mHippoE-14 cell line E2 treatment significantly increased expression levels of phospho-STAT3 by 46% and phospho-Akt by 43% after 5 min (Fig. 2A, B). E2 also increased

phospho-STAT3 by 80% and phospho-Akt by 35% after 5 min in the mHippoE-18 cell line (Fig. 2D, E). Expression levels of phospho-Akt and phospho-STAT3 returned to baseline in both cell lines 15 min after E2 exposure. On the contrary, E2 had no effect on the expression levels of phospho-CREB (ser133) in either the mHippoE-14 or mHippoE-18 cell lines (Fig. 2C, F). These results indicate that estrogen mediates rapid signaling in the hippocampal cell lines.

Estrogen protects against glutamate-induced neurotoxicity

Estrogen has been shown to confer neuroprotection in several *in vivo* models of ischemia (Simpkins et al., 1997; Dubal et al., 1998; Lebesgue et al., 2010) and *in vitro* excitotoxicity models (Goodman et al., 1996; Singer et al., 1999). We sought to determine whether estrogen provides neuroprotection in our immortalized, clonal hippocampal cell lines against glutamate-induced neurotoxicity. In order to establish the neurotoxicity paradigm, we treated mHippoE-14 and mHippoE-18 with increasing concentrations of glutamate for 24 h and assessed cell viability using the MTT assay. We observed that after 24 h glutamate at a concentration of 25 mM, the neurons had significantly reduced cell viability by 48% and 53% in the mHippoE-14 and mHippoE-18 cell lines respectively (Fig. 3A, C). In general, immortalized cell lines are more robust than primary cultures; thus it would be expected that relatively higher levels of glutamate would be required to induce cell death. We next investigated whether pretreatment with E2 (10 or 100 nM) for 24 h would offer protection against glutamate-induced neurotoxicity. E2 was found to significantly reduce cell death from glutamate treatment in both the mHippoE-14 and mHippoE-18 cell lines. Interestingly, a concentration of 100 nM was required to significantly reduce cell death from 37% (glutamate plus vehicle) to 20% (glutamate plus estrogen) in the mHippoE-14 cell line, while E2 at 10 nM significantly reduced cell death from 40% (glutamate plus vehicle) to 9% (glutamate plus E2) in the mHippoE-18 cell line (Fig. 3B, D). Finally, we investigated whether E2 would also provide neuroprotection against glutamate neurotoxicity in the mHippoA-mix cell line. Unlike the embryonic clonal cell lines above, E2 was not effective in protecting these cells against glutamate-induced cell death (27% cell death, glutamate plus vehicle; 24% cell death, glutamate plus E2 10 nM; and 28% cell death, glutamate plus E2 100 nM) (Fig. 3E).

Activation of ER α protects against glutamate neurotoxicity and is blocked by ER antagonist ICI 162780

In order to further investigate the neuroprotective effects of estrogen against glutamate neurotoxicity we next selectively activated the classical ERs, ER α and ER β , using the specific ER agonists, PPT and DPN, respectively. mHippoE-14 and mHippoE-18 were pretreated with PPT or DPN (10–1000 μ M) for 24 h followed by co-treatment of PPT or DPN plus glutamate for 24 h. Surprisingly, in the mHippoE-14 cells neither PPT nor DPN pre-

Fig. 2. Acute estrogen treatment activates STAT3 and AKT kinases in the hippocampal cell lines mHippoE-14 and mHippoE-18. E2 (10 nM) increases pSTAT3 (A) and pAkt (B) after 5 min, but not pCREB (C) in the mHippoE-14 cells and in the mHippoE-18 cells (D–F). Hippocampal cells were serum starved for 4 h before treatment with E2 (10 nM) or vehicle alone for 5, 15, 30 and 60 min. Results shown are relative to corresponding control protein levels (vehicle at 5 min set to 1.0) and expressed as mean \pm SEM ($n=5$ independent experiments). Phospho-proteins are normalized to G protein-beta. * $P<0.05$.

treatment provided neuroprotection against glutamate-induced neurotoxicity (Fig. 4A, B). PPT (10 and 100 nM) protected against glutamate neurotoxicity in the mHippo-E18

by reducing cell death from 51% with glutamate plus vehicle to 36% cell death with glutamate plus PPT (10 or 100 nM) (Fig. 4C). In addition, DPN at a much higher concen-

