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Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan and lindane in primary neuronal cultures

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ABSTRACT

The organochlorine chemicals endosulfan, dieldrin, and γ-hexachlorocyclohexane (γ-HCH, lindane) are persistent pesticides to which people are exposed mainly via diet. Their antagonism of the γ-aminobutyric acid-A ($GABA_A$) receptor makes them convulsants. They are also endocrine disruptors because of their interaction with the estrogen receptor (ER). Here, we study the effects of dieldrin, endosulfan and lindane on ERs in primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC). All the compounds tested inhibited the binding of [^{3}H]-estradiol to the ER in both CN and CGC, with dieldrin in CGC showing the highest affinity. We also determined the effects of the pesticides on protein kinase B (Akt) and extracellular-regulated kinase 1 and 2 (ERK1/2) phosphorylation. Dieldrin and endosulfan increased Akt phosphorylation in CN, which was inhibited by the ERβ antagonist PHTPP. Instead, Akt and ERK1/2 phosphorylation induced by dieldrin in CGC was mediated by multiple activation of ERα, ERβ and G-protein coupled receptor 30. Lindane did not activate these pathways but it inhibited estradiol-mediated Akt and ERK1/2 activation. In CN, all the chemicals activated ERK1/2 through a mechanism involving $GABA_A$ and glutamate receptors. Long-term exposure to these pesticides reduced the levels of ERα, but not of ERβ. Moreover, extracts of CN treated with endosulfan, dieldrin or lindane induced cell proliferation in MCF-7 human breast cancer-derived cells, whereas only extracts of CGC treated with dieldrin induced MCF-7 cell proliferation. Overall, the observed alterations on ER-mediated signaling and ER levels in neurons might contribute to the neurotoxicity of these organochlorine pesticides.

Keywords: Pesticides; estrogen receptor; protein kinase B (Akt); extracellular-regulated kinase (ERK); $GABA_A$ receptor; neuronal cultures

INTRODUCTION

Dieldrin, endosulfan and hexachlorocyclohexane (HCH) are organochlorine chemicals that have been used in agriculture and pharmaceutical applications for many years. They all persist in the environment and bioaccumulate in animals and plants, leading to instances of food contamination (Gonzalez *et al.*, 2003; Hites *et al.*, 2004) and eventually dietary exposure in humans (Mariscal-Arcas *et al.*, 2010). Because of their persistence and toxicity, dieldrin and γ-HCH (lindane) are listed in the Stockholm Convention on Persistent Organic Pollutants (http://www.pops.int); while the status of endosulfan is currently under review. Despite the uses and production of many organochlorine pesticides (OCPs) are banned in most developed countries, the general population is still exposed to them (Bouvier *et al.*, 2006). Furthermore, dieldrin and lindane have been detected in post-mortem human brain from Parkinson Disease patients at higher levels than in control ones (Corrigan *et al.*, 2000; Fleming *et al.*, 1994).

Estrogens represent an important class of hormones that can promote the development, maturation and function of the central nervous system (CNS). The wide distribution of estrogen receptors (ERs) in brain tissue supports the view that estrogens are important to the functioning of the CNS. For instance, 17β-estradiol (E2) plays a crucial role in synaptogenesis, reproductive behavior and neuronal survival (Garcia-Segura *et al.*, 2001; Micevych and Dominguez, 2009) and different roles of ERα and ERβ in mediating these effects have been reported (Morissette *et al.*, 2008; Zhao and Brinton, 2007). The effects of E2 have often been said to be mediated via ERs located in the cytoplasm or the nuclear membrane and thus the effect of E2 on the regulation of target genes has been studied. However, recent findings indicate that E2 also acts on plasma membrane-bound ERs to initiate intracellular signaling pathways and regulate cellular

functions; these are known as non-genomic actions. Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways are believed to be involved in such ER-mediated neuroendocrine events and neuroprotection (Mendez *et al.*, 2005; Morissette *et al.*, 2008; Ogiue-Ikeda *et al.*, 2008; Zhao and Brinton, 2007). In addition, the membrane G protein-coupled receptor 30 (GPR30) has been described to mediate some of the non genomic actions of E2 in non-neuronal cells, such as activation of MAPK (Filardo *et al.*, 2000). Although GPR30 is widely distributed in the rodent brain (Hazell *et al.*, 2009), its involvement in the neuroendocrine effects of estrogens is poorly understood.

The estrogenic activity of dieldrin, endosulfan and lindane has been demonstrated *in vitro* in human breast cancer-derived cells and recombinant cell lines (Lemaire *et al.*, 2006; Maranghi *et al.*, 2007; Soto *et al.*, 1994; Soto *et al.*, 1995). Moreover, the three OCPs have been shown to act as antagonists of the androgen receptor, while endosulfan and lindane inhibit aromatase activity (Andersen *et al.*, 2002; Li *et al.*, 2008; Nativelle-Serpentini *et al.*, 2003), the enzyme responsible for E2 synthesis. Prolonged exposure to these pollutants modifies the expression of both ERα and ERβ in MCF-7 human-derived breast cancer cells (Grunfeld and Bonefeld-Jorgensen, 2004). Studies of endocrine disruptors have largely focused on the genomic pathways. However, there is growing concern about the non-genomic responses to these compounds. For example, OCPs have been reported to increase extracellular-regulated kinase 1 and 2 (ERK1/2) and Akt phosphorylation by activating ER in cell lines (Bulayeva and Watson, 2004; Li *et al.*, 2006). Furthermore, some endocrine disruptors (including OCPs) have been described to activate GPR30 in cell lines (Thomas and Dong, 2006). However, whether dieldrin, endosulfan or lindane bind and activate neuronal ERs remains to be determined.

Several studies have reported that animals chronically exposed to OCPs have learning and behavioral deficits and alterations in locomotor activity (Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). Also, they can affect the development of several neurotransmitter systems, including aminoacidergic (GABAergic and glutamatergic) and monoaminergic (serotonergic) (Briz *et al.*, 2010; Cabaleiro *et al.*, 2008; Liu *et al.*, 1997). Some of the latest effects have been attributed to their inhibitory action at the $GABA_A$ receptor (Pomes *et al.*, 1994; Vale *et al.*, 2003). However, the molecular mechanisms underlying such cognitive impairments are not yet fully understood. (Ogiue-Ikeda *et al.*, 2008) recently reported that some xenoestrogens are able to modulate synaptic plasticity and spinogenesis in hippocampal neurons. Nevertheless, little attention has been paid on their endocrine disrupting activity in the CNS (Bulayeva and Watson, 2004; Schantz and Widholm, 2001).

The objective of the present work was to examine the potential activity of dieldrin, endosulfan and lindane on neuronal ERs in order to evaluate them as possible targets of OCPs in the CNS. Two different neuronal cultures were used: cerebellar granule cells (CGC) and cortical neurons (CN), because the cortex and cerebellum are known to be involved in important brain functions such as memory processes and motor coordination. Furthermore, ERα and ERβ are present in CGC and in the mice cortex, and show different patterns of expression during development (Belcher, 1999; Prewitt and Wilson, 2007). We aimed to study the effects of these OCPs on two of the major intracellular signaling pathways associated with ER activation: MAPK and PI3K/Akt, both in the absence and presence of E2. We also wanted to address the involvement of the different ERs in these estrogenic actions of OCPs, by using specific antagonists of ERα, ERβ and GPR30. The effects of prolonged exposure to these pollutants on ERα and ERβ levels were also studied. Finally, to further characterize the short- and long-

term estrogenic effects on neurons of the chemicals tested, we used the E-Screen assay, in which MCF-7 human breast cancer-derived cells were incubated with extracts of CGC and CN that had previously been exposed to these pollutants.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16th gestational day) and mice pups (7th postnatal day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multiwell plates were from Nunc™ (Rockilde, Denmark). Fetal bovine serum (FBS) was obtained from Gibco (Invitrogen, Barcelona, Spain). Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Biochrom (Berlin, Germany) and phenol red-free DMEM was from Thermo Scientific HyClone (Logan, UT, USA). Isofluorane (FORANE®) was from Abbot Laboratories (Madrid, Spain). Trypsin, soybean trypsin inhibitor, DNAse, bovine serum albumin (BSA), charcoal, ethylenediaminetetraacetic acid (EDTA), DMSO, dieldrin (97.9% of purity), α-endosulfan (99.6% of purity), 17β-estradiol (E2), methyl-piperidino-pyrazole (MPP) dihydrochloride, picrotoxinin, (+)-MK-801 hydrogen maleate (MK-801) and sulphorhodamine-B (SRB) were from Sigma (St. Louis, MO, USA). β-HCH (99% of purity) was from LGC (Teddington Middlesex, U.K.). Lindane (99% of purity) was from the Institute of Industrial Organic Chemistry (Warsaw, Poland). ICI182780, (3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G-15), 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) were from Tocris Cookson (Bristol, U.K.). Dextran T-70 was from Pharmacia-LKB (Uppsala, Sweden). [2,4,6,7-

^{3}H]-17β-estradiol ([^{3}H]-E2, 88 Ci/mmol) was from Amersham Biosciences (GE Healthcare, Buckinghamshire, U.K.). Optiphase 'Hisafe'2 liquid scintillation cocktail was from Wallac Oy (Turku, Finland).

