0022-3565/06/3193-1124–1132$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 319:1124–1132, 2006

Vol. 319, No. 3
109504/3152323
*Printed in U.S.A.*



# Estrogenic Agonist Activity of ICI 182,780 (Faslodex) in Hippocampal Neurons: Implications for Basic Science Understanding of Estrogen Signaling and Development of Estrogen Modulators with a Dual Therapeutic Profile

Liqin Zhao, Kathleen O'Neill, and Roberta Diaz Brinton

*Department of Molecular Pharmacology and Toxicology, Norris Foundation Laboratory for Neuroscience Research, School of Pharmacy, University of Southern California, Los Angeles, California*

Received June 21, 2006; accepted August 31, 2006

## ABSTRACT

The present study sought to determine the characteristics of ICI 182,780 (Faslodex) action in rat primary hippocampal neurons. We first investigated the neuroprotective efficacy of ICI 182,780 against neurodegenerative insults associated with Alzheimer's disease and related disorders. Dose-response analyses revealed that ICI 182,780, in a concentration-dependent manner, significantly promoted neuron survival following exposure to either excitotoxic glutamate (200 $\mu$M)- or $\beta$-amyloid$_{1-42}$ (1.5 $\mu$M)-induced neurodegeneration of hippocampal neurons. At a clinically relevant concentration of 50 ng/ml, ICI 182,780 exerted nearly maximal neuroprotection against both insults with efficacy comparable with that induced by the endogenous estrogen 17$\beta$-estradiol. Thereafter, we investigated the impact of 50 ng/ml ICI 182,780 on mechanisms of 17$\beta$-estradiol-inducible neuronal plasticity and neuroprotection. Results of these analyses demonstrated that ICI 182,780 directly induced a series of rapid intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) oscillations in a pattern comparable with that of 17$\beta$-estradiol. In addition, ICI 182,780 exerted dual regulation of the glutamate-induced rise in $[Ca^{2+}]_i$ identical to that induced by 17$\beta$-estradiol. Further analyses demonstrated that ICI 182,780 induced significant activation of extracellular signal-regulated kinase 1/2 and Akt (protein kinase B) and significantly increased expression of spinophilin and Bcl-2, with efficacy comparable with neurons treated with 17$\beta$-estradiol. Taken together, results of these in vitro analyses of ICI 182,780 provide direct evidence of an estrogenic agonist profile of ICI 182,780 action in rat hippocampal neurons. Therapeutic development of neuroselective estrogen receptor modulators that mimic ICI 182,780 is discussed with respect to the potential of safe and efficacious alternatives to estrogen therapy for the prevention of postmenopausal cognitive decline and late-onset Alzheimer's disease.

ICI 182,780 (Faslodex) is a 7$\alpha$-alkylsulfinyl analog of the endogenous estrogen 17$\beta$-estradiol and binds to both estrogen receptor (ER) subtypes with a comparable affinity to 17$\beta$-estradiol (Wakeling et al., 1991). ICI 182,780 is an efficacious antagonist of the ER-dependent proliferative actions of estrogen in reproductive organs such as the breast and uterus (Howell et al., 2000). Moreover, it is a "pure" antiestrogen lacking estrogen-like agonist activity in these organs, as seen with tamoxifen (TMX) (Howell et al., 2000). Partial agonist activity of TMX is believed to be the major cause of the development of resistance to TMX therapy for the treatment of estrogen-dependent breast cancers (Howell et al., 2000). The absence of estrogenic activity and the observations that TMX-resistant tumors remain sensitive to ICI 182,780 treatment led to Food and Drug Administration approval for the use of ICI 182,780 as an adjuvant endocrine therapy to treat ER-positive metastatic breast cancers in postmenopausal women with disease progression following the first line antiestrogen therapy, i.e., TMX-resistant breast cancers (Bross et al., 2002).

The ER antagonism of ICI 182,780 in the breast and uterus

This work was supported by grants from the National Institutes of Mental Health, the National Institutes of Aging, the K. T. and E. L. Norris Foundation, the L. K. Whittier Foundation, and the Stanley Family Trust (to R.D.B.).
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.109504.

**ABBREVIATIONS:** ICI, ICI 182,780 (Faslodex); ER, estrogen receptor; TMX, tamoxifen; AF, activation function; SERM, selective estrogen receptor modulator; ERK, extracellular signal-regulated kinase; NBM, neurobasal medium; DIV, day(s) in vitro; HBS, HEPES-buffered saline; Akt, protein kinase B; PBS, phosphate-buffered saline; AM, acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; HRP, horseradish peroxidase; NE, neuroprotective efficacy; CREB, cyclic AMP response element-binding protein; RAL, raloxifene; ICI 164,384, (*N*-*n*-butyl-*N*-methyl-11-[3,17$\beta$-dihydroxyestra-1,3,5(10)-trien-7$\alpha$-yl]-undecanamide; E2, 17$\beta$-estradiol.

has been associated with its "down-regulation" of ER, resulting from its disruption of the shuttling of ER between the cytoplasm and the nucleus, a constitutively active process involving ER in both the absence and presence of 17$\beta$-estradiol (Dauvois et al., 1993). The accumulated ER presence in the cytoplasm undergoes elevated receptor turnover and degradation (Linstedt et al., 1986). Inhibition of nuclear uptake of ER induced by ICI 182,780 and its structural analogs has been proposed to be related to the terminal portion of the 7$\alpha$ side chains of these compounds, which protrude out of the ligand binding pocket and interfere with ER dimerization, a structural prerequisite for the translocation of ER into the nucleus (Fawell et al., 1990). In addition, ICI 182,780 has been demonstrated to fully abolish ER transcription regulated by both activation function AF-1 and AF-2 domains, which is distinct from the ER partial agonist/antagonist TMX or its active metabolite, 4-hydroxyl-tamoxifen, where only the AF-2 domain is blocked, and the estrogenic agonist activity of these selective estrogen receptor modulators (SERMs) can be achieved through the activation of the other ligand-independent AF-1 domain (Wakeling et al., 1991).

Although a large number of studies demonstrated that a high concentration of ICI 182,780 was an antiestrogen in brain (Purves-Tyson and Keast, 2004; Miller et al., 2005), there is a lack of research on ICI 182,780 action at a relatively low pharmacological and clinically relevant concentration. Several reports indicated estrogenic activity of ICI 182,780 in select brain tissue/cells. For example, in vitro ICI 182,780 induced a significant increase in phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in neonatal rat primary cerebellar neurons (Wong et al., 2003). Another in vitro study demonstrated that ICI 182,780 failed to block the activation of ERK1/2 induced by 17$\beta$-estradiol in basal forebrain cholinergic neurons (Dominguez et al., 2004). The ineffectiveness of ICI 182,780 in inhibiting 17$\beta$-estradiol-induced agonistic effects in the brain tissue was also indicated in studies on hypothalamic neurons and our own studies in hippocampal neurons (Cambiasso and Carrer, 2001). These novel findings of "deviated" properties from the conventional notion of ICI 182,780 acting as a "full" estrogen antagonist led us to pursue in more detail the pharmacological characteristics of ICI 182,780 action in neurons vulnerable to neurodegeneration associated with Alzheimer's disease and related disorders. In the present study, we sought to determine the estrogen agonist and/or antagonist properties of ICI 182,780 action on indicators widely accepted as key outcomes and mechanisms of estrogenic action in hippocampal neurons. Results of our in vitro analyses indicate that ICI 182,780 effectively and significantly induced estrogenic outcomes and activated underlying biochemical mechanisms consistent with estrogen actions, supporting its estrogenic agonist activity in these neurons.

## Materials and Methods

**Chemicals.** 17$\beta$-Estradiol was purchased from Steraloids (Newport, RI). ICI 182,780 was purchased from Tocris Cookson (Ellisville, MO). The sources of other materials are indicated in the experimental methods described below.

**Neuronal Cultures.** The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Southern California. Primary cultures of rat hippocampal neurons were prepared according to the method described previously (Zhao et al., 2004). In brief, hippocampi were dissected from the brains of embryonic day 18 Sprague-Dawley rat fetuses, treated with 0.02% trypsin in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM $KH_2PO_4$, 0.34 mM $Na_2HPO_4{\cdot}7H_2O$, 10.0 mM glucose, and 10.0 mM HEPES) at 37°C for 5 min and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. For intracellular $Ca^{2+}$ imaging analyses, approximately $10^4$ cells were seeded onto poly-D-lysine (10 $\mu$g/ml)-coated 22-mm coverslips in covered 35-mm Petri dishes. For neuroprotection and Western immunoblotting analyses, approximately $10^6$ cells/ml were seeded onto poly-D-lysine-coated solid black and clear bottom 96-well culture plates and 60-mm Petri dishes, respectively. Cells were grown in phenol-red free neurobasal medium (NBM; Invitrogen, Carlsbad, CA) supplemented with B27, 5 U/ml penicillin, 5 $\mu$g/ml streptomycin, 0.5 mM glutamine, and 25 $\mu$M glutamate at 37°C in 10% $CO_2$ for the first 3 days and NBM without glutamate afterward. Cultures grown in serum-free NBM yields approximately 99.5% neurons and 0.5% glial cells.