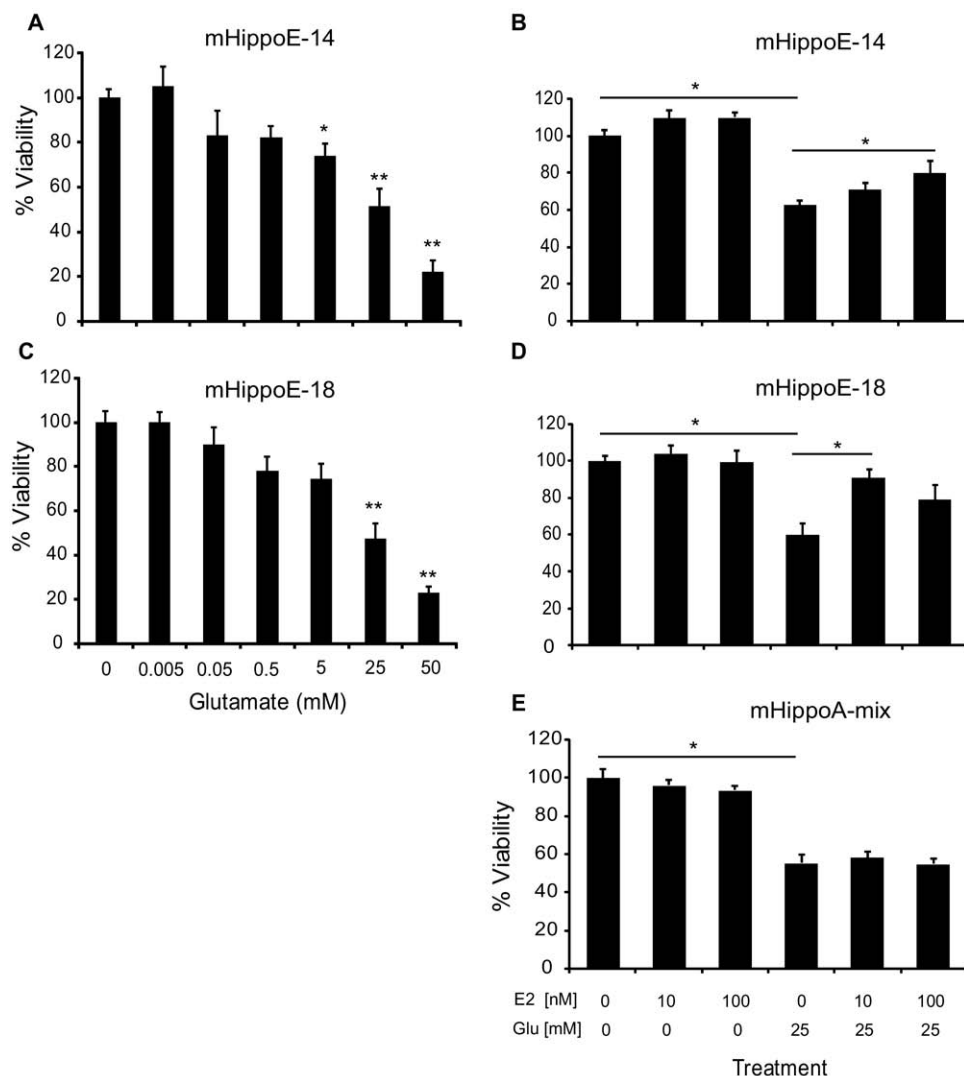


Fig. 3. Estrogen protects mHippoE-14 and mHippoE-18 cells from glutamate neurotoxicity. Effect of 24 h glutamate treatment (5 nM–50 mM) on cell viability of the mHippoE-14 (A) and mHippoE-18 (C) cells as determined by the MTT assay. mHippoE-14 (B), mHippoE-18 (D), and mHippoA-mix (E) cell lines were pre-treated with E2 (10 and 100 nM) followed by co-treatment with glutamate (25 mM) for 24 h. Results shown are relative to vehicle treated cultures which are set to 100% viability and expressed as mean \pm SEM ($n=3-12$ independent experiments). * $P<0.05$, ** $P<0.001$.

tration of 1 μ M also significantly reduced cell death in the mHippoE-18 cell line from 51 to 34% cell death (Fig. 4D). Because we have shown that E2 does significantly attenuate cell death by glutamate treatment in the mHippoE-14 cells, but selective activation of either ER α or ER β does not, the effect of activating both receptor subtypes by pretreating the cells with DPN and PPT together prior to glutamate treatment was investigated. Similarly, pretreatment with both DPN and PPT together did not protect mHippoE-14 cells from glutamate-induced neurotoxicity (Fig. 4E). This would suggest an ER-independent mechanism for neuroprotection.