Neuronal Cultures

Primary cultures of CN and of CGC were prepared from cerebral cortices of 16th-gestational-day mice fetuses and from cerebellum of 7-day-old mice pups, respectively, as previously described (Babot *et al.*, 2007; Briz *et al.*, 2010). For CN, pregnant animals were anesthetized with isofluorane, killed by cervical dislocation and the fetuses extracted. Cortices were dissected with forceps, mechanically minced and cells were then dissociated by mild trypsinization [0.02% (w/v)] at 37ºC for 10 min followed by trituration in a DNAse solution [0.004% (w/v)] containing soybean trypsin inhibitor [0.05% (w/v)]. The cells were then suspended in DMEM containing 5mM KCl, 31mM glucose and 0.2mM glutamine supplemented with p-aminobenzoate, insulin, penicillin and 10% fetal calf serum. The cell suspension (1.5 x 10^{6} cells ml^{-1}) was seeded in 6- or 24-well plates precoated with poly-D-lysine and incubated for at least 8 days in a humidified 5% CO_2/95% air atmosphere at 37ºC. A mixture of 5µM 5-fluoro-2'-deoxyuridine and 20µM uridine was added after 1-2 days *in vitro* (DIV) to prevent glial proliferation. In the case of CGC, the cerebellum was removed after decapitation and cells were dissociated by mild trypsinization [0.025% (w/v)] at 37ºC for 15 min followed by trituration in a DNAse solution [0.004% (w/v)] containing soybean trypsin inhibitor [0.06% (w/v)]. From this step on, the procedure is the same as for CN, but the DMEM contains 25mM KCl. Animals were handled in compliance with protocols approved by the Generalitat de Catalunya regional authority, Spain, following the EU guidelines.

MCF-7 cell line

Cloned MCF-7 human breast cancer cells were grown in DMEM supplemented with 10% FBS in an atmosphere of 5% CO2/95% air under saturating humidity at 37ºC. The cells were subcultivated at weekly intervals using a mixture of 0.05% trypsin and 0.01% EDTA.

Charcoal-dextran treatment of serum to remove sex steroids

Sex steroids were removed from FBS by charcoal-dextran (CD) stripping. Briefly, a suspension of 5% charcoal with 0.5% dextran T-70 was prepared. Aliquots of the CD suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1,000 x *g* for 10 min. Supernatants were aspirated and the serum aliquots were mixed with the charcoal pellets. This CD-serum mixture was maintained in suspension by rolling (6 cycles/min) at 37ºC for 1 h. The suspension was centrifuged at 2000 x *g* for 20 min, and the supernatant was then filtered through a 0.22-mm filter (Millipore). CD-treated FBS (CD-FBS) was stored at −20ºC until needed. For some experiments, a commercial CD-treated serum was used (Gibco).

Chemical Treatments

Stock solutions for each compound were prepared in DMSO and frozen in aliquots of 100 µl. The final concentration of DMSO in the culture medium was < 0.5%. To avoid cross-contamination between different wells in the same plate, DMSO or OCPs were each always added to separate plates. Cultured neurons were exposed for short periods of time (≤ 5 h) to OCPs alone or in combination with the ER agonist/antagonist in Hank's solution (1.3mM CaCl, 5.4mM KCl, 0.4mM KH_2PO_4, 0.5mM MgCl, 0.4mM $MgSO_4$, 137mM NaCl, 4.2mM $NaHCO_3$, 0.3mM Na_2HPO_4, 8mM HEPES, 5.5mM glucose, adjusted to pH 7.4), unless otherwise stated. The concentrations of OCPs used in these experiments were generally chosen ranging from the lowest observed effect concentration (LOECs) up to the half-inhibitory concentration (IC_{50}), both obtained

from the [^{3}H]-E2 binding assay (Table 1). In contrast, for prolonged exposure periods, cells were treated after 1-2 DIV by adding the stock pesticide solution or DMSO to the culture medium. The medium was not changed until the experiments were performed, generally at 7-8 DIV. In this case, the concentrations used were those previously reported to have long-term effects in neuronal endpoints in these cultures (Babot *et al.*, 2007; Briz *et al.*, 2010). In order to examine the effects of the cultured neuronal cells exposed to OCPs on MCF-7 cell proliferation, CGC and CN were grown in 6-well plates and the medium was replaced for phenol red-free DMEM containing 5% CD (CD-DMEM) after 1-2 DIV. CGC and CN were treated with the chemicals for different exposure times. At the end of the exposure time (5 h or 48 h), the cells were washed three times with 1.5 ml of sterile phosphate-buffered solution (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4), scrapped and collected in 200 µl/well of sterile PBS. They were stored at –80ºC until used.

[^{3}H]-E2 binding assay

[^{3}H]-E2 binding experiments were performed on intact cultured neurons grown for at least 8 DIV in 24-well plates. Cells were washed three times with Hank's solution and incubated with [^{3}H]-E2 in Hank's solution for 5 h at 37ºC. Cells were washed three times with 1.5 ml of cold Hank's solution and then incubated with 0.2 ml of ethanol for 30 min at room temperature. Ethanol extracts were collected and then their radioactivity was measured by liquid scintillation counting (with Optiphase 'Hisafe'2 cocktail). Saturation curves were obtained by using different [^{3}H]-E2 concentrations (0.02–4nM) whereas a fixed [^{3}H]-E2 concentration (0.5–1nM) and 6–8 concentrations of OCPs were used to obtain the competition curves. Non-specific binding was determined in the presence of 50µM of unlabeled E2. Specific binding was calculated by subtracting the non-specific binding. Apparent K_d and B_{max} parameters and half-inhibitory concentration

(IC_{50}) of each pesticide were determined by adjusting specific [^{3}H]-E2 binding into a one-site saturation and competition binding curve, respectively.

Western Blot

Neuronal cultures grown in 6-well plates were washed twice with cold Hank's solution and cells were harvested with 0.2 ml of loading buffer [62.5mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 50mM dithiothreitol] and briefly sonicated. After boiling for 5 min and centrifugation at 16,100 x g for 5 min, 15-25 µg of protein from each sample were subjected to SDS-PAGE using 10-12% polyacrilamide resolving gel at 60 mA for 1.5-2 h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% non-fat dry milk in TBS-T (20mM Tris-HCl (pH 7.6), 140mM NaCl, 0.1% Tween-20). Membranes were incubated overnight at 4 ºC with the following primary antibodies: rabbit polyclonal anti-Akt, rabbit monoclonal anti-p44/42 MAPK, anti-phospho-Akt (Ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (All 1:2000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-ERα or anti-ERβ (both 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All primary antibodies were diluted in TBS-T containing 5% BSA, except anti-ERβ which was diluted in TBS-T containing 5% non-fat dry milk. After the membranes were washed, they were incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated (HRP) secondary antibody (1:4000, Jackson InmunoResearch, West Grove, PA, USA). On all the membranes, a monoclonal anti-actin (1:10000, Sigma) or anti-GAPDH (1:4000, Assay Designs, Ann Arbor, MI, USA) and a secondary HRP-linked anti-mouse (1:8000, Jackson InmunoResearch) antibodies were used as a control of the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP Kit, Bio-Rad, Hercules, CA, USA). Luminescence was quantified with a Versadoc Imagine

System (Bio-Rad). Digital images were then quantified by using the Quantity One software (Bio-Rad).

E-Screen bioassay

MCF-7 cells were used in the test of estrogenicity according to a technique slightly modified from that originally described in (Soto *et al.*, 1994). Briefly, MCF-7 cells were trypsinized and seeded in 24-well plates at initial concentrations of 1 x 10^4 cells per well in 10% FBS in DMEM. Cells were allowed to attach for 24 h; then, the seeding medium was removed and replaced by the experimental medium (10% CD-FBS-supplemented phenol red-free DMEM). Extracts of CGC and CN, treated as described above with the chemicals for different exposure times, were sonicated using a Sonopuls HD 200 sonicator (Bandelin Electronic, Berlin, Germany) three times for 4 s bursts each at 50% power on ice. Extracts were resuspended in 4 ml of experimental medium, vigorously shaken and left at rest for 10 min, then filtered through a 0.22-µm filter and tested on MCF-7 cells. In each experiment, a dose-response curve (0.1-1,000pM) of E2, and a negative control (cell treated only with hormone-free medium) were included. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, fixing the cells and staining them with SRB. In brief, the cells were treated with cold 10% trichloracetic acid (TCA) and incubated at 4ºC for 30 min, washed five times with tap water and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10mM Tris base (pH 10.5) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in Titertek Multiscan apparatus (Flow, Irvine, CA, USA) at 492 nm. Linearity of the SRB assay with cell number was verified prior to the cell growth experiments. The proliferative effect was calculated as the ratio between the highest cell yield obtained with 100pM of E2 and the

proliferation of hormone-free control cells [MCF-7 cell proliferation (fold-over control)].

Gas chromatography and Electron Capture Detector (GC-ECD) of OCPs

Cells were grown in 6-well plates and treated with OCPs for 6 DIV as indicated previously. After rinsing three times with PBS, the cells were scrapped in PBS and briefly sonicated. The homogenates were then extracted by gentle agitation with n-hexane (200 µl) for 10 min and store at - 20ºC until use.