**Glutamate Exposure.** Primary hippocampal neurons grown on solid black and clear bottom 96-well culture plates for 7 days in vitro (DIV) were pretreated with vehicle alone or steroids for 48 h, followed by exposure to 200 $\mu$M glutamate at room temperature for 5 min in HEPES-buffered saline (HBS) solution (containing 100 mM NaCl, 2.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM $NaH_2PO_4$, 4.2 mM $NaHCO_3$, 10.0 mM glucose, and 12.5 mM HEPES). Immediately following glutamate exposure, cultures were washed once with HBS and replaced with fresh NBM containing test steroids. Cultures were returned to the culture incubator and allowed to incubate for an additional 24 h before neuronal viability measurements on the following day.

**$\beta$-Amyloid$_{1-42}$ Exposure.** $\beta$-Amyloid$_{1-42}$ (American Peptide Company, Sunnyvale, CA) was dissolved in 10 mM HCl at 1 mM and stored at −20°C as a stock solution. Aggregation of $\beta$-amyloid$_{1-42}$ was prepared by incubating the stock solution in 0.1 M phosphate-buffered saline (PBS) solution (3.42 ml of 1 M $Na_2HPO_4$ and 1.58 ml of 1 M $NaH_2PO_4$ in 50 ml of double-distilled $H_2O$) at room temperature for 3 days before use, which yielded a concentration of $\beta$-amyloid$_{1-42}$ at 100 $\mu$M. Primary hippocampal neurons grown on solid black and clear-bottom 96-well culture plates for 7 DIV were pretreated with vehicle alone or steroids for 48 h, followed by exposure to freshly prepared 1.5 $\mu$M $\beta$-amyloid$_{1-42}$ in NBM in the presence of vehicle alone or test steroids at 37°C for 3 days before neuronal viability measurement.

**Neuronal Viability.** Neuronal viability was determined with calcein acetoxymethyl ester (AM; Molecular Probes, Eugene, OR) staining, which indicates the metabolically live cells in the cultures (Zhao et al., 2004). Following glutamate or $\beta$-amyloid$_{1-42}$ exposure, cultures were rinsed with warm PBS twice and incubated with 1 $\mu$M calcein AM in PBS at room temperature for 30 min. For cultures exposed to glutamate, the amount of fluorescence generated by calcein, a green fluorescent product derived from enzymatic esteration of calcein AM by esterases in live cells, was measured on a SpectraMax microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at excitation/emission of 485/530 nm. For cultures exposed to $\beta$-amyloid$_{1-42}$, fluorescent micrographs were captured with a Marianas digital microscopy workstation (Intelligent Imaging Innovations, Denver, CO), and the number of live cells in the cultures indicated by the green fluorescence staining was counted blind to the experimental condition with the image analysis program SlideBook 4.0 (Intelligent Imaging Innovations).

**Intracellular $Ca^{2+}$ Imaging.** The intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in hippocampal neurons was measured by ratiometric $Ca^{2+}$ imaging with the $Ca^{2+}$-sensitive fluorescent dye, fura-2 AM (Molecular Probes), on an InCyt2 fluorescence imaging system (Intracellular Imaging, Cincinnati, OH). Before imaging, hippocampal neurons grown on poly-D-lysine-coated coverslips for 7 DIV were loaded with 2 $\mu$M fura-2 AM in HBS (containing 100 mM NaCl, 2.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $NaH_2PO_4$, 4.2 mM

$NaHCO_3$, 10.0 mM glucose, and 12.5 mM HEPES) at 37°C for 40 min. Excess fura-2 AM dye was removed by washing with HBS twice, and then the cultures were incubated in HBS at 37°C for 30 min to equilibrate. The coverslip with fura-2 AM-loaded neurons was mounted in a perfusion chamber placed on an inverted microscope (IMT-2; Olympus Optical, Melville, NY) equipped with epifluorescence optics (20×; Nikon, Kanagawa, Japan). The perfusion system connected to the perfusion chamber was balanced using two variable speed pumps. The cultures were perfused with HBS at a flow rate of 2 ml/min. Fura-2 AM was sequentially excited by a xenon light source at 340 and 380 nm by means of two narrow beam bandpass filters selected by a computer-controlled filter wheel. The emitted fluorescence was filtered through a 520-nm filter. A pair of images, one at 340-nm excitation and one at 380 nm, were captured with an intensified charge-coupled device camera (COHU, San Diego, CA) and analyzed with the program InCyt Im2 (Intracellular Imaging). As the $[Ca^{2+}]_i$ rises, the fluorescence intensity generated at 340-nm excitation increases, whereas the intensity at 380-nm excitation decreases. The relative $[Ca^{2+}]_i$ was determined by taking the ratio of emission intensity at 340- to 380-nm excitation.

**Western Immunoblotting.** Primary hippocampal neurons grown on poly-D-lysine-coated culture dishes for 7 DIV were treated with vehicle alone or steroids for 25 min [for analyses on ERK1/2 and protein kinase B (Akt) expression] or 48 h (for analyses on spinophilin and Bcl-2 expression). Cultures were washed with ice-cold PBS once and scraped off the dish in 1 ml of PBS. Cells were then centrifuged at 5000 rpm for 5 min, and the pellets were dissolved in the radioimmunoprecipitation assay lysis buffer (PBS, 1% Triton, 0.2% SDS, and protease and phosphatase inhibitor cocktail set I; Calbiochem, San Diego, CA) and suspended by passage through a 200-$\mu$l pipette tip. Following incubation at 4°C for 45 min, the samples were centrifuged at 12,000 rpm for 10 min, and the supernatants were collected as the whole-cell protein extracts. Protein concentration was determined by the bicinchoninic acid method. Twenty-microgram protein samples were diluted in 15 $\mu$l of 2× SDS containing sample buffer, and the final volume was 30 $\mu$l with water. After denaturation on a hot plate at 95 to 100°C for 5 min, 20 $\mu$l of the mixture was loaded per lane on 10% SDS-polyacrylamide minigels followed by electrophoresis at 90 V. A high-range Precision Protein Standard (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein size. The proteins were then electrotransferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) from the gels. Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20.

For detection of *pERK1/2* expression, membranes were incubated with the primary antibody against phospho-ERK1/2 ($pTpY^{185/187}$, polyclonal; Biosource International, Camarillo, CA) at 1:760 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:3300 dilution; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. After scanning, membranes were stripped and reprobed with the primary antibody against ERK2 (polyclonal, 1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight followed by incubation with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 1 h.

For detection of *pAkt* expression, membranes were incubated with the primary antibody against phospho-Akt ($pS^{473}$, monoclonal; Cell Signaling Technology, Beverly, MA) at 1:2000 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories). After scanning, membranes were stripped and reprobed with the primary antibody against Akt (polyclonal, 1:1000 dilution; Cell Signaling Technology) at 4°C overnight followed by incubation with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 1 h.

For detection of *spinophilin* expression, membranes were incubated with the primary antibody against spinophilin (polyclonal; Upstate, Charlottesville, VA) at 1:750 dilution in PBS-Tween at 4°C overnight and then incubated with the anti-rabbit HRP-conjugated secondary antibody (1:10,000 dilution; Vector Laboratories) at room temperature for 1 h. For detection of *Bcl-2* expression, membranes were incubated with the primary antibody against Bcl-2 (monoclonal; BD Bioscience, San Jose, CA) at 1:250 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 2 h. After scanning, membranes were stripped and reprobed with the primary antibody against $\beta$-actin (monoclonal, 1:250 dilution; Santa Cruz Biotechnology) at room temperature for 2 h followed by incubation with the anti-mouse HRP-conjugated secondary antibody (1:10,000 dilution; Vector Laboratories) at room temperature for 1 h.

All the membranes were developed with a 3,3′,5,5′-tetramethylbenzidine peroxidase substrate kit (Vector Laboratories). Relative intensities of the immunoreactive bands were quantified by optical density analysis using an image digitizing software, Un-Scan-It version 5.1 (Silk Scientific, Orem, UT).