To prove that the protective effects of estrogen with the 24 h pretreatment were specific to ERs or perhaps to GPR30, we used the well-established ER antagonist ICI 182780 or selective GPR30 antagonist G-15. Cells were pretreated for 1 h with ICI 182780 (100 nM) or G-15 (1 μ M)

to block the receptors, followed by a 24 h co-treatment with estrogen (100 nM, mHippoE-14, 10 nM, mHippoE-18), as was determined in previously (Fig. 4). This was followed by a 24 h exposure to glutamate (25 mM) to induce neurotoxicity, in combination with estrogen and antagonist. We find that ICI 182780 was able to completely block the protective effect of estrogen in both the mHippoE-14 and mHippoE-18 neurons (Fig. 5). Interestingly, G-15 was only able to have block the protective effect of E2 on the mHippoE-14 neurons. The compounds alone did not alter the viability of the neurons.

Acute estrogen treatment protects against glutamate-induced neurotoxicity

Estrogen has been shown to protect neurons against cell death through rapid kinase signaling pathways (Ci-

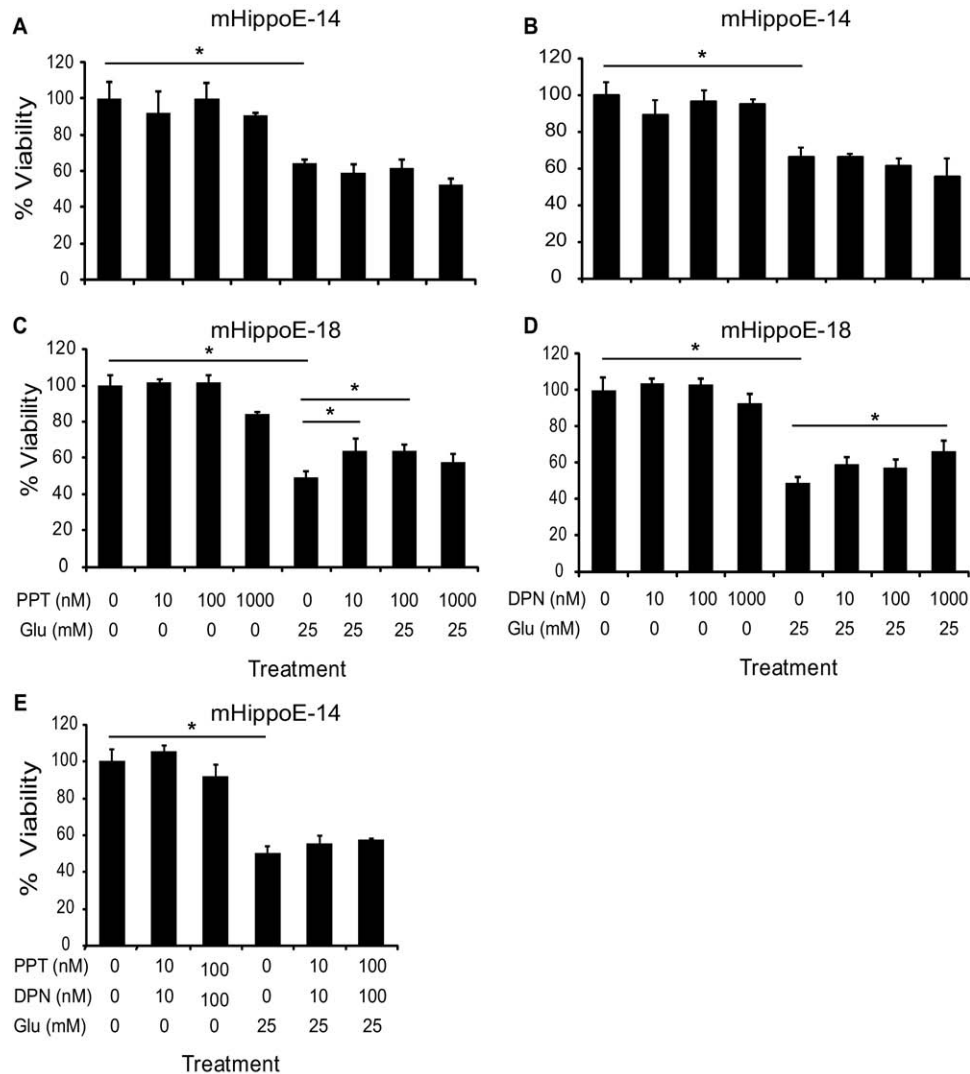


Fig. 4. Effect of the selective ER agonists, PPT and DPN on glutamate-induced neurotoxicity in mHippoE-14 and mHippoE-18 cell lines. Cells were pre-treated for 24 h with PPT (10–1000 nM) (A, C), DPN (10–1000 nM) (B, D), or a combination of PPT and DPN (10 and 100 nM) (E) followed by a 24 h co-treatment with glutamate (mM) and cell viability was determined using the MTT assay. Results shown are relative to vehicle treated cultures (set to 100%) and are expressed as mean \pm SEM ($n=4-5$ independent experiments). * $P<0.05$.

marosti et al., 2005; Dziennis et al., 2007; Jover-Mengual et al., 2007; Zhao and Brinton, 2007). Because we showed that estrogen rapidly activates the STAT3 and PI3K/Akt kinase pathways, we were interested in determining whether short-term estrogen treatment could protect the clonal hippocampal cell lines from glutamate neurotoxicity. mHippoE-14 and mHippoE-18 cells were pretreated with a dose curve of E2 (100 nM, 1 μ M, or 10 