Clean up. The extracts were cleaned up by elution through 15 ml chromatographic columns packed with 2 g of ISOLUTE Florisil (Biotage AB, Uppsala, Sweden). The columns were preconditioned with 7 ml of n-hexane. The solvent in the column was removed under a low vacuum (-0.1bar) to dry by extraction with a vacuum manifold station (J.T. Baker, Deventer, The Netherlands). The eluates were disposed in harzardous waste. The extracts for analysis (200 µl) were loaded onto the column and eluted with 5 ml n-hexane, followed by 5 ml n-hexane-ethyl acetate (4:1, v/v). The eluates were collected in 40 ml reservoirs and concentrated under low vacuum (-0.1bar). These extracts were concentrated to 0.5 ml and transferred to GC vials where they were further concentrated under a gentle stream of nitrogen (the last drop solution). These mixtures were reconstituted to 200 µl with iso-octane. Recoveries were calculated with three spiking concentrations, 5, 50 and 500ng/ml, respectively. Mean recoveries were 70%, 85.6% and 85.5% for lindane, endosulfan and dieldrin, respectively. These values were considered when calculating OCP concentrations. Unspiked Florisil SPE columns were also prepared and analyzed following the same cleanup method. These samples were analyzed as blanks. No trace levels of these chemicals were observed.

Instrumental analysis. Instrumental analysis was performed on a 6890N Agilent Gas Chromatograph coupled to a 63Ni Electron Capture Detector (Agilent Technologies,

Avondale, PA, USA) with a 7683 Agilent Autosampler. The samples were injected (2ul) in splitless mode at 280°C into a 30 m × 0.25 mm HP-5 MS UI capillary column containing 5% phenyl methyl siloxane (0.25 μm film thickness). The temperature program was from 90°C (held for 2 min) to 130°C at 15°C/min, then from 130°C to 290°C (held for 18 min) at 4°C/min. The carrier gas was helium and was kept at 2 ml/min constant flow. Detector temperature was 320°C. Nitrogen was used as make-up gas at 60 ml/min constant flow. Solvent standards were prepared in iso-octane in a concentration range between 0.5 and 500 ng/ml. These standards were used to create calibration curves for each compound. Linearity in the concentration range studied was $r^2 > 0.999$. Analyte concentrations in the samples were determined by the external standard method using these curves. Limits of detection (LODs) and quatification (LOQs) were calculated as the minimum amounts of analytes which producing peaks with signal-to-noise ratios equal to 3 and 10, respectively. LODs were 0.17, 0.13 and 0.24 injected pg and LOQs 0.93, 0.78 and 1.55 injected pg for lindane, endosulfan and dieldrin, respectively.

Data analysis

Data are shown as mean ± SE. Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. For MCF-7 cell proliferation assays, mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. Individual dose-response curves were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, Graph-Pad Software Inc., San Diego, CA, USA). Statistical comparisons were made by one-way ANOVA followed by Dunnett's post-comparison test when comparing more than two

groups, and two-way ANOVA followed by the Bonferroni post-test when comparing two factors.

RESULTS

OCPs inhibit [^{3}H]-E2 binding in primary neuronal cultures.

Saturation curves of [^{3}H]-E2 binding were derived from primary cultures of CGC and CN in order to characterize the binding parameters (K_d and B_{max}) of these cells. Values for apparent K_d were 1.1 ± 0.4nM and 2.5 ± 0.8nM, respectively, and those for B_{max} were 479 ± 93 fmol/mg protein and 282 ± 38 fmol/mg protein, respectively (N = 3-5). However, these cultures differed in the relative expression of the two ER isoforms. ERα was expressed more in CGC than in CN, whereas the opposite occurred with ERβ (Supplementary Fig. 1).

The OCPs dieldrin, endosulfan and lindane caused a concentration-dependent inhibition of [^{3}H]-E2 binding in both CGC and CN (Fig. 1). Table 1 summarizes the IC_{50} and LOEC values of the three pesticides in each culture. Dieldrin was the inhibitory agent with highest affinity for ER in CGC ($p < 0.05$ versus endosulfan and lindane), whereas no differences were observed between the three compounds in CN. Moreover, dieldrin showed greater affinity for ER in CGC than in CN ($p < 0.01$). In contrast, β-HCH up to 50μM did not displace [^{3}H]-E2 from binding to ER in CN (Fig. 1C) or CGC (data not shown).

Dieldrin, endosulfan and lindane differently activate Akt and ERK1/2 phosphorylation in neurons.

We aimed to test whether OCPs could activate two of the major intracellular signaling pathways associated with the ER-dependent non-genomic effects of E2: the MAPK and PI3K/Akt pathways. Exposure to dieldrin or endosulfan for 5 h enhanced Akt

phosphorylation at concentrations close to their IC_{50} values against the ER in CGC and CN (Fig. 2A-B). Likewise, ERK1/2 phosphorylation was increased in CN after exposure to these compounds; however, only dieldrin produced a significant effect on CGC (Fig. 2C-D). In contrast, lindane induced ERK1/2 phosphorylation in CN but not in CGC after 5 h of exposure. Nevertheless, a higher concentration of lindane than of dieldrin or endosulfan was required to observe a similar effect (Fig. 2).

Next we studied the effects of OCPs on E2-induced activation of ERK1/2 and Akt. Because E2 induces a rapid activation of both kinases that peaks after 30-60 min (Mannella and Brinton, 2006; Minano *et al.*, 2007), cells were treated with 10nM E2 both in the absence and in the presence of the OCPs for 1 h. E2 increased ERK1/2 phosphorylation in CGC and CN, and Akt phosphorylation in CN but not in CGC (Fig. 3). Under the above conditions, dieldrin and endosulfan activated Akt phosphorylation in both cultures both in the presence and absence of E2. Instead, lindane did not modify phospho-Akt levels within 1 h, but it inhibited E2-induced Akt activation in CN (Fig. 3A-B). Dieldrin enhanced phospho-ERK1/2 levels to a similar extent as 10nM E2 did; moreover, no additive effects were observed when the two compounds were present. In contrast, endosulfan and lindane did not affect ERK1/2 phosphorylation within 1 h of treatment; however, they inhibited E2-mediated ERK1/2 activation in both cell types (Fig. 3C-D).

OCP-induced Akt and ERK1/2 phosphorilation is inhibited by ER antagonists.

In order to confirm that the effects of the pesticides on Akt and ERK1/2 phosphorylation are mediated through ER activation, we used the ER antagonist ICI182780 (ICI) and specific antagonists for ERα (MPP), ERβ (PHTPP) and GPR30 (G-15). Cells were previously treated with the respective ER antagonists (all at 1μM) for 30 min, and then exposed to OCPs for 5 h in the presence of the ER antagonists. ICI

prevented dieldrin-induced Akt phosphorylation in both CGC and CN (Fig. 4A-B). Similar results were obtained with endosulfan (Fig. 4C-D). Likewise, the increase in phospho-ERK1/2 caused by dieldrin treatment in CGC was significantly reduced by the ER antagonist ICI (Fig. 5A). In contrast, dieldrin-induced ERK1/2 activation in CN was unaffected by ICI (Fig. 5B). The $GABA_A$ receptor antagonist bicuculline has been shown to increase ERK1/2 phosphorylation through an activity-dependent glutamate receptor activation (Chen *et al.*, 2007). Therefore, it is possible that OCPs can also activate the MAPK pathway as a consequence of their blockade of the $GABA_A$ receptor (Pomes *et al.*, 1994). In order to test this hypothesis in our cultures, we used the non-competitive $GABA_A$ receptor antagonist picrotoxinin (PTX). Exposure to 100μM PTX increased ERK1/2 phosphorylation in CN after 5 h of exposure (Fig. 5B). In contrast, PTX did not affect phospho-ERK levels in CGC (Fig. 5A), suggesting different mechanisms in OCP-mediated activation of the MAPK pathway between the cultures studied. Moreover, PTX- (Supplementary Fig. 2) and dieldrin- (Fig. 5D) induced activation of ERK1/2 was inhibited by a cocktail of glutamate receptor antagonists.

Finally, we used MPP, PHTPP and G15 (alone and combined) to find out which ER is responsible for the effects of OCPs on Akt and ERK1/2. All the ER antagonists reduced the increase on Akt and ERK phosphorylation induced by dieldrin in CGC, but statistical significance was only reached with G-15. Furthermore, combinations of these antagonists completely abolished dieldrin-induced Akt (Fig. 4A) and ERK1/2 (Fig.5C) activation. In contrast, the activation of Akt induced by this OCP in CN was only blocked by PHTPP (Fig. 4B). On the other hand, the effects of endosulfan on Akt were selectively inhibited by PHTPP in both CGC and CN (Fig. 4C-D), indicating a specific action of this OCP on ERβ. Instead, none of these ER antagonists reversed the effects of dieldrin (data not shown), endosulfan or lindane (Supplementary Fig. 3) on ERK1/2

phosphorylation in CN. These results rule out the involvement of the ERs on OCP-induced MAPK activation in CN.

Estrogenic effects of CGC and CN extracts in the E-Screen bioassay.

We used the E-Screen bioassay, a method widely used to assess the estrogen-like activity of environmental pollutants (Soto *et al.*, 1994; Soto *et al.*, 1995), to estimate the potential estrogenicity of primary cultures of CGC and CN pre-exposed to OCPs for short and long periods of time (5 and 48 h, respectively). This test is based on the proliferation of the human-derived breast cancer cell line MCF-7 in response to chemicals that activate ERα. Extracts of naive and DMSO-treated neurons similarly increased basal proliferation of MCF-7 cells (data not shown). Therefore, the statistical comparisons for OCPs were made with respect to neuronal cultures treated with DMSO. Extracts of CGC pre-exposed to dieldrin for short and long periods of time significantly enhanced the proliferation of MCF-7 cells (with respect to DMSO-treated neurons) (Fig. 6A-C). Furthermore, pre-exposure of extracts of CN to dieldrin, endosulfan or lindane for 48 h increased the proliferative effect of DMSO-treated neurons (Fig. 6D). However, this effect was not observed at shorter exposure times (Fig. 6B).