**Statistical Analyses.** Data are presented as group means ± S.E.M. Statistically significant differences were determined by a one-way analysis of variance followed by a Newman-Keuls post hoc analysis.

## Results

**ICI 182,780 Promoted Neuronal Survival against Neurodegenerative Insults in Rat Primary Hippocampal Neurons.** Multiple in vitro and in vivo studies indicate that 17$\beta$-estradiol is a highly efficacious neuroprotective agent against a wide variety of neurodegenerative insults when administered prior to the insults (Brinton, 2001). In the course of attempts to use ICI 182,780 as an estrogen antagonist in our previous experiments, we discovered that ICI 182,780 did not function as an antagonist in neurons (L. Zhao and R. D. Brinton, unpublished data). To determine in detail the estrogen agonist and/or antagonist properties of ICI 182,780 action in neurons, we first investigated the impact of ICI 182,780 on neuronal viability when challenged by neurodegenerative insults, glutamate and $\beta$-amyloid$_{1-42}$, in a prevention model paradigm using rat primary hippocampal neurons. Hippocampal neurons grown on poly-D-lysine-coated 96-well culture plates for 7 DIV were pretreated with vehicle alone or ICI 182,780 at multiple concentrations (1, 10, 50, 100, 500, and 1000 ng/ml) for 48 h before exposure to 200 $\mu$M glutamate for 5 min followed by recovery for 24 h (Fig. 1A) or 1.5 $\mu$M $\beta$-amyloid$_{1-42}$ for 3 days (Fig. 1B). 17$\beta$-Estradiol (10 ng/ml; 36.7 nM) was used as a positive control and analyzed in parallel. Neuronal viability was determined by calcein AM staining, which is indicative of metabolically active cells. Results are presented as neuroprotective efficacy (NE), which is defined as the percentage of the neurotoxin-induced cell death prevented by experimental treatment and quantitated by the equation: $NE = (V_{treatment} - V_{neurotoxin})/(V_{control} - V_{neurotoxin}) \times 100\%$, where $V_{treatment}$ is the individual value from experimental-treated cultures, $V_{neurotoxin}$ is the mean value from neurotoxin alone-treated cultures, $V_{control}$ is the mean value from vehicle-treated control cultures, and neurotoxin refers to glutamate or $\beta$-amyloid$_{1-42}$. Exposure of neurons to either 200 $\mu$M glutamate or 1.5 $\mu$M $\beta$-amyloid$_{1-42}$ induced a significant decline in neuronal viability as demonstrated by reduced fluorescence intensity or reduced number of live cell staining in the cultures, respectively (Fig. 1, **, $P < 0.01$ compared with vehicle alone-treated control cultures). Pretreatment with ICI

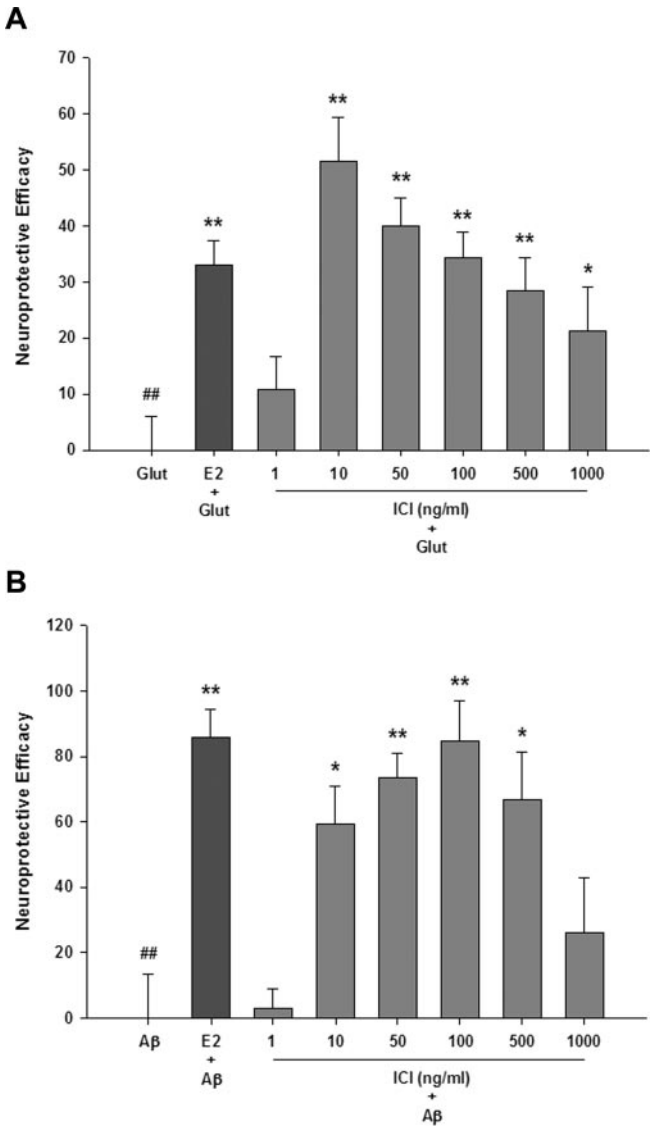


**Fig. 1.** ICI 182,780 promoted neuronal survival against excitotoxic glutamate- (A) and $\beta$-amyloid$_{1-42}$-induced (B) neurodegeneration in a concentration-dependent manner in rat primary hippocampal neurons. Hippocampal neurons grown for 7 DIV were pretreated with vehicle alone or steroids for 48 h followed by an exposure to 200 $\mu$M glutamate for 5 min followed by a 24-h recovery (A) or an incubation with 1.5 $\mu$M $\beta$-amyloid$_{1-42}$ for 3 days (B). Neuronal viability was determined with calcein AM staining to indicate metabolically live cells in the cultures. For cultures exposed to glutamate, the amount of fluorescence generated by calcein, a green fluorescent product derived from enzymatic esteration of calcein AM by esterases in live neurons, was measured at excitation/emission of 485/530 nm. For cultures exposed to $\beta$-amyloid$_{1-42}$, fluorescent micrographs were captured, and the number of live neurons was quantitated blind to the experimental condition. Results are presented as NE, which is defined as the percentage of the neurotoxin-induced cell death prevented by the steroid treatment and quantitated by the equation: $NE = (V_{treatment} - V_{neurotoxin})/(V_{control} - V_{neurotoxin}) \times 100\%$, where $V_{treatment}$ is the individual value from experimentally treated cultures, $V_{neurotoxin}$ is the mean value from neurotoxin alone-treated cultures, and $V_{control}$ is the mean value from vehicle-treated control cultures; neurotoxin refers to glutamate or $\beta$-amyloid$_{1-42}$. Data are expressed as mean $\pm$ S.E.M., $n \geq 4$. **, $P < 0.01$ compared with vehicle-treated control cultures; *, $P < 0.05$; **, $P < 0.01$ compared with neurotoxin alone-treated cultures. Glut, glutamate; A$\beta$, $\beta$-amyloid$_{1-42}$.

182,780 significantly increased neuron survival against both excitotoxic glutamate- and $\beta$-amyloid$_{1-42}$-induced neurotoxicity. The neuroprotective potency of ICI 182,780 against both insults was comparable, and 10 ng/ml was the minimally effective concentration at which near 60% of neurons survived from both insults. Against excitotoxic glutamate, 10 ng/ml ICI 182,780 exerted the greatest neuroprotection (Fig. 1A, 51.4 $\pm$ 7.8% increase in neuronal viability compared with glutamate alone-treated cultures; **, $P < 0.01$), whereas 100 ng/ml was the $EC_{100}$ against $\beta$-amyloid$_{1-42}$-induced cell death (Fig. 1B, 84.8 $\pm$ 12.3% increase in neuronal viability compared with $\beta$-amyloid$_{1-42}$ alone-treated cultures; **, $P < 0.01$). There was no statistically significant difference in neuroprotective efficacy among 10, 50, and 100 ng/ml ICI 182,780. Based on the equal efficacy of 10, 50, and 100 ng/ml, we selected 50 ng/ml as the optimal dose for further analyses because ICI 182,780 50 ng/ml exhibited nearly maximal neuroprotection against both glutamate and $\beta$-amyloid$_{1-42}$-induced neurotoxicity (Fig. 1A, 39.9 $\pm$ 5.2% increase in neuronal viability compared with glutamate alone-treated cultures; **, $P < 0.01$; Fig. 1B, 73.6 $\pm$ 7.3% increase in neuronal viability compared with $\beta$-amyloid$_{1-42}$ alone-treated cultures; **, $P < 0.01$). Moreover, 50 ng/ml ICI 182,780 induced a neuroprotective efficacy comparable with that induced by 17$\beta$-estradiol (Fig. 1A, 33.1 $\pm$ 4.2% increase in neuronal viability compared with glutamate alone-treated cultures; **, $P < 0.01$; Fig. 1B, 85.9 $\pm$ 8.6% increase in neuronal viability compared with $\beta$-amyloid$_{1-42}$ alone-treated cultures; **, $P < 0.01$). Based on these analyses, we selected 50 ng/ml (82.4 nM), a relatively low and clinically relevant concentration of ICI 182,780 (Howell et al., 1996; Robertson et al., 2004), for subsequent comparative analyses of the impact of ICI 182,780 on the molecular mechanisms underlying estrogen-inducible neurotrophism and neuroprotection (Zhao et al., 2005).