μ M for both mHippoE-14 and mHippoE-18 cells) for 1 h prior to 24 h co-treatment with glutamate or controls of E2 and glutamate for 24 h. Unlike the 24 h E2 pretreatment, short term estrogen exposure only protected the neurons against glutamate neurotoxicity in the mHippoE-14 and mHippoE18 cell lines at a higher concentration of 10 μ M (Fig. 6).

Activation of GPR30 protects against glutamate neurotoxicity

GPR30 is the most recently identified estrogen receptor and has been described to mediate the effects of estrogen in several physiological systems including the CNS; reviewed in detail (Maggiolini and Picard, 2010; Olde and Leeb-Lundberg, 2009). We were interested in determining whether activation of GPR30 confers neuroprotection in hippocampal neurons. To address this mHippoE-14 and mHippoE-18 cell lines were exposed to either a 24 or 1 h pretreatment with G-1 (10 and 100 nM) followed by co-treatment with glutamate for 24 h. It was observed that 24 h pretreatment with the GPR30 agonist G-1 did not protect the cells against glutamate

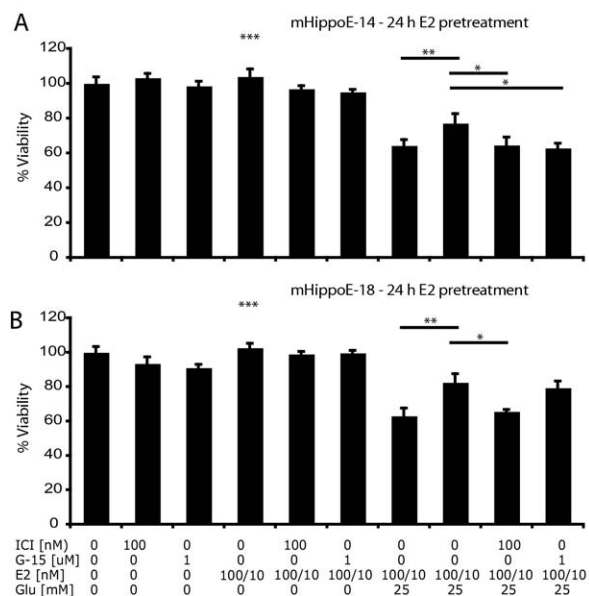


Fig. 5. Effect of ER antagonist, ICI 182780, and selective GPR30 antagonist, G-15, on estrogen protection of glutamate-induced neurotoxicity in mHippoE-14 and mHippoE-18 cell lines. mHippoE-14 (A) and mHippoE-18 (B) cells were pretreated for 1 h with ICI 182780 (100 nM) or G-15 (1 μM), followed by a 24 h co-treatment with estrogen (100 nM, mHippoE-14, 10 nM, mHippoE-18). Then, a 24 h treatment with glutamate (25 mM), estrogen and antagonist, in combination. Cell viability was determined using the MTT assay. Results shown are relative to vehicle treated cultures (set to 100%) and are expressed as mean±SEM ($n=4$ independent experiments). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

neurotoxicity in both the cell lines (Fig. 7A, B). On the contrary, 1 h pretreatment with G-1 (100 nM) significantly attenuated cell death induced by glutamate, 61–49% cell death in the mHippoE-14 cells and 47–34% cell death in the mHippoE-18 cells (Fig. 7C, D).