We also studied the direct effects of dieldrin, endosulfan and lindane on MCF-7 cell proliferation to evaluate the potential endocrine-disrupting activity of these compounds. In this cell line, 5μM of either dieldrin or endosulfan significantly induced cell proliferation. Conversely, lindane did not have any effect up to 50μM (Supplementary Fig. 3). These effects are consistent with our previous observations, but now we show that the lowest observed effect concentration (LOEC) for both dieldrin and endosulfan on the E-screen bioassay is 5μM (Supplementary Fig. 4) instead of 10μM (Soto *et al.*, 1994; Soto *et al.*, 1995).

Long-term exposure to OCPs reduces ERα but not ERβ levels in CGC and CN.

Since prolonged treatment with E2 and several xenoestrogens, including endosulfan and dieldrin, differently modulates the expression of ERα and ERβ in epithelial cells (Grunfeld and Bonefeld-Jorgensen, 2004), we addressed the question of whether this effect also occurred in our neuronal cultures. Long-term exposure to dieldrin for 6 DIV reduced the levels of ERα in both CGC and CN. Nevertheless, this effect was evident at 0.06μM dieldrin in CN, whereas a higher dieldrin concentration (3μM) was required to see a similar reduction in CGC (Fig. 7A-B). Accordingly, long-term exposure to 0.2μM dieldrin in CN reduced apparent ER B_{max} from 321 ± 49 fmol/mg prot to 169 ± 29 fmol/ mg prot ($p < 0.05$, N = 3) without affecting apparent K_d (3.7 ± 0.7nM and 2.9 ± 0.9nM, respectively). Similarly, long-term exposure to endosulfan and lindane reduced ERα protein levels in CGC and CN to a similar extent as 1nM E2 did (Fig. 7A-B). None of the OCPs tested significantly modified ERβ protein levels (Fig. 7C-D).

We have previously reported that long-term exposure of CN and CGC to dieldrin concentrations like those used here results in decreased functionality of the $GABA_A$ receptor (Babot *et al.*, 2007; Briz *et al.*, 2010). Again, the effects of OCPs on CN were mimicked by the $GABA_A$ receptor antagonist PTX. Long-term exposure to PTX reduced ERα levels (Fig. 7B) without affecting ERβ levels (data not shown). In contrast, exposure to PTX for 6 DIV did not modify ERα protein levels in CGC (Fig. 7A). Finally, we aimed to determine the intracellular incorporation in our cultures after 6 DIV of exposure to OCPs. Table 2 shows the actual intracellular concentration of OCPs in CN. A similar yield of accumulation (around 10%) was found for all the pesticides.

DISCUSSION

The present study shows that dieldrin, endosulfan and lindane have endocrine-disrupting activity in two different neuronal populations, CGC and CN, through their direct interaction with neuronal ERs. All three OCPs inhibited [^{3}H]-E2 binding to ER in CGC and CN, whereas the β-HCH isomer did not affect it. We found that dieldrin possesses greater affinity to ER than endosulfan and lindane in CGC. In addition, dieldrin was a significantly more potent inhibitor of [^{3}H]-E2 binding in CGC than in CN. The binding affinities for E2 (apparent K_d) and the IC_{50} values for the OCPs are similar to those observed in previous studies using recombinant ERs from different species, including humans (Gale *et al.*, 2004; Scippo *et al.*, 2004). Furthermore, IC_{50} values for dieldrin and endosulfan are of the same order of magnitude as the concentrations of pesticides that elicit positive effects on proliferation and transactivation assays in estrogen-sensitive cell lines (this work, Supplementary Fig. 3; (Andersen *et al.*, 2002; Lemaire *et al.*, 2006; Soto *et al.*, 1994). Dieldrin has been shown to possess greater affinity for ERα than endosulfan and lindane (Scippo *et al.*, 2004; Sumbayev *et al.*, 2005) and it also has a higher affinity for ERα than for ER? (Gale *et al.*, 2004). Thus, the different relative expression of the two ER isoforms in the cultures studied (higher for ERα in CGC and for ER? in CN; Supplementary Fig. 1) might explain the observed differences in IC_{50} values (Table 1). In addition, we found that dieldrin is able to activate intracellular signaling pathways in CGC through a multiple action on ERα, ER? and GPR30 (Fig. 5). This promiscuity can also explain the highest inhibitory potency of dieldrin in the [^{3}H]-E2 binding assay with respect to endosulfan and lindane. Few studies have examined the effects of lindane on ER. The results showed here are consistent with recent findings that attribute potential endocrine-disrupting activity to lindane through its action on either ERα or ERβ (Li *et al.*, 2008; Maranghi *et al.*, 2007). In contrast, β-HCH did not displace E2 from its binding to ER in

our cultures, as previously reported in MCF-7 cells (Coosen and van Velsen, 1989; Steinmetz *et al.*, 1996). Nevertheless, this pollutant is considered an endocrine disruptor in human breast cancer cells through a non classic ER-dependent mechanism (Steinmetz *et al.*, 1996).

Among the non-genomic effects of E2, the activation of MAPK and PI3K/Akt pathways have been shown to be crucial in most of the neuronal functions regulated by estrogens (Mendez *et al.*, 2005; Ogiue-Ikeda *et al.*, 2008). In our experimental conditions, E2 enhanced both Akt and ERK1/2 phosphorylation in CN whereas ERK1/2 was activated by E2 treatment in CGC, but Akt was not (Fig. 3, Table 2). These effects are in agreement with those previously described in the same cultures (Belcher *et al.*, 2005; Mannella and Brinton, 2006; Minano *et al.*, 2007). E2-mediated ERK1/2 activation in CGC has been reported to involve protein kinase A, Src-kinase and a G protein-coupled receptor (Belcher *et al.*, 2005). Accordingly, dieldrin-induced ERK1/2 activation was inhibited by the GPR30 antagonist G-15. The inability of endosulfan (whose effects appear to be mediated just through ERβ) to activate ERK1/2 in CGC supports the involvement of GPR30 in ERK1/2 activation in these cells. Nevertheless, ERα and ERβ also contribute to these effects because their respective antagonists further reduced dieldrin-induced ERK1/2 activation when combined with G-15. Likewise, the effects of dieldrin on Akt phosphorylation in CGC were only completely suppressed when all ERs were blocked by their respective antagonists. In contrast, dieldrin increases Akt phosphorylation in CN as a result of its interaction with ER?. Similarly, activation of both ERK1/2 and Akt induced by E2 in CN has been described to be dependent on the interaction of ER with PI3K (Mannella and Brinton, 2006). Thus, the fact that ERβ is more expressed in CN than in CGC may underlie the observed differences regarding Akt activation by dieldrin.

Although ICI 182780 is a commonly used ER antagonist, it has been reported to transiently activate ERK1/2 phosphorylation in CGC and other cells through a GPR (Belcher *et al.*, 2005; Filardo *et al.*, 2000). However, in our experimental conditions this compound did not affect the MAPK pathway. Moreover it inhibited rather than enhanced the effects of OCPs in Akt and ERK1/2. In contrast, OCP-mediated ERK1/2 activation in CN was not inhibited by ER antagonists. $GABA_A$ receptor antagonists are able to increase ERK1/2 phosphorylation in hippocampal neurons, being this effect inhibit by NMDA receptor antagonists (Chen *et al.*, 2007). Although OCPs does not acutely interfere with NMDA receptor activity (Babot *et al.*, 2007; Briz *et al.*, 2010), exposure to OCPs for 5 h could increase the activation of glutamate receptors as a consequence of $GABA_A$ receptor blockade . The results obtained here using the $GABA_A$ receptor antagonist PTX and a cocktail of glutamate receptor antagonists support the involvement of $GABA_A$ and glutamate receptors in OCP-induced ERK1/2 activation in these cells. These observations confirm that the mechanisms underlying the activation of the MAPK pathway by OCPs differ depending on the neuronal cell type.

The effects of endosulfan on Akt and those of dieldrin on Akt and ERK1/2 were unaltered by co-treatment with E2. In addition, the lack of additive effects on Akt phosphorylation when co-exposed to E2 suggests that dieldrin and endosulfan act as agonists at the ER ligand-binding site. Non-genomic effects of several OCPs (including dieldrin and endosulfan) have been linked to ERα activation in a pituitary cell line (Bulayeva and Watson, 2004). In the present work, we found that dieldrin was also able to activate ER?. Instead, endosulfan had specific ERβ agonism without any activity on ERα, at least regarding Akt phosphorylation. This may be accounted for different expression levels and/or localization of ERα between cell lines and primary neuronal cultures. On the other hand, lindane has been proposed as ERα antagonist and ERβ

agonist (Li *et al.*, 2008; Maranghi *et al.*, 2007). In the present work, lindane acts as ER antagonist rather than an agonist because it not only failed to activate Akt and ERK1/2, but it actually inhibited the effects of E2 on these protein kinases. Nevertheless, it would seem controversial to claim that dieldrin and endosulfan activated Akt through an ER-dependent mechanism in CGC while E2 did not. Altogether, these results suggest that the classic view of endocrine disruptors as agonists or antagonists of ER may be too simplistic to describe the estrogenic effects of OCPs, at least in systems in which different ERs are present. In this sense, it has been demonstrated that OCPs induce a unique pattern of conformational changes in both ERα and ERβ, which is a combination of the patterns induced by E2 and the partial ER antagonist 4-hydroxy-tamoxifen (Sumbayev *et al.*, 2005). In turn, this would allow the ligand-activated ER to bind different co-regulators or associated proteins, and therefore different effects on the signaling pathways associated can be expected depending on the relative expression of ERα, ERβ and GPR30 and on the specific affinity for each ER that a given OCP has. For this reason, environmental estrogens could potentially affect brain development and behavior in very different ways (Schantz and Widholm, 2001).