**ICI 182,780 Directly Induced a Series of Rapid But Irregular $[Ca^{2+}]_i$ Oscillations in a Manner Comparable with 17$\beta$-Estradiol in Rat Primary Hippocampal Neurons.** Although the full spectrum of molecular mechanisms that underlie estrogen promotion of neuronal plasticity and survival remains to be elucidated, a number of cellular responses have been well documented (Zhao et al., 2005). Key among these is 17$\beta$-estradiol regulation of intracellular $Ca^{2+}$ signaling (Zhao et al., 2005). Our previous studies demonstrated that 17$\beta$-estradiol induces intracellular $Ca^{2+}$ influx through voltage-dependent L-type $Ca^{2+}$ channels, which is required for downstream activation of Src and mitogen-activated protein kinase signaling pathways. This signaling pathway is required for estrogen induction of neurotrophic and neuroprotective outcomes in neurons (Wu et al., 2005; Zhao et al., 2005).

In this experiment, we determined whether ICI 182,780 would directly induce a rapid rise in $[Ca^{2+}]_i$ in neurons comparable with that induced by 17$\beta$-estradiol. Primary hippocampal neurons grown on poly-D-lysine-coated coverslips for 7 DIV were loaded with the $Ca^{2+}$-sensitive dye, fura-2 AM, followed by ratiometric intracellular $Ca^{2+}$ imaging analyses. The relative change in $[Ca^{2+}]_i$ in neurons was determined by monitoring the change in the ratio of emission fluorescence intensity at 340- to 380-nm excitation. Results of these analyses indicated that similar to with 17$\beta$-estradiol (10 ng/ml, Fig. 2A), direct perfusion of neurons with ICI 182,780 (50 ng/ml, Fig. 2B) induced a series of rapid $[Ca^{2+}]_i$ oscillations in approximately 40 to 50% of the neurons within the 30-min observation period. Eight responsive neurons are

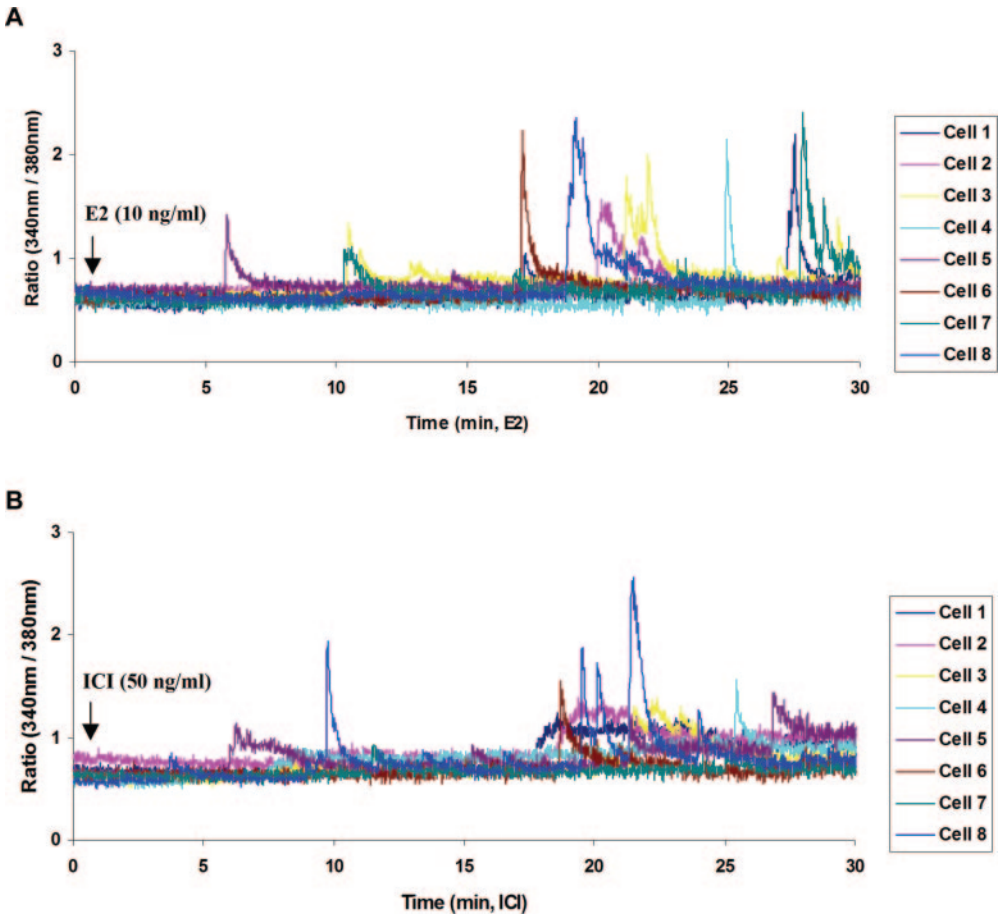


**Fig. 2.** ICI 182,780 (B, 50 ng/ml) directly induced rapid $[Ca^{2+}]_i$ oscillations in a manner comparable with that induced by 17$\beta$-estradiol (A, 10 ng/ml) in rat primary hippocampal neurons. Hippocampal neurons grown for 7 DIV were loaded with fura-2 AM before perfusion with HBS containing vehicle alone or steroids. The $[Ca^{2+}]_i$ change in neurons was recorded with the Incyt2 intracellular $Ca^{2+}$ imaging system. Results are presented as the change of the ratio of fluorescence intensities generated at 340 and 380 nm, respectively. Throughout the 30-min observational period, approximately 50% of the neurons in the same culture responded to the steroid perfusion with unique response patterns in both response time and magnitude. For illustration, eight responding neurons from the same culture are presented to demonstrate the heterogeneity of the $[Ca^{2+}]_i$ change among neurons. No response occurred in neurons perfused with vehicle alone-containing control HBS.

shown to provide a representative profile of the heterogeneity of the $[Ca^{2+}]_i$ change in both the temporal characteristics and the magnitude of response (Fig. 2). Approximately 10 to 20% of the neurons responded at an intermediate level within the first 15 min (Fig. 2). At 15 to 30 min, both 17$\beta$-estradiol and ICI 182,780-perfused cultures exhibited a greater number of responding neurons with a greater magnitude of response (Fig. 2). Overall, ICI 182,780 induced a comparable average change (0.9) in the ratio of fluorescence intensities generated at 340- and 380-nm excitation, respectively, in responding neurons, to that (1.2) induced by 17$\beta$-estradiol (Fig. 2). A change in $[Ca^{2+}]_i$ was not observed in neurons perfused with vehicle alone within the same observation period (30 min, data not shown).