We then wanted to determine if the acute protective effects of E2 on glutamate toxicity were due to the direct action of specific estrogen receptors. Again, we used pretreatment with the ER antagonist ICI 182780 or selective GPR30 antagonist G-15. The neurons were pretreated with the antagonists and then exposed to an acute 1 h treatment with 10 μM E2 before cotreatment with glutamate in combination with E2 and antagonists. We found that only the GPR30 antagonist G-15 was effective to significantly block the neuroprotective effect of estrogen in both mHippoE-14 and mHippoE-18 neurons (Fig. 8A, B). The ER antagonist ICI 182780 did not have any effect on the E2 protection, and further none of the compounds alone, including antagonists, had any effect on the basal proliferative properties of the neurons (Fig. 8).

DISCUSSION

We report here the generation of novel mouse embryonic and adult immortalized hippocampal cell lines using SV40 T-antigen retroviral transfer into primary mouse hippocampal neurons. Four clonal embryonic lines, mHippoE-2, mHippoE-5, mHippoE-14, mHippoE-18, and one mixed

adult line, mHippoA-mix, were all found to exhibit neuronal morphologies with neurite extensions and expression of neuronal markers, while each also displayed unique gene expression profiles. These findings suggest that the array of newly developed hippocampal cell lines represent distinctive cells types and may originate from different regions within the hippocampus. A variety of steroid hormone receptors were shown to be expressed in the cell lines, with ERα and ERβ expressed in all, GPR30 was expressed to some degree in all lines, except mHippoE-5, and AR was expressed in only the mHippoA-mix and mHippoE-14 cell lines. In addition, each cell line also uniquely expresses an array of genes involved in energy homeostasis. Because steroid hormones and neuropeptides play an important role in hippocampal physiology, these cell lines will prove to be excellent models for further investigation into their role and delineating the cellular and molecular mechanisms of their actions in hippocampal cells. Similar to any model system, including *in vivo* manipulations, clonal cell lines come with inherent limitations. Therefore, prudence must be exercised when attempting to directly extrapolate to whole animal physiology. Yet, the ability to dissect complex cellular regulatory mechanisms and the information gained from the use of these cell models will allow a more focused approach in the intact animal. Additional characterization of the subclones and further functional analyses

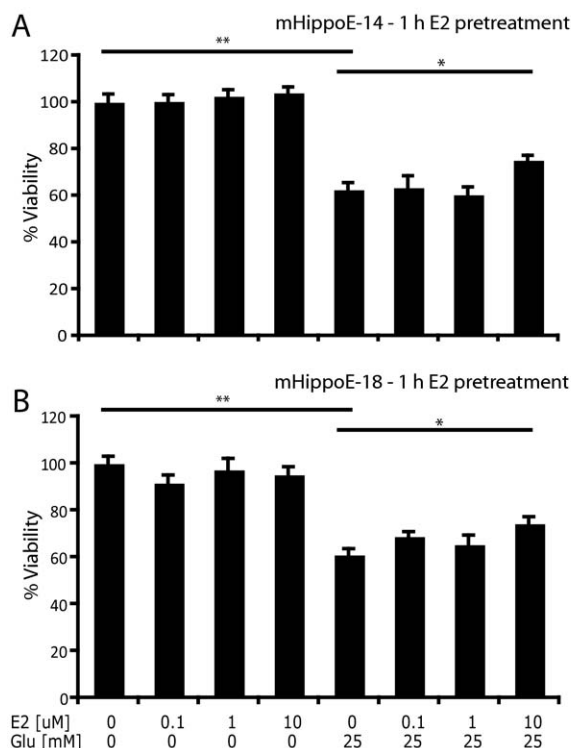


Fig. 6. Acute estrogen exposure protects mHippoE-14 and mHippoE-18 cells from glutamate-induced neurotoxicity. mHippoE-14 (A) and mHippoE-18 (B) cells were pre-treated for 1 h with E2 (100 nM, 1 or 10 μM) followed by co-treatment for 24 h with glutamate (25 mM) and cell viability was determined using the MTT assay. Results shown are relative to vehicle treated cultures (set to 100%) and are expressed as mean±SEM ($n=4$ independent experiments). * $P<0.05$; ** $P<0.001$.