E2 is *de novo* synthesized in the brain through the rate-limiting enzyme aromatase, which is present in cortex and cerebellum at moderate levels (Amateau *et al.*, 2004; Roselli *et al.*, 1984). Although it is believed that Purkinje cells are the main source of E2 in the cerebellum (Sakamoto *et al.*, 2003), active aromatase has been detected in a cerebellar granule progenitor cell line (Gottfried-Blackmore *et al.*, 2007). Therefore, it is not surprising that neuronal extracts from naïve and DMSO-treated CN and CGC were positive in the E-Screen bioassay. Interestingly, neuronal cultures previously exposed to OCPs for 48 h showed an additional proliferative effect in MCF-7 cells (Fig. 6). It is unlikely that these effects are due to alterations in the intracellular levels of

endogenous estrogens, since endosulfan slightly inhibits rather than activates aromatase, and dieldrin has no effect on aromatase activity (Andersen *et al.*, 2002). Instead, we observed that long-term exposure to OCPs causes them to accumulate inside the neurons during the time in culture (Table 2), and as a result of their persistence they probably preserve their estrogen-like activity for days after the treatment. However, it is controversial to claim that neurons exposed to lindane had a significantly higher proliferative effect than non-treated cultures if we consider that this compound was inactive in the E-Screen assay (this work, Supplementary Fig. 3; Soto *et al.*, 1995). Nevertheless, it has been reported that lindane enhances aromatase activity at short exposure times (< 6 h) followed by mild inhibition at longer times in cell lines (Nativelle-Serpentini *et al.*, 2003). In addition, aromatase activity has been shown to be dependent on neuronal excitability (Hojo *et al.*, 2004), which can be increased by OCPs. Therefore, we can expect fluctuations in E2 levels in our cultures that could eventually lead to increased estrogenicity after 48 h of lindane exposure. According to that observed in MCF-7 cells (Grunfeld and Bonefeld-Jorgensen, 2004), we found that prolonged exposure to OCPs specifically reduced the expression of ERα without affecting that of ERβ. In our study, these effects seemed to be mediated through their interaction with ER rather than with the $GABA_A$ receptor in CGC, since PTX exposure did not modify ERα levels in these cells. In contrast, the effects of OCPs on cortical ERα were observed at concentrations more than 100 times lower than their respective affinities to ER. Moreover, prolonged exposure to PTX in CN significantly reduced ERα but not ERβ, suggesting that the effects of OCPs on ER levels are due to the blockade of $GABA_A$ receptor in CN. Similar changes in ERα expression have been observed in the rat hippocampus after status epilepticus and in the brains of patients with temporal lobe epilepsy (Killer *et al.*, 2009; Tokuhara *et al.*, 2005). It is worth

noting that the ER-dependence of both ERK1/2 activation and ERα levels differs in a similar way between the cultures studied. A cross-talk between ERK1/2 activation and the genomic regulation of several ER targets (including ER) in MCF-7 cells after treatment with E2 or OCPs has been recently suggested (Silva *et al.*, 2010). In addition, the PI3K/Akt pathway has been shown to regulate ERα protein stability (Mendez and Garcia-Segura, 2006). However, further studies are needed to confirm a direct correlation between the activation of these signaling pathways by OCPs and their regulation of ER protein levels.

In summary, the present study shows that OCPs have estrogenic effects in primary cultures of CGC and CN through interaction with neuronal ERs, but more interestingly that these effects persist during the time in culture and may represent an important issue of OCP-induced neurotoxicity. Although the concentrations described here to have estrogenic effects suggest that OCPs are mild xenoestrogens; however, we observed that long-term exposure to dieldrin reduced ERα levels at concentrations close to those found in human brain (Corrigan *et al.*, 2000), suggesting that this OCP could potentially act as endocrine disruptor in the CNS. Environmental estrogens have been reported to rapidly modulate synaptic plasticity and affect cognition (Ogiue-Ikeda *et al.*, 2008; Schantz and Widholm, 2001). However, the mechanisms underlying such effects are not completely understood. The PI3K/Akt and MAPK pathways are well known to be involved in synaptic plasticity and synaptogenesis. Therefore, the alterations in the physiological activation of these signaling pathways or on ER levels caused by OCPs and by other endocrine disruptors (Bulayeva and Watson, 2004; Fan *et al.*, 2010), might contribute to their cognitive and behavioral effects (Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). A better understanding of the molecular mechanism of OCP action in the CNS may be useful to predict and

eventually prevent the neurotoxicity of OCPs. Therefore, future investigations should be designed to determine the specific role of the different ERs in the neurological alterations associated with OCP exposure.

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FIGURE LEGENDS

Fig. 1. Concentration-dependent inhibition of [^{3}H]-17?-estradiol ([^{3}H]-E2) binding by OCPs in primary neuronal cultures. Competition curves of specific [^{3}H]-E2 binding for dieldrin (A), endosulfan (B) and HCH isomers (C). Different concentrations of OCPs were incubated with a fix [^{3}H]-E2 concentration both in CN (■) and in CGC (▲). C) ?-HCH competition curve (●) was performed in CN.

Fig. 2. Effect of OCPs on Akt and ERK1/2 phosphorylation in primary neuronal cultures. CGC and CN were exposed to DMSO (control or C), 3μM or 10μM dieldrin (D3 or D10), 3μM or 10μM endosulfan (E3 or E10) and 30μM lindane (L30) for 5 h and then the levels of total and phosphorylated Akt, ERK1 and ERK2 were quantified by Western Blot. Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to four independent experiments. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ versus control.

Fig. 3. Effect of OCPs on E2-mediated Akt and ERK1/2 phosphorylation. Cultures were exposed to DMSO (Control or C), 10μM dieldrin (D), 10μM endosulfan (En) or 30μM

lindane (Ln) for 1 h both in the absence and presence of 10nM E2. Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three independent experiments. Statistical comparisons were made by Two-way ANOVA: * $p < 0.05$, ** $p < 0.01$ versus control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus E2-treated cells.

Fig. 4. Effect of ER antagonists on OCP-induced Akt phosphorylation. Cells were exposed to DMSO (Control or C), 10μM dieldrin (D) or 10μM endosulfan (E) for 5 h both in the absence and presence of ICI, MPP, PHTPP (PH) and G-15 (all at 1μM) and combinations of them (D+All represent D+MPP+PH+G-15). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to four independent experiments. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ versus control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus OCP-treated cells.

Fig. 5. Effect of ER antagonists on OCP-induced ERK1/2 phosphorylation. Cultures were exposed to DMSO (Control or C) or 10μM dieldrin (D) for 5 h alone or in the presence of the respective ER antagonists. A-B) Cultures were pre-treated with 1μM ICI for 30 min and then exposed to dieldrin. The treatment with picrotoxinin (PTX) was performed at 100μM for 5 h. C) Cultures were pre-treated with MPP, PHTPP (PH), G-15 (all at 1μM) and combinations of them (D+All represent D+MPP+PH+G-15) for 30 min and then exposed to dieldrin. D) Cells were treated with the glutamate receptor antagonists MK-801 and NBQX (M+N, both at 10μM) together with DMSO or dieldrin. Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each

panel. Data are mean ± SE of three independent experiments. Statistical comparisons were made by *t*-test (for PTX), One-way ANOVA (panel C) or Two-way ANOVA (panels A-B and D): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus dieldrin-treated cells; $^{\$\$}$ $p < 0.01$ versus ICI-treated cells.

Fig. 6. Proliferative effect of extracts from primary neuronal cultures pre-exposed to OCPs in MCF-7 cells. A-B) Cultured neurons were exposed to DMSO or OCPs (all at 30μM) for 5 h. C-D) Neurons were exposed to DMSO or the indicated concentrations of OCPs for 48 h. MCF-7 cells were incubated for 6 DIV in the presence of extracts of CN and of CGC. Results are expressed as proliferative effect (calculated as the ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Data are mean ± SE of three independent experiments, each one performed in triplicates. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus DMSO-treated neurons.

Fig. 7. Effect of long-term exposure to OCPs on ERα (panels A and B) and ERβ (panels C and D) levels in primary neuronal cultures. CGC and CN were exposed to DMSO (Control or C), 100μM picrotoxinin (PTX) or the indicated concentrations (μM) of dieldrin (D), endosulfan (En) and lindane (Ln) for 6 DIV. The treatment with E2 was performed at 1nM for 48 h. Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to six independent experiments. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ versus control.