**ICI 182,780 Differentially Regulated $[Ca^{2+}]_i$ Rise in Response to Glutamate in a Manner Comparable with 17$\beta$-Estradiol in Rat Primary Hippocampal Neurons.** Previous findings from our group demonstrated that estrogens, including 17$\beta$-estradiol and conjugated equine estrogens, exerted a dual regulation of the intracellular $Ca^{2+}$ dynamics in the presence of exogenous glutamate in neurons, depending on whether the concentration of glutamate is in the tolerable physiological range or in the excitotoxic range (Nilsen et al., 2002). At a physiological concentration of glutamate (25 $\mu$M) (Nilsen et al., 2002), estrogens potentiated the glutamate-induced $[Ca^{2+}]_i$ rise, a cellular response leading to estrogen promotion of neuronal morphogenesis and new synapse formation (Nilsen et al., 2002). In contrast, at an excitotoxic level of glutamate (200 $\mu$M), estrogens attenuated the glutamate-induced $[Ca^{2+}]_i$ rise in neurons, a cellular response considered as a fundamental event leading to estrogen promotion of neuronal defense and survival (Nilsen et al., 2002). Therefore, we pursued whether ICI 182,780 would regulate hippocampal neuron intracellular $Ca^{2+}$ dynamics in a manner comparable with estrogens. Primary hippocampal neurons grown on poly-D-lysine-coated coverslips for 7 DIV were pretreated with vehicle alone, 17$\beta$-estradiol (10 ng/ml), or ICI 182,780 (50 ng/ml) for 48 h prior to ratiometric intracellular $Ca^{2+}$ imaging analyses by fura-2 AM. 17$\beta$-Estradiol induced an average of 34.3% potentiation and an average 32.5% attenuation of the $[Ca^{2+}]_i$ rise induced by 25 and 200 $\mu$M glutamate, respectively (Fig. 3A, 34.3 $\pm$ 3.1% increase; Fig. 3B, 32.5 $\pm$ 3.2% reduction compared with vehicle alone-pretreated control cultures; $*$, $P < 0.05$; $**$, $P < 0.01$). ICI 182,780 induced a comparable direction and magnitude of the $[Ca^{2+}]_i$ change with those induced by 17$\beta$-estradiol. Neurons pretreated with ICI 182,780 and subsequently exposed to 25 $\mu$M glutamate exhibited an average 39.1% potentiation of the $[Ca^{2+}]_i$ rise induced by glutamate (Fig. 3A; 39.1 $\pm$ 2.8% increase compared with vehicle alone-pretreated control cultures; $*$, $P < 0.05$). Consistent with neuronal response to 17$\beta$-estradiol, ICI 182,780 protected neurons from excess intracellular $Ca^{2+}$ with an average 32.9% attenuation of the $[Ca^{2+}]_i$ rise induced by 200 $\mu$M excitotoxic glutamate (Fig. 3B; 32.9 $\pm$ 3.9% reduction compared with vehicle alone-pretreated control cultures; $**$, $P < 0.01$).

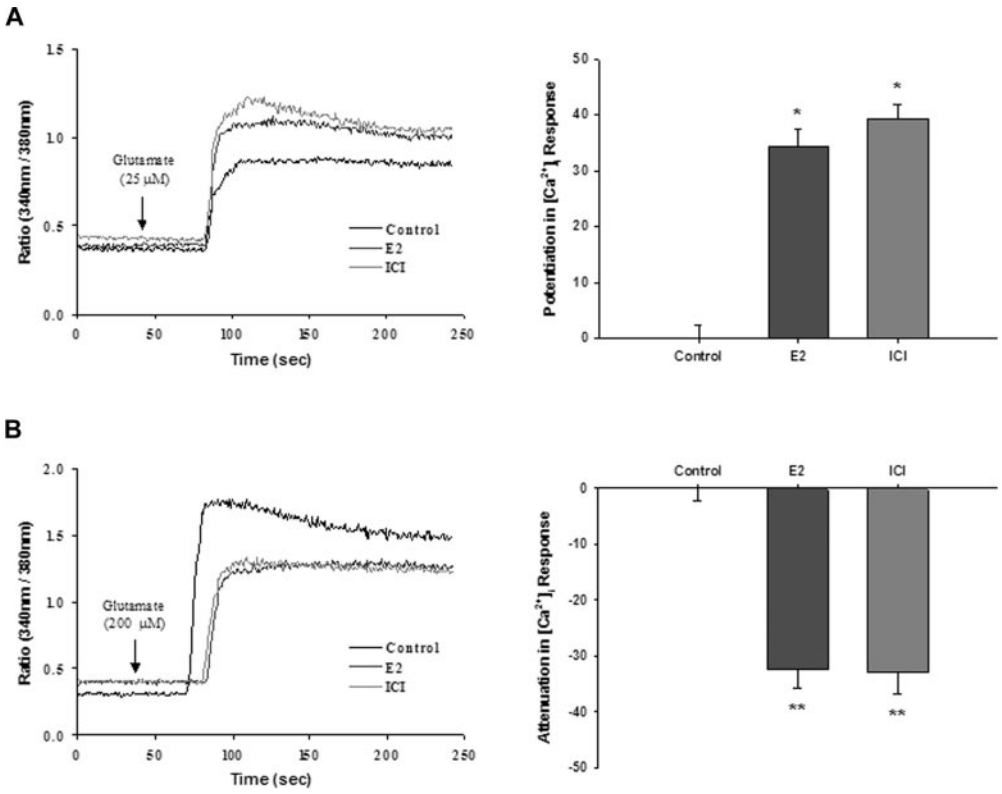


**Fig. 3.** ICI 182,780 (50 ng/ml) differentially regulated $[Ca^{2+}]_i$ rise in response to a physiological (A, 25 $\mu$M) or a neurotoxic concentration (B, 200 $\mu$M) of glutamate, in a manner comparable with that induced by 17$\beta$-estradiol (10 ng/ml) in rat primary hippocampal neurons. Hippocampal neurons grown for 7 DIV were pretreated with vehicle alone or steroids for 48 h prior to intracellular $Ca^{2+}$ imaging. Neurons were loaded with fura-2 followed by perfusion with control HBS for 40 s and then HBS containing 25 or 200 $\mu$M glutamate. The $[Ca^{2+}]_i$ change in neurons was recorded with Incyt2 intracellular $Ca^{2+}$ imaging system. In both A and B, left panels are the time-lapse $[Ca^{2+}]_i$ change in response to perfusion with glutamate, which are presented as the change of the ratio of fluorescence intensities generated at 340 and 380 nm, respectively. Right panels are the percentage of potentiation (A) and attenuation (B) of $[Ca^{2+}]_i$ rise in response to 25 or 200 $\mu$M glutamate, respectively, induced by the steroid treatment compared with vehicle alone-treated control cultures, *, $P < 0.05$; **, $P < 0.01$. Data are expressed as the mean ± S.E.M., $n \geq 3$.

**ICI 182,780 Rapidly Increased ERK1/2 and Akt Phosphorylation with Efficacy Comparable with 17$\beta$-Estradiol in Rat Primary Hippocampal Neurons.** One of the key downstream signaling events initiated by estrogen-induced intracellular $Ca^{2+}$ rise and required for activation of the transcription factor, the cyclic AMP response element-binding protein (CREB), which results in increased transcription of various neurotrophic and neuroprotective genes, is the rapid activation of Src and ERK1/2 (Wu et al., 2005; Zhao et al., 2005). In parallel, through a unified upstream mechanism, estrogen binding to ER and interaction with phosphatidylinositol-3-kinase activates the Akt signaling pathway, which plays a pivotal role in altering the expression and function of the Bcl-2 family proteins (Znamensky et al., 2003; Mannella and Brinton, 2006). Based on these findings, we determined whether ICI 182,780 would activate these same signaling mechanisms in rat primary hippocampal neurons. Neurons grown on poly-D-lysine-coated culture dishes for 7 DIV were B27 supplement-deprived for 45 min prior to incubation with vehicle alone, 17$\beta$-estradiol (10 ng/ml), ICI 182,780 (50 ng/ml), or 17$\beta$-estradiol (10 ng/ml) plus ICI 182,780 (50 ng/ml) for 25 min prior to harvesting of proteins for detection of phosphorylated ERK and Akt expression by Western immunoblotting analyses. Total ERK and Akt expression levels in the same protein samples were also detected and used as the loading controls. Results of these analyses indicate that exposure of neurons to ICI 182,780 rapidly induced a significant increase in phosphorylation of both ERK2 and Akt (Fig. 4A, 28.6 ± 5.6%; Fig. 4B, 33.1 ± 5.0% increase compared with vehicle alone-treated control cultures, respectively; **, $P < 0.01$), with efficacy comparable with that induced by 17$\beta$-estradiol (Fig. 4A, 55.0 ± 13.6%; Fig. 4B, 25.8 ± 2.3% increase compared with vehicle alone-treated control cultures, respectively; **, $P < 0.01$). The presence of ICI 182,780 failed to block the effect of 17$\beta$-estradiol and combined use of both steroids exerted a comparable impact on both pERK1/2 and pAkt (Fig. 4A, 43.0 ± 9.6%; Fig. 4B, 30.9 ± 6.9% increase compared with vehicle alone-treated control cultures, respectively; **, $P < 0.01$). There were no statistically significant differences among the 17$\beta$-estradiol, ICI 182,780, and 17$\beta$-estradiol plus ICI 182,780 treatment groups.