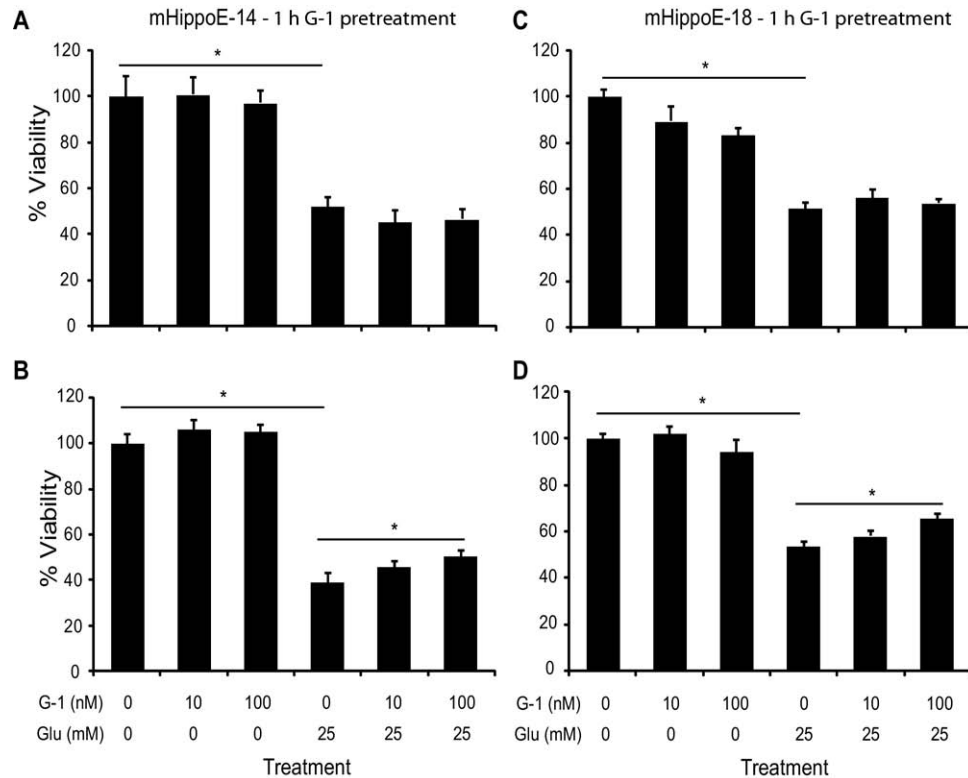


Fig. 7. Activation of GPR30 protects mHippoE-14 and mHippoE-18 cells from glutamate-induced neurotoxicity. Cells were pre-treated with the GPR30 agonist, G-1 (10 and 100 nM) for 24 h (A, C) or 1 h (B, D) followed by co-treatment with glutamate (25 mM) for 24 h and cell viability was determined using the MTT assay. Results shown are relative to vehicle treated cultures (set to 100%) and are expressed as mean \pm SEM ($n=4-6$ independent experiments). * $P<0.05$.

is of great interest, as this will uncover other avenues of study for which these cell lines will be useful.

Not only do these novel hippocampal cell lines morphologically appear to be neurons and exhibit neuronal markers, but functional analysis also demonstrated they exhibit neuron-like responses. mHippoE-14 and mHippoA-mix cells were activated, as determined by cFOS mRNA expression, following acute serum treatment, a response indicative of neuronal activation. Acute estrogen treatment was shown to activate STAT3 and PI3K/Akt kinase signaling cascades in the mHippoE-14 and mHippoE-18 cells, again responses that mirrored neurons *in vivo* (Dziennis et al., 2007) and in hippocampal slice cultures (Cimarosti et al., 2005).

The hippocampus has been targeted to study neurodegeneration and neuroprotective therapies, such as estrogen, which has received a great deal of attention due to its promising neuroprotective potential. Unlike the hippocampus *in vivo* or *in vitro*, which contains an assortment of cell types including endothelial cells, glia and neurons of which there are several types, our clonal hippocampal cell lines provide the unique opportunity to study cell specific aspects of neuroprotection due to the homogenous population of a single neuronal cell type. We focused our attention on studying the neuroprotective effects of estrogen in the cell lines; mHippoE-14, mHippoE-18, and mHippoA-mix. The mHippoE-14 cell line originates from embryonic female hippocampus, as determined by the lack of expres-

sion of the male chromosome marker, SSTR1, while mHippoE-18 originate from embryonic male (express SSTR1 mRNA), and mHippoA-mix originate from adult male hippocampus. Our findings demonstrate that all 3 cell lines were equally susceptible to glutamate-induced neurotoxicity resulting in 40–60% cell death. Interestingly, estrogen provided varying degrees of neuroprotection in each of the hippocampal cell lines. Both embryonic-derived cell lines were protected by estrogen, although this effect was stronger in the male mHippoE-18 cells as they were protected by a lower concentration of estrogen (10 nM) compared to the 100 nM of estrogen that was required to rescue mHippoE-14 neurons from cell death. Interestingly, quantitative RT-PCR demonstrated that the mHippoE-14 line expressed lower levels of ER α , which may have a direct effect on the levels of estrogen required to exhibit neuroprotection.