Culture	CGC	CN	CGC	CN
Test compound	**IC50 (µM)**		**LOEC (µM)**	
Dieldrin	$2.2 \pm 0.5^{*, \#\#}$	34.0 ± 4.4	1^{**}	3^{**}
Endosulfan	21.7 ± 4.2	35.0 ± 8.3	3^{*}	10^{*}
Lindane	24.8 ± 5.4	36.2 ± 2.4	10^{**}	30^{**}

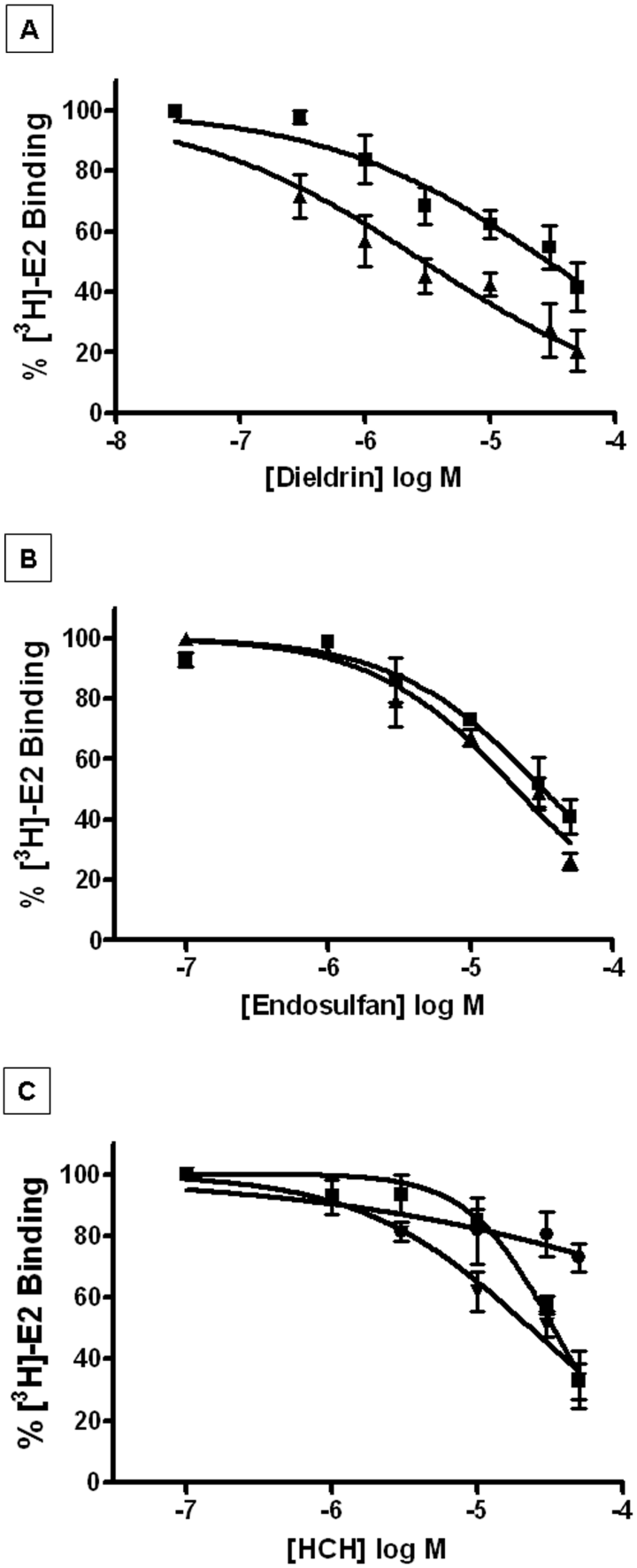
Table 1: IC_{50} and LOEC values of organochlorine pesticides on [^{3}H]-E2 binding in primary cultures of cerebellar granule cells (CGC) and cortical neurons (CN).

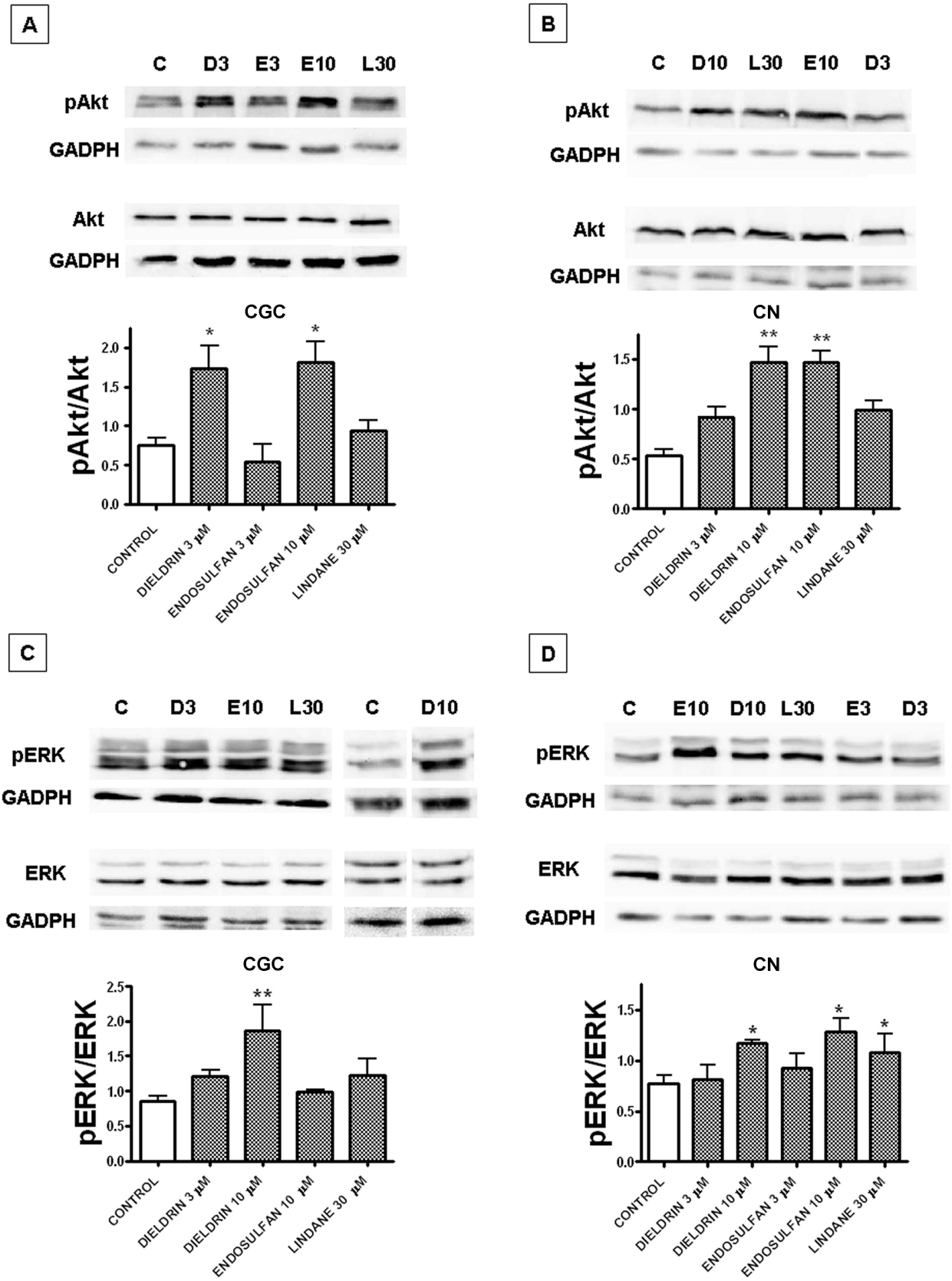
Note: IC50 values were calculated from the concentration-dependent inhibition of [^{3}H]-E2 binding and are mean ± SE of three independent experiments. Statistical differences between IC50 values were obtained for dieldrin with respect to the other pesticides in CGC (*P<0.05, two-way ANOVA) and for dieldrin comparing CGC and CN ($^{\#\#}$P<0.01, two-way ANOVA). LOEC values were the lowest concentrations used that resulted in statistically different [^{3}H]-E2 binding with respect to control (*P<0.05, **P<0.01, One-way ANOVA).