**ICI 182,780 Increased Spinophilin and Bcl-2 Expression with Efficacy Comparable with 17$\beta$-Estradiol in Rat Primary Hippocampal Neurons.** Spinophilin, a protein that is enriched in the heads of neuronal dendritic spines, has been demonstrated to play a significant role in modulating both dendritic morphology and glutamatergic synaptic activity (Feng et al., 2000; Lee et al., 2004). We have shown previously that activation of CREB initiated by estrogen activation of the intracellular $Ca^{2+}$/Src/ERK signaling cascade led to increased expression of spinophilin in hippocampal neurons, which is consistent with estrogen-inducible promotion of neuronal morphogenesis and synaptoplasticity (Zhao et al., 2005).

In light of these findings, we conducted Western immunoblotting analyses to determine the impact of ICI 182,780 on the expression of spinophilin in rat primary hippocampal neurons. We used $\beta$-actin as an internal loading control in our analyses because we determined that $\beta$-actin does not change in response to either 17$\beta$-estradiol or progesterone even though $\beta$-actin is enriched in spines and interacts with spinophilin. Hippocampal neurons grown for 7 DIV and treated with 50 ng/ml ICI 182,780 for 48 h had a significant increase in spinophilin expression (Fig. 5A, 44.8 ± 8.9% increase compared with vehicle alone-treated control cultures; **, $P < 0.01$), which was statistically comparable with that induced by 10 ng/ml 17$\beta$-estradiol (Fig. 5A, 29.5 ± 7.4%

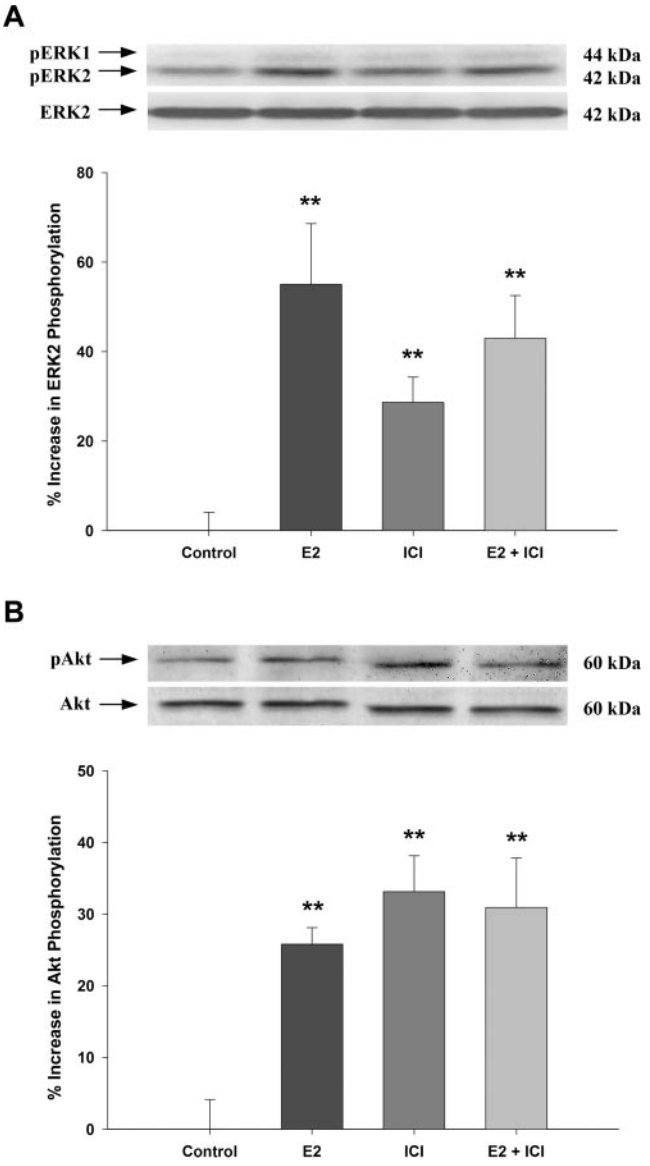


**Fig. 4.** ICI 182,780 (50 ng/ml) rapidly increased both ERK2 (A) and Akt phosphorylation (B), with efficacy comparable with 17$\beta$-estradiol (10 ng/ml) in rat primary hippocampal neurons. Hippocampal neurons grown for 7 DIV were treated with vehicle alone or steroids for 25 min followed by Western immunoblotting analyses on phosphorylated and total ERK2 and Akt expression in the whole-cell lysate preparation of neurons. pERK2 and pAkt levels were normalized against the levels of total ERK2 and Akt, respectively. Results are presented as percent increase in pERK2 (A) and pAkt (B) expression compared with vehicle alone-treated control cultures and expressed as mean $\pm$ S.E.M., $n \geq 3$; $**$, $P < 0.01$.

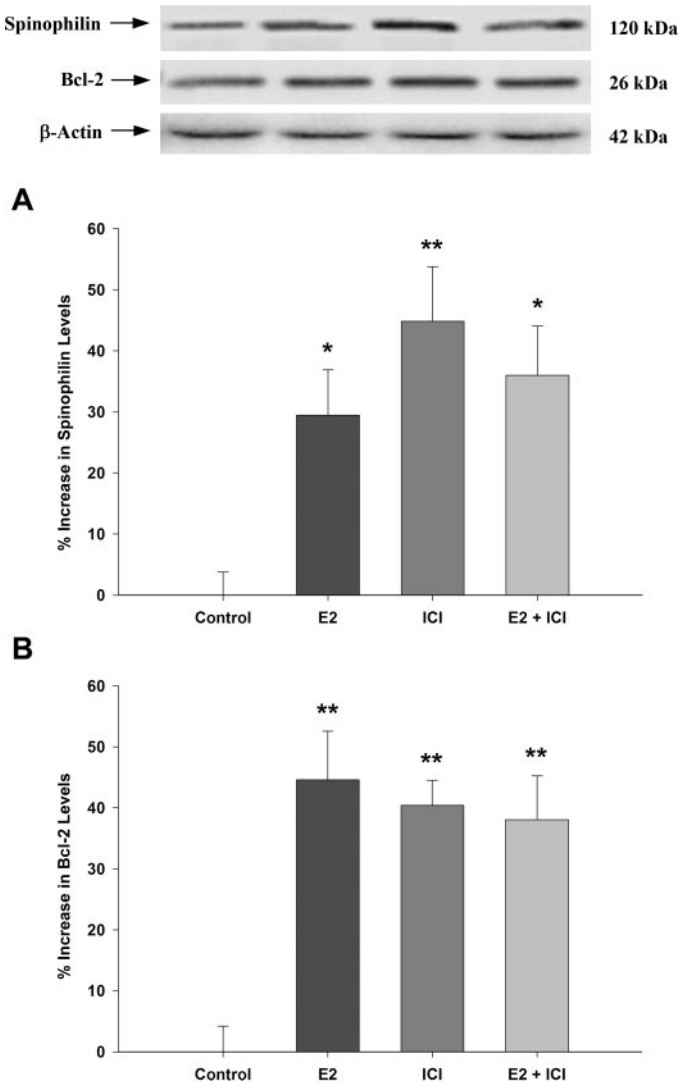


**Fig. 5.** ICI 182,780 (50 ng/ml) increased both spinophilin (A) and Bcl-2 (B) expression, with efficacy comparable with 17$\beta$-estradiol (10 ng/ml), in rat primary hippocampal neurons. Hippocampal neurons grown for 7 DIV were treated with vehicle alone or steroids for 48 h followed by Western immunoblotting analyses for spinophilin and Bcl-2 expression in whole-cell lysate preparation. $\beta$-Actin was used as an internal loading control protein. Results are presented as percent increase in the test protein expression compared with vehicle alone-treated control cultures and expressed as mean $\pm$ S.E.M., $n \geq 3$; $*$, $P < 0.05$; $**$, $P < 0.01$.

increase compared with vehicle alone-treated control cultures; $*$, $P < 0.05$). Coadministration of 17$\beta$-estradiol (10 ng/ml) and ICI 182,780 (50 ng/ml) resulted in a comparable effect with that induced by either 17$\beta$-estradiol or ICI 182,780 alone (Fig. 5A, 36.0 $\pm$ 8.1% increase compared with vehicle alone-treated control cultures; $*$, $P < 0.05$).

Estrogen activation of both intracellular $Ca^{2+}$/Src/ERK/CREB and Akt signaling cascades leads to up-regulation of the Bcl-2 family antiapoptotic proteins, such as Bcl-2 and Bcl-xl, has been proposed as a critical component underlying estrogen promotion of neuronal survival (Nilsen and Brinton, 2003). Estrogen-inducible elevation in Bcl-2 and Bcl-xl expression in neurons enhances mitochondrial $Ca^{2+}$ load tolerability induced by neurotoxic insults, leading to estrogen neuroprotection through sustained mitochondrial function (Nilsen and Brinton, 2003).