On the other hand, neuroprotection studies carried out in primary cultures pool together neurons from both sexes and potential differences in gender are therefore not investigated. However, a study using primary hippocampal cultures from male and female rats, also showed that estrogen more potently protected male cultures from hypoxia-induced neuronal cell death (Heyer et al., 2005). One might speculate that intrinsic differences in the cells due to gender, such as changes in the ratio of ERs following estrogen and/or glutamate treatment, may account for these differences. On the contrary, estrogen had no pro-

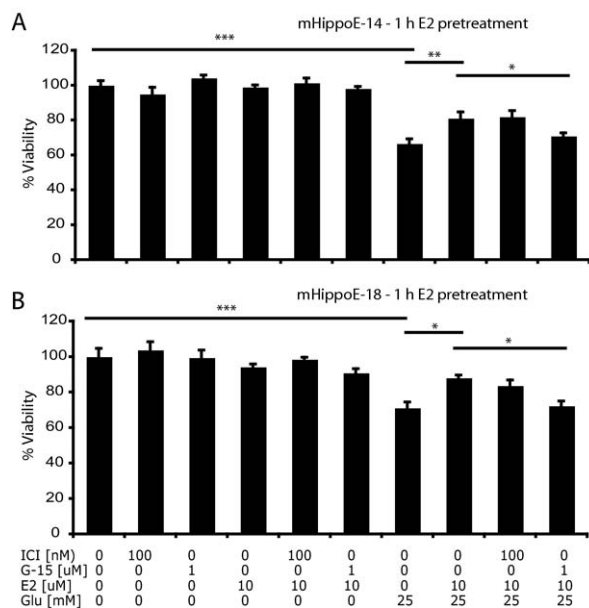


Fig. 8. Effect of ER antagonist, ICI 182780, and selective GPR30 antagonist, G-15, on acute estrogen protection of glutamate-induced neurotoxicity in (A) mHippoE-14 and (B) mHippoE-18 cell lines. Cells were pretreated for 1 h with ICI 182780 (100 nM) or G-15 (1 μM), followed by a 1 h co-treatment with estrogen (10 μM). The mHippoE-14 and mHippoE-18 were then treated for 24 h with glutamate (25 mM), estrogen and antagonists in combination. Cell viability was determined using the MTT assay. Results shown are relative to vehicle treated cultures (set to 100%) and are expressed as mean ± SEM ($n=4$ independent experiments). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

tective effect against glutamate neurotoxicity in the mHippoA-mix cells. We speculate that the mHippoA-mix cell line originates from the dentate gyrus region of the hippocampus formation, because in order to immortalize primary cultures with retroviral transfer of the SV40 T-antigen cells must be proliferating. The only region within the adult hippocampus that undergoes lifelong neurogenesis is the dentate gyrus (Altman and Das, 1965; Kaplan and Hinds, 1977; Cameron et al., 1993). The dentate gyrus is not often used to study neuroprotective effects of estrogen in comparison to the more vulnerable CA1–CA3 regions; however, two *in vivo* studies have found that estrogen may protect cells in the dentate gyrus from kainic acid-induced cell death (Azcoitia et al., 1999; Hilton et al., 2004). We speculate that our adult-derived cell line likely originated from the dividing cell within the dentate gyrus, and may therefore not be as susceptible to the protective effects of estrogen to glutamate stress, as those cells originating from the CA1–CA3 regions of the hippocampus.

BDNF has been shown to confer neuroprotective properties, and has been suggested to play a role in mediating the neuroprotective effects of estrogen, since its gene promoter contains an estrogen response element (Sohrabji et al., 1995). This was recently demonstrated as the BDNF scavenger TrkB-Fc attenuated neuroprotection by estrogen in hippocampal slice cultures (Aguirre and Baudry, 2009). We investigated whether BDNF would offer neuroprotection against glutamate neurotoxicity in the

mHippoE-14 and mHippoE-18 cell lines and found that BDNF did not protect either cell line from glutamate-induced cell death, suggesting that BDNF likely does not play a role in the estrogen-mediated neuroprotective effect in these cell lines (data not shown). Further studies using increasing concentrations of BDNF and functional analysis of BDNF receptors will be undertaken to further understand the role of BDNF in these cell lines.