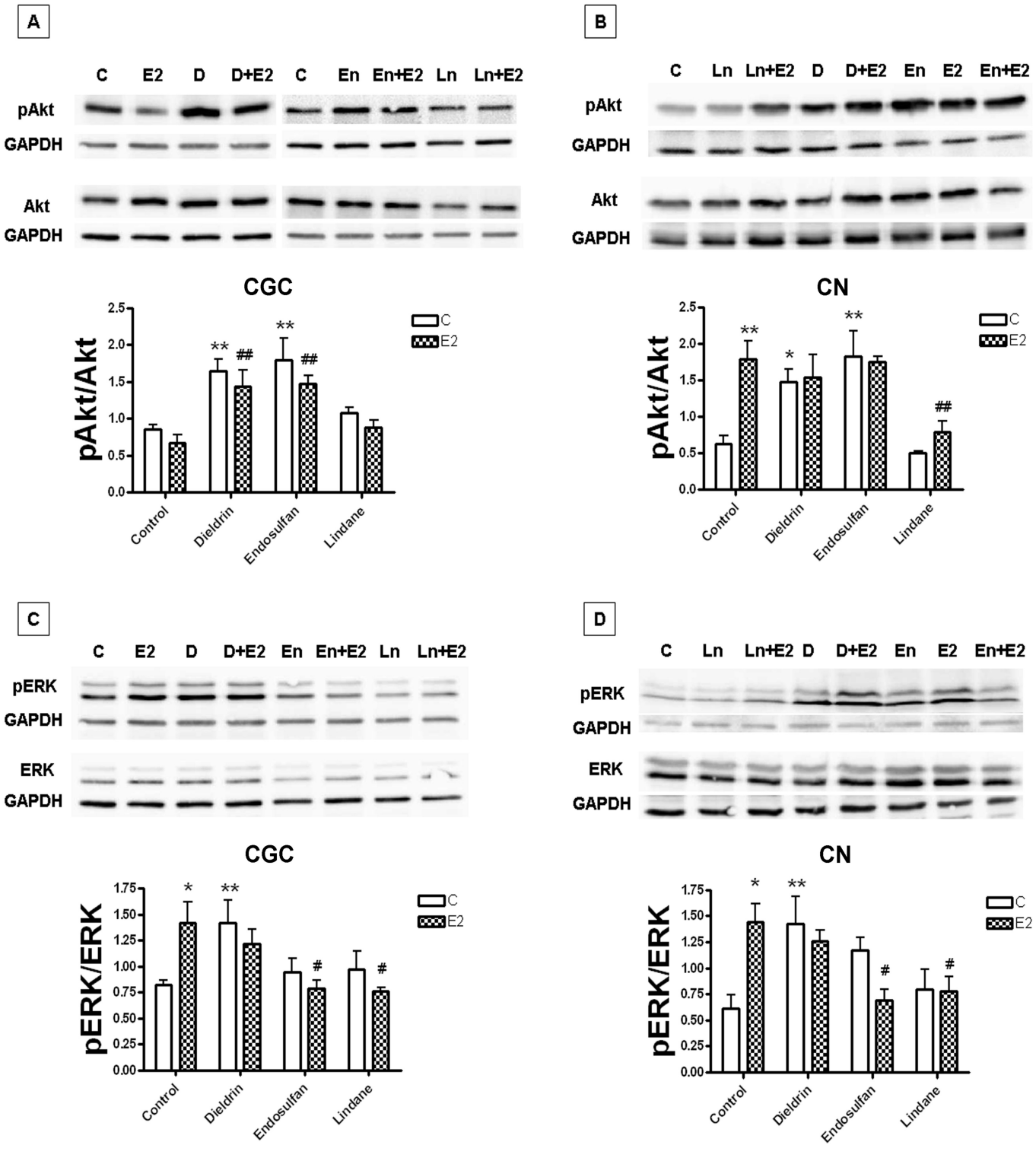
OCP	Treatment concentration (µM)	Intracelular concentration (ng/ mg protein)	Intracellular accumulation yield (%)
Dieldrin	0.1	35.1 ± 2.7	11.5 ± 0.9
Endosulfan	1	326.1 ± 13.5	10.0 ± 0.4
Lindane	10	2517± 174.2	10.8 ± 0.7

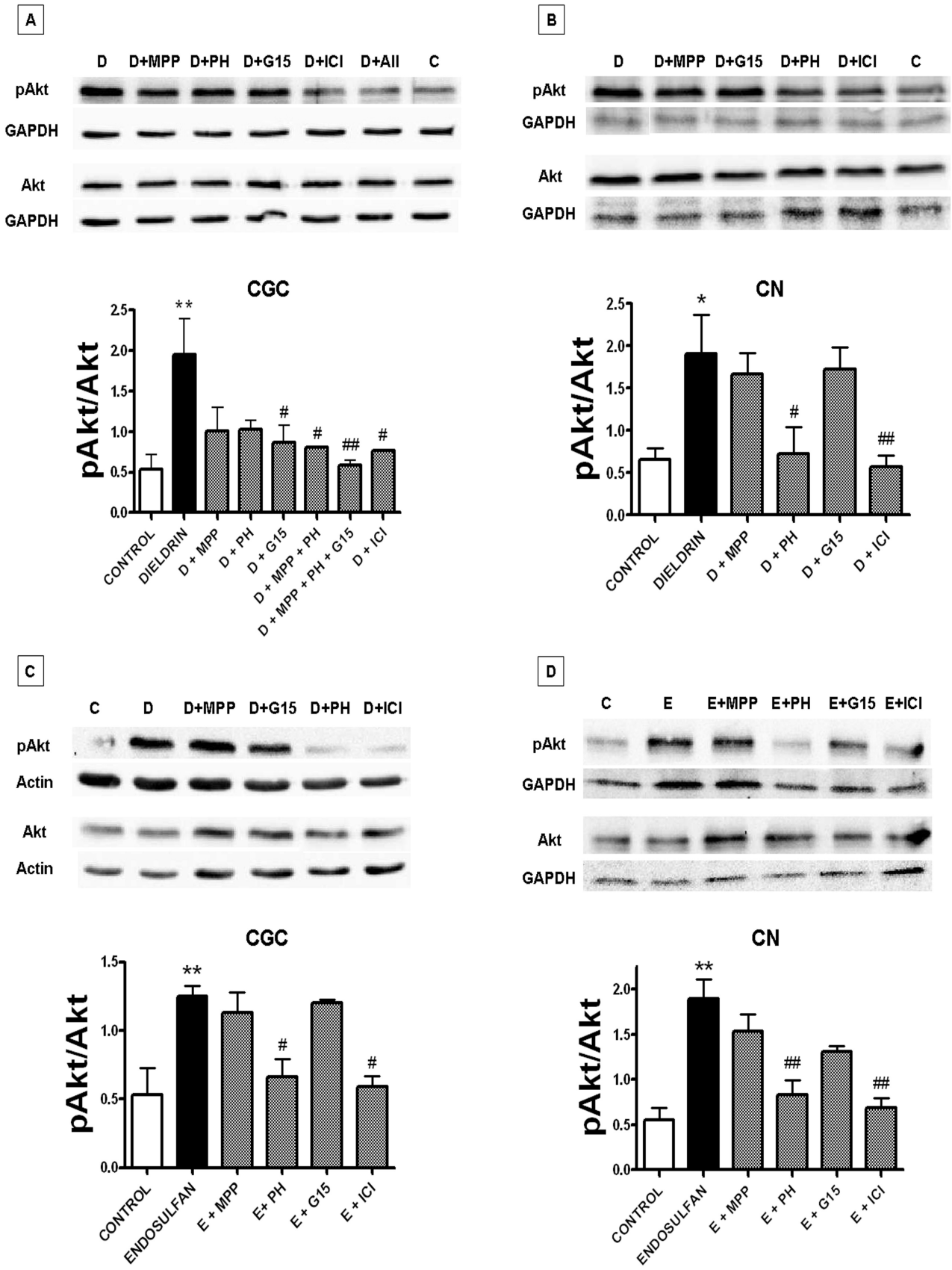
Table 2: Intracellular levels of OCPs in cortical neurons after 6 DIV of exposure

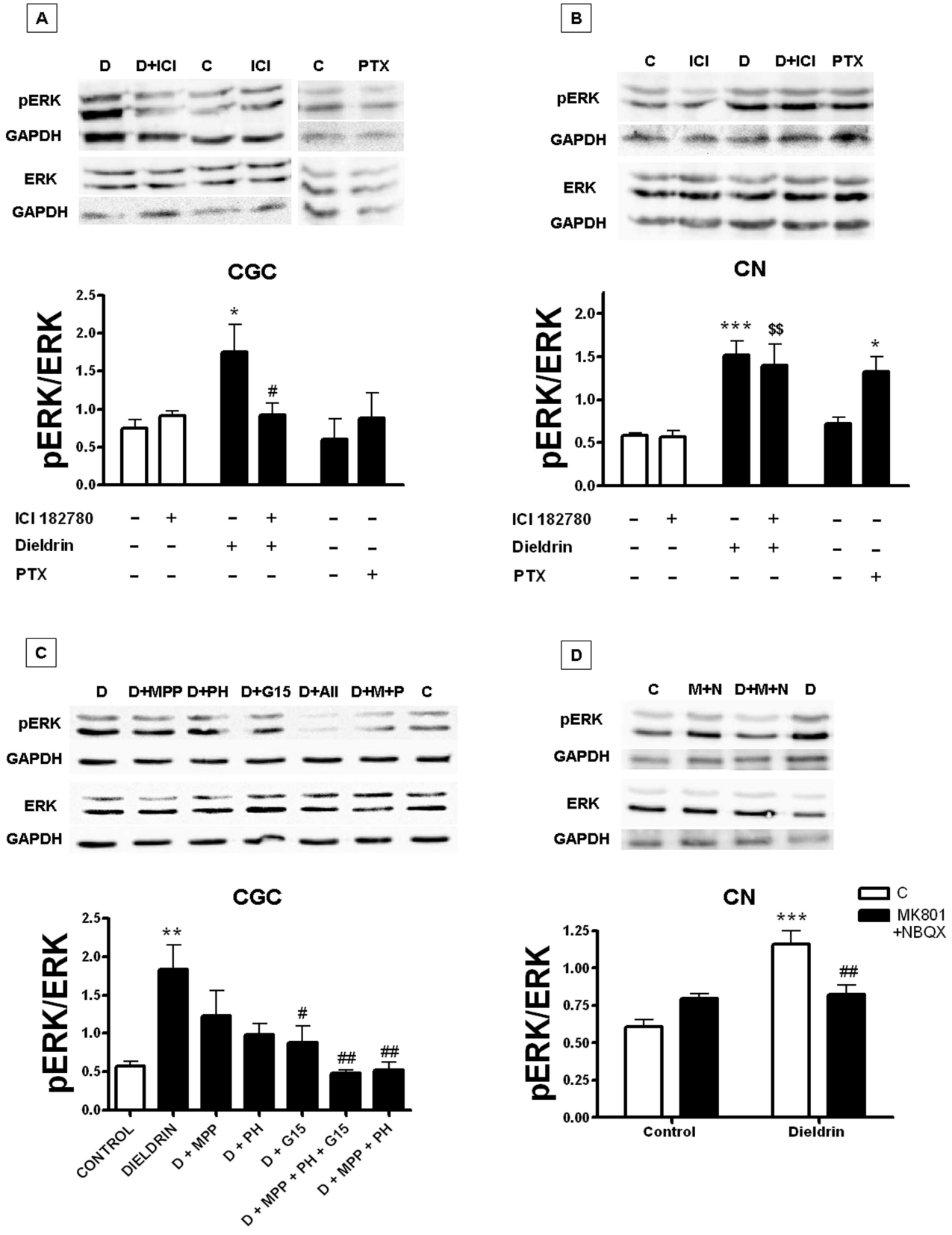
Note: Primary cultures of cortical neurons were treated for 6 DIV with OCPs. Chemicals from the intracellular extracts were separated by gas chromatography and chemical concentration was determined by using an Electron Capture Detector. Values represent mean ± SD of two independent measures.