Based on these earlier findings, we evaluated the impact of ICI 182,780 on expression of Bcl-2 protein in rat primary hippocampal neurons. Neurons grown for 7 DIV were treated with vehicle alone, 17$\beta$-estradiol (10 ng/ml), ICI 182,780 (50 ng/ml), or 17$\beta$-estradiol (10 ng/ml) plus ICI 182,780 (50 ng/ml) for 48 h followed by Western immunoblotting analyses. Results of these analyses indicated that both 17$\beta$-estradiol and ICI 182,780 significantly and comparably increased Bcl-2 expression in neurons (Fig. 5B; 44.6 $\pm$ 8.0% and 40.4 $\pm$ 4.1% increase compared with vehicle alone-treated control cultures, respectively; $**$, $P < 0.01$). The presence of ICI 182,780 not only failed to block the effect of 17$\beta$-estradiol, but the combined use of 17$\beta$-estradiol and ICI 182,780 induced a

comparable increase in Bcl-2 expression with that induced by either 17β-estradiol or ICI 182,780 alone (Fig. 5B; 38.0 ± 7.2% increase compared with vehicle alone-treated control cultures; **, $P < 0.01$). There were no statistically significant differences among the 17β-estradiol, ICI 182,780, and 17β-estradiol plus ICI 182,780 treatment groups.

## Discussion

Data presented herein provide in vitro evidence indicating an estrogenic agonist profile of ICI 182,780 action, at a clinically relevant concentration, in rat primary hippocampal neurons. First, the data demonstrate that ICI 182,780 was an efficacious neuroprotective agent against neurodegenerative insults associated with Alzheimer's disease and related disorders. Second, ICI 182,780 significantly activated biochemical mechanisms required for estrogen promotion of neuronal plasticity and neuroprotection. The data further demonstrate that ICI 182,780-induced neuronal responses were of comparable magnitude with those induced by 17β-estradiol. Moreover, the presence of ICI 182,780 did not inhibit 17β-estradiol-induced neural responses.

Our findings are in contrast to the conventional classification of ICI 182,780 as a "full" antiestrogen in ER-positive tissues. In addition to our data derived from hippocampal neurons, studies by the Simpkins group demonstrated that ICI 182,780 was protective against β-amyloid$_{25–35}$ or $H_2O_2$ toxicity in SK-N-SH and human lens cells (Wang et al., 2003, 2006; Kumar et al., 2005). Estrogen agonist properties of ICI 182,780 have also been observed in bone where ICI 182,780 promoted bone growth (Sibonga et al., 1998). Taken together, these earlier investigations along with our present analyses suggest that as with other SERMs, exemplified by TMX and raloxifene (RAL), ICI 182,780 should also be classified as a mixed estrogen agonist/antagonist.

We propose that one factor to account for the "inversed" pharmacological properties exhibited by ICI 182,780 in distinct tissues/cells could be the nature of an estrogen-binding receptor involved. Estrogen antagonism of ICI 182,780 in reproductive tissues has been associated with the conformational remodeling of the classical ER induced by its bulky side chain substituted on the 7α position of the 17β-estradiol core structure. The terminal portion of this long tail structure, present as an additional structure absent in both TMX and RAL, binds along the coactivator recruitment site in the AF-2 cleft, which in turn precludes helix 12 from adopting either the characteristic agonist orientation, as seen with 17β-estradiol, in which helix 12 is aligned over the ligand-binding pocket (Brzozowski et al., 1997), or the AF-2 antagonist orientation, as seen with TMX and RAL, in which helix 12 orients along the coactivator binding surface in AF-2 (Brzozowski et al., 1997; Shiau et al., 1998). One functional consequence derived from this conformational remodeling of ER induced by ICI 182,780 is the impaired dimerization of ER, a structural prerequisite for the translocation of ER into the nucleus, resulting in the inhibition of the ER-DNA binding (Fawell et al., 1990; Brzozowski et al., 1997). The estrogenic agonist activity of ICI 182,780 in parallel to 17β-estradiol in hippocampal neurons suggests that these two ER ligands, although they exhibit opposite pharmacological profiles in some ER-positive tissues, in particular reproductive tissues, are able to activate the same molecular target and the downstream signaling cascades, leading to their estrogen agonist actions in neurons. In view of the unique characteristics of ICI 182,780 interaction with ER, these analyses may suggest that the parallel estrogenic agonist activity of 17β-estradiol and ICI 182,780 in hippocampal neurons is likely mediated by a nonclassical signaling pathway separate from proliferative actions of estrogen in reproductive tissues.

Increasing evidence indicates that there is a plethora of structural variants of ERα and ERβ enriched in the brain (Brinton and Nilsen, 2001; Toran-Allerand, 2004), which may carry out distinct functions from the classical nuclear receptors and serve as the molecular basis to confer ICI 182,780-induced agonist activity in neurons. In support of this hypothesis, specific mutations in the ER could reverse the antiestrogenic property of an ER antagonist to that representing an estrogen agonist. For instance, a study conducted by Mahfoudi et al. (1995) demonstrated that both TMX and ICI 164,384 acted as potent agonists in HeLa cells expressing ER mutants introduced in a conserved hydrophobic region of AF-2. Such mutations also promoted the stability and nuclear localization of the receptor compared with the wild-type ER following ICI 164,384 treatment (Mahfoudi et al., 1995). Based on this observation, it is conceivable that there might exist ER variants (e.g., with the structural alteration in AF-2) in neurons; binding of ICI compounds could induce the repositioning of helix 12 distinct from that observed in the full-length receptor and lead to exposure of the AF-2 without affecting the dimerization and nuclear uptake capability of the receptors.

Alternatively, studies from multiple laboratories, including our own, indicate that a membrane-associated estrogen-binding receptor induces the rapid actions of 17β-estradiol and ICI 182,780 in neurons (Brinton, 2001; McEwen, 2002). Based on our recent observations that both ERα-selective agonist PPT and ERβ-selective agonist DPN were effective in activating the neurotrophic and neuroprotective outcomes and the same underlying mechanisms, we propose that this membrane-associated estrogen-binding receptor could be ERα or ERβ or their structural analogs (Zhao et al., 2004). This hypothesis is partially supported by increasing evidence indicating the subcellular localization of ERα and ERβ in non-nuclear compartments including membranes in rat and mouse cortical and hippocampal neurons (Milner et al., 2001; Nishio et al., 2004; Kalita et al., 2005). In addition, our observation that PPT and DPN compete for the same membrane binding site as the BSA-17β-estradiol conjugate (BSA-17β-estradiol-fluorescein isothiocyanate) in rat hippocampal neurons provides indirect evidence for membrane localization of ERα and ERβ or at least ERα- and ERβ-related receptors (Wu et al., 2005). Functionally, it is likely that these membrane-associated classical ER-related receptors could associate and act with other membrane structures, leading to initiation of signaling cascades (Norman et al., 2004; Mannella and Brinton, 2006).

An alternative hypothesis for a membrane-associated estrogen-binding receptor in neurons could be a structurally distinct class of receptors independent of the classical ER. In support of this hypothesis, recent discoveries demonstrated that a transmembrane G protein-coupled receptor, GPR30, although it remains controversial whether it localizes to the plasma membrane or exclusively to the endoplasmic reticulum, can be activated by a number of the same ER ligands

and responsible for estrogen-induced rapid signaling independently of classical ER in some cell types, including ER-negative cell types (Revankar et al., 2005; Thomas et al., 2005). ICI 182,780 exhibits high binding affinity to GPR30 and acts as an agonist of GPR30 (Thomas et al., 2005), which has important implications for a membrane-associated estrogen-binding receptor in mediating the consistent agonistic effects of 17$\beta$-estradiol and ICI 182,780 in hippocampal neurons.

From a therapeutic development perspective, our findings that ICI 182,780 acts as an estrogen agonist in neurons provides a translational opportunity for development of an ideal brain-selective ER modulator that mimics the beneficial effects of estrogen agonists in brain while lacking or antagonizing activation of estrogenic proliferative responses in reproductive organs (neuro-SERMs) (Zhao et al., 2005). Because ICI 182,780 does not readily cross the blood-brain barrier (Howell et al., 2000), design and development of a brain-accessible ICI-like molecule that is structurally and functionally analogous to ICI 182,780 while possessing improved chemical features that promote blood-brain barrier penetration is required. The availability of such a molecule will provide a valuable pharmacological tool to further decipher the in vivo activity of this category of ER ligands in the brain. Proof of principle of the in vivo efficacy of ICI to mimic neuro-SERMs is anticipated to provide a dual therapeutic profile to treat ER-positive breast cancers while simultaneously preventing estrogen deficiency-associated cognitive decline and neurodegeneration in postmenopausal women.