The role of estrogen receptors in mediating the neuroprotective effect of estrogen remains controversial. Some report that ER α is responsible (Dubal et al., 2001), while others report that ER β (Carswell et al., 2004) is involved, and finally there is some evidence that both ERs are mediators (Zhao et al., 2004; Miller et al., 2005; Zhao and Brinton, 2007). Our findings suggest that in the mHippoE-18 cell line ER α is responsible for mediating the neuroprotective effect of estrogen as PPT, a selective ER α agonist, confers neuroprotection against glutamate-induced neurotoxicity at concentrations of 10 and 100 nM. Further, the use of ICI 182780 indicated that these effects are directly through ERs. Although we observed that the selective ER β agonist, DPN, at a concentration of 1 μM significantly protected against glutamate-induced cell death, we interpret this finding cautiously as this concentration been shown to also activate ER α (Harrington et al., 2003). Surprisingly, neither PPT nor DPN alone or in combination protected mHippoE-14 cells from glutamate-induced cell death suggesting that estrogen acts through an ER-independent mechanism to protect these cells from neurotoxicity, a finding that has been reported by others (Liu et al., 2002; Yi et al., 2008). Activation of protein kinases, including STAT3 (Dziennis et al., 2007) and PI3K/Akt (Honda et al., 2000; Harms et al., 2001; Cimarosti et al., 2005) by estrogen has been shown to mediate the neuroprotective effects of estrogen. We demonstrated that in the mHippoE-14 cell line estrogen activates the STAT3 and PI3K/Akt pathways, and thus we speculate that direct activation of these kinases may be responsible for the estrogen-mediated neuroprotective effect in the mHippoE-14 cell line.

Estrogen receptors located at the membrane carry out rapid non-genomic actions of estrogen and several studies have shown that acute estrogen treatment is effective in conferring neuroprotective effects (Cimarosti et al., 2005; Gulino et al., 2006; Sandstrom and Rowan, 2007; Sribnick et al., 2009; Lebesgue et al., 2010). Which receptor(s) are responsible for the non-genomic effects of estrogen is still debatable, but may be ER α , ER β , or GPR30. We found that estrogen pre-treatment for 1 h required a relatively higher concentration of E2 leading to neuroprotection against glutamate-induced cell death, as determined by a dose curve. On the contrary, we observed that 1 h pre-treatment with G-1, the GPR30 agonist, did significantly protect both mHippoE-14 and mHippoE-18 cells from glutamate-induced neurotoxicity. The longer 24 h pre-treatment with G-1 was not effective at conferring neuroprotection. This is the first *in vitro* study to show that activation of GPR30 protects against neurodegeneration. An *in vivo* study earlier this year by Lebesgue et al. (2010) demon-

strated that when G-1 and estrogen were administered immediately after ischemia and the number of surviving CA1 neurons was measured 7 days following ischemia, both compounds protected against neurodegeneration (Lebesgue et al., 2010). Together with our finding, this study provides evidence that activation of GPR30 is involved in neuroprotection of the hippocampus. The selective GPR30 antagonist G-15 was linked to the acute neuroprotection by estrogen, confirming that activation of GPR30 is required for the estrogen-mediated protection of the hippocampal neurons *in vitro*.

CONCLUSION

In conclusion, studies on the neuroprotective effects of estrogen using *in vivo* and *in vitro* models of primary or slice cultures have been crucial to our current understanding of the neuroprotective potential of estrogen. However, by using immortalized populations of hippocampal neurons, described herein, we can further dissect the cellular signaling mechanisms mediating the neuroprotective effects of estrogen in individual cell types. Furthermore, the potential applications of these cell lines should not be limited to neurodegenerative studies, as they will prove to be invaluable models for studying other aspects of hippocampal physiology, including the cellular mechanisms involved in long term potentiation and adult neurogenesis. Further, how neuropeptides and hormone action contribute to the overall function of the hippocampus is not yet understood. We expect that the use of the hippocampal cell models will contribute to our understanding of the overall role of neuroendocrine components to hippocampal neuronal responses and can be more easily approached in this manner.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neuroscience.2010.06.076](https://doi.org/10.1016/j.neuroscience.2010.06.076).

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