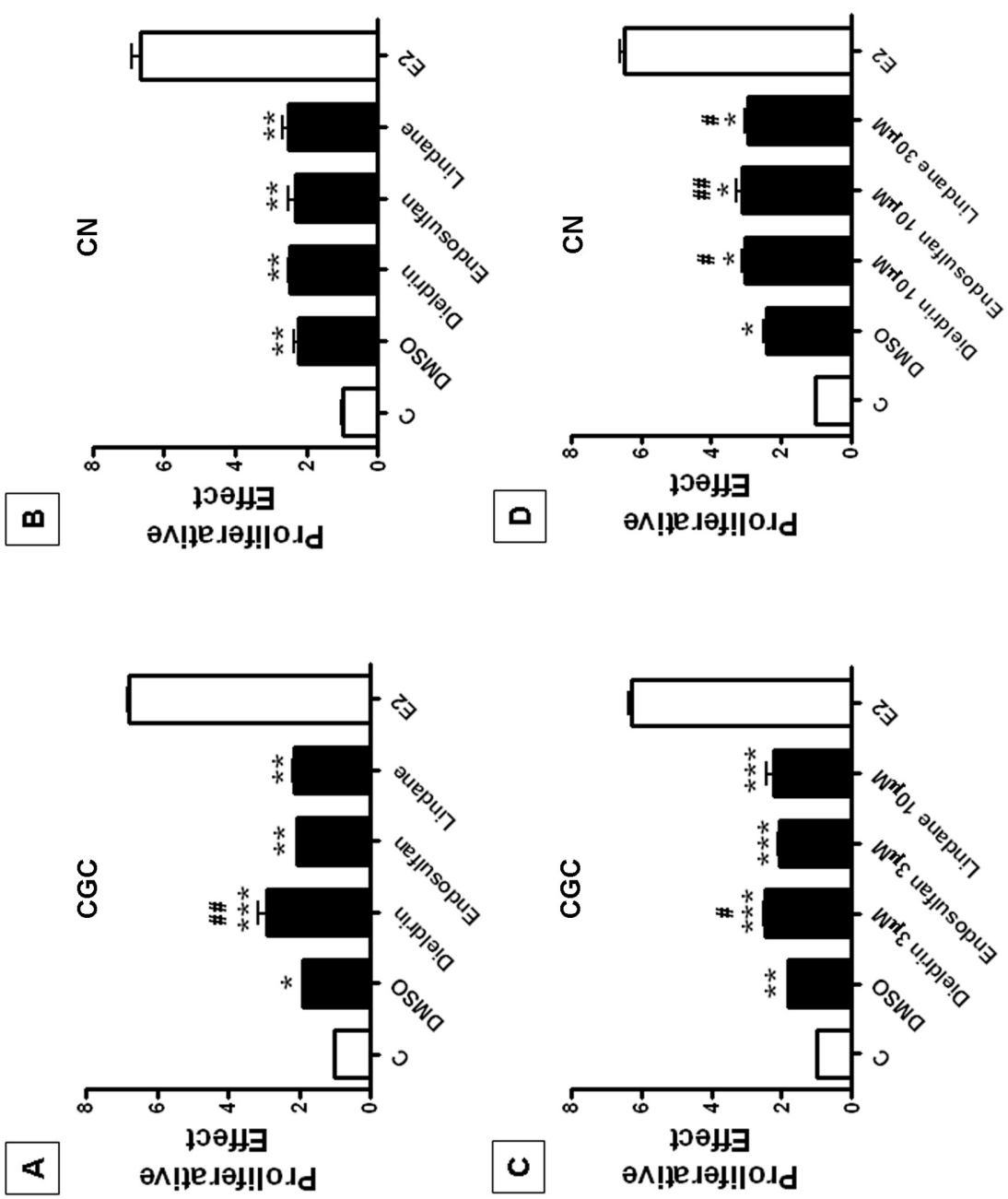


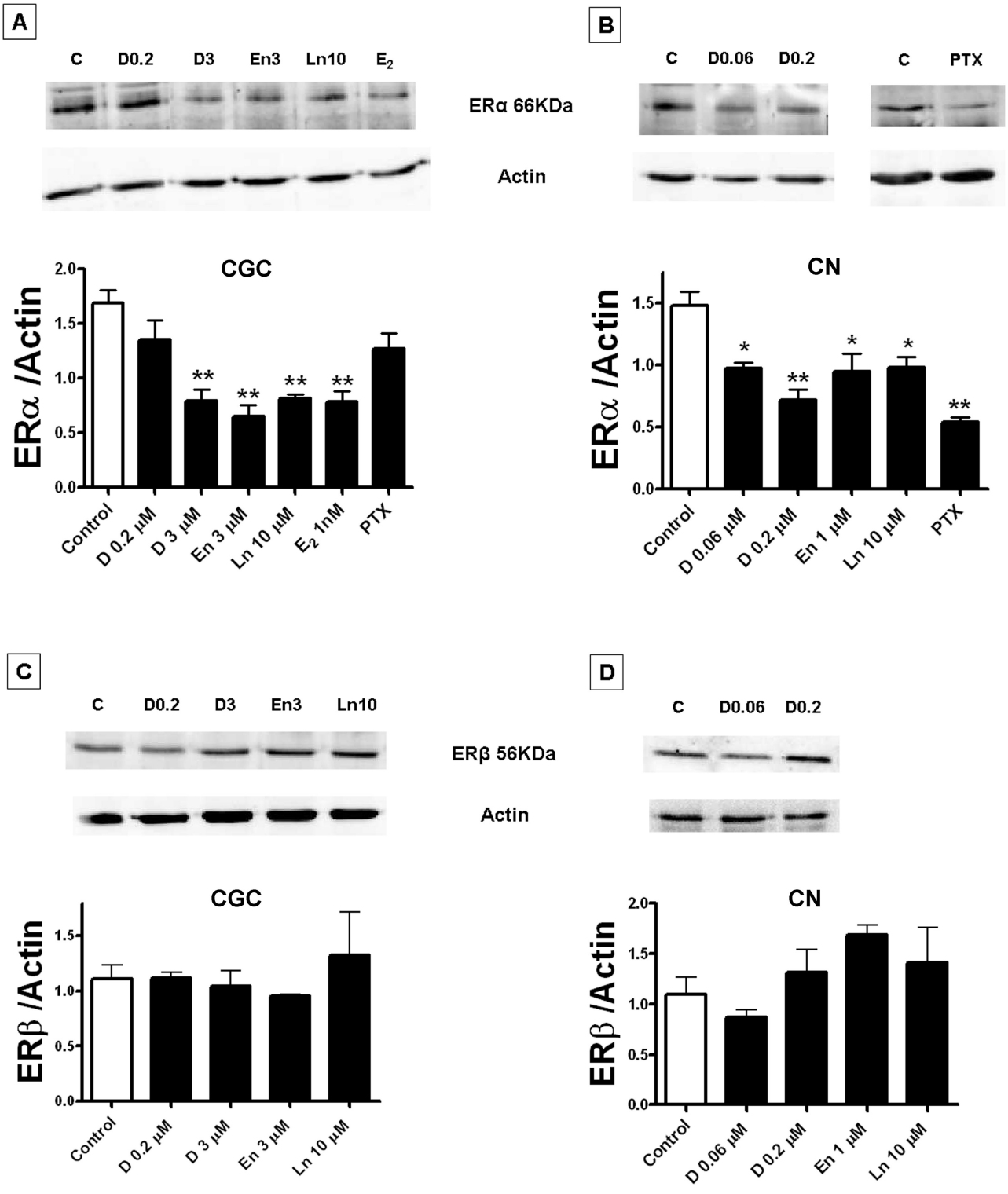