**Acknowledgments**

We gratefully thank Sean Iwamoto for providing excellent technical support.

**References**

Brinton RD (2001) Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: recent insights and remaining challenges. *Learn Mem* **8:**121–133.

Brinton RD and Nilsen J (2001) Sex hormones and their brain receptors. *Int Encyclopedia Soc Behav Sci* **21:**13946–13951.

Bross PF, Cohen MH, Williams GA, and Pazdur R (2002) FDA drug approval summaries: fulvestrant. *Oncologist* **7:**477–480.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature (Lond)* **389:**753–758.

Cambiasso MJ and Carrer HF (2001) Nongenomic mechanism mediates estradiol stimulation of axon growth in male rat hypothalamic neurons in vitro. *J Neurosci Res* **66:**475–481.

Dauvois S, White R, and Parker MG (1993) The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *J Cell Sci* **106:**1377–1388.

Dominguez R, Jalali C, and de Lacalle S (2004) Morphological effects of estrogen on cholinergic neurons in vitro involves activation of extracellular signal-regulated kinases. *J Neurosci* **24:**982–990.

Fawell SE, White R, Hoare S, Sydenham M, Page M, and Parker MG (1990) Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. *Proc Natl Acad Sci USA* **87:**6883–6887.

Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC, and Greengard P (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc Natl Acad Sci USA* **97:**9287–9292.

Howell A, DeFriend DJ, Robertson JF, Blamey RW, Anderson L, Anderson E, Sutcliffe FA, and Walton P (1996) Pharmacokinetics, pharmacological and antitumour effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. *Br J Cancer* **74:**300–308.

Howell A, Osborne CK, Morris C, and Wakeling AE (2000) ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. *Cancer* **89:**817–825.

Kalita K, Szymczak S, and Kaczmarek L (2005) Non-nuclear estrogen receptor beta and alpha in the hippocampus of male and female rats. *Hippocampus* **15:**404–412.

Kumar DM, Perez E, Cai ZY, Aoun P, Brun-Zinkernagel AM, Covey DF, Simpkins JW, and Agarwal N (2005) Role of nonfeminizing estrogen analogues in neuroprotection of rat retinal ganglion cells against glutamate-induced cytotoxicity. *Free Radic Biol Med* **38:**1152–1163.

Lee SJ, Romeo RD, Svenningsson P, Campomanes CR, Allen PB, Greengard P, and McEwen BS (2004) Estradiol affects spinophilin protein differently in gonadectomized males and females. *Neuroscience* **127:**983–988.

Linstedt AD, West NB, and Brenner RM (1986) Analysis of monomeric-dimeric states of the estrogen receptor with monoclonal antiestrophilins. *J Steroid Biochem* **24:**677–686.

Mahfoudi A, Roulet E, Dauvois S, Parker MG, and Wahli W (1995) Specific mutations in the estrogen receptor change the properties of antiestrogens to full agonists. *Proc Natl Acad Sci USA* **92:**4206–4210.

Mannella P and Brinton RD (2006) Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J Neurosci* **26:**9439–9447.

McEwen B (2002) Estrogen actions throughout the brain. *Recent Prog Horm Res* **57:**357–384.

Miller NR, Jover T, Cohen HW, Zukin RS, and Etgen AM (2005) Estrogen can act via estrogen receptor alpha and beta to protect hippocampal neurons against global ischemia-induced cell death. *Endocrinology* **146:**3070–3079.

Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, and Alves SE (2001) Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J Comp Neurol* **429:**355–371.

Nilsen J and Brinton RD (2003) Mechanism of estrogen-mediated neuroprotection: regulation of mitochondrial calcium and Bcl-2 expression. *Proc Natl Acad Sci USA* **100:**2842–2847.

Nilsen J, Chen S, and Brinton RD (2002) Dual action of estrogen on glutamate-induced calcium signaling: mechanisms requiring interaction between estrogen receptors and src/mitogen activated protein kinase pathway. *Brain Res* **930:**216–234.

Nishio M, Kuroki Y, and Watanabe Y (2004) Subcellular localization of estrogen receptor beta in mouse hippocampus. *Neurosci Lett* **355:**109–112.

Norman AW, Mizwicki MT, and Norman DP (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* **3:**27–41.

Purves-Tyson TD and Keast JR (2004) Rapid actions of estradiol on cyclic amp response-element binding protein phosphorylation in dorsal root ganglion neurons. *Neuroscience* **129:**629–637.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science (Wash DC)* **307**:1625–1630.

Robertson JF, Erikstein B, Osborne KC, Pippen J, Come SE, Parker LM, Gertler S, Harrison MP, and Clarke DA (2004) Pharmacokinetic profile of intramuscular fulvestrant in advanced breast cancer. *Clin Pharmacokinet* **43:**529–538.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95:**927–937.

Sibonga JD, Dobnig H, Harden RM, and Turner RT (1998) Effect of the high-affinity estrogen receptor ligand ICI 182,780 on the rat tibia. *Endocrinology* **139:**3736–3742.

Thomas P, Pang Y, Filardo EJ, and Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146**:624–632.

Toran-Allerand CD (2004) Minireview: a plethora of estrogen receptors in the brain: where will it end? *Endocrinology* **145:**1069–1074.

Wakeling AE, Dukes M, and Bowler J (1991) A potent specific pure antiestrogen with clinical potential. *Cancer Res* **51:**3867–3873.

Wang X, Dykens JA, Perez E, Liu R, Yang S, Covey DF, and Simpkins JW (2006) Neuroprotective effects of 17beta-estradiol and nonfeminizing estrogens against H2O2 toxicity in human neuroblastoma SK-N-SH cells. *Mol Pharmacol* **70**:395–404.

Wang X, Simpkins JW, Dykens JA, and Cammarata PR (2003) Oxidative damage to human lens epithelial cells in culture: estrogen protection of mitochondrial potential, ATP, and cell viability. *Investig Ophthalmol Vis Sci* **44:**2067–2075.

Wong JK, Le HH, Zsarnovszky A, and Belcher SM (2003) Estrogens and ICI182,780 (Faslodex) modulate mitosis and cell death in immature cerebellar neurons via rapid activation of p44/p42 mitogen-activated protein kinase. *J Neurosci* **23:**4984–4995.

Wu T-W, Chen S, Nilsen J, and Brinton RD (2005) Estradiol induction of intracellular calcium rise and ERK activation via membrane estrogen binding sites in single hippocampal neurons, in *Endocrine Society Annual Meeting*; 2005 June 4–7; San Diego, CA.. The Endocrine Society, Chevy Chase, MD.

Wu T-W, Wang JM, Chen S, and Brinton RD (2005) 17beta-Estradiol induced $Ca^{2+}$ influx via L-type calcium channels activates the Src/ERK/cyclic-AMP response element binding protein signal pathway and BCL-2 expression in rat hippocampal neurons: a potential initiation mechanism for estrogen-induced neuroprotection. *Neuroscience* **135:**59–72.

Zhao L, Chen S, Wang JM, and Brinton RD (2005) 17beta-Estradiol induces $Ca^{2+}$ influx, dendritic and nuclear $Ca^{2+}$ rise and subsequent cyclic AMP response element-binding protein activation in hippocampal neurons: a potential initiation mechanism for estrogen neurotrophism. *Neuroscience* **132:**299–311.

Zhao L, O'Neill K, and Brinton RD (2005) Selective estrogen receptor modulators (SERMs) for the brain: current status and remaining challenges for developing neuroSERMs. *Brain Res Brain Res Rev* **49:**472–493.

Zhao L, Wu T-W, and Brinton RD (2004) Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. *Brain Res* **1010:**22–34.

Znamensky V, Akama KT, McEwen BS, and Milner TA (2003) Estrogen levels regulate the subcellular distribution of phosphorylated Akt in hippocampal CA1 dendrites. *J Neurosci* **23:**2340–2347.

**Address correspondence to:** Dr. Roberta Diaz Brinton, Molecular Pharmacology and Toxicology, Neuroscience, and Biomedical Engineering, University of Southern California, 1985 Zonal Avenue, PSC 503, Los Angeles, CA 90089. E-mail: rbrinton@usc.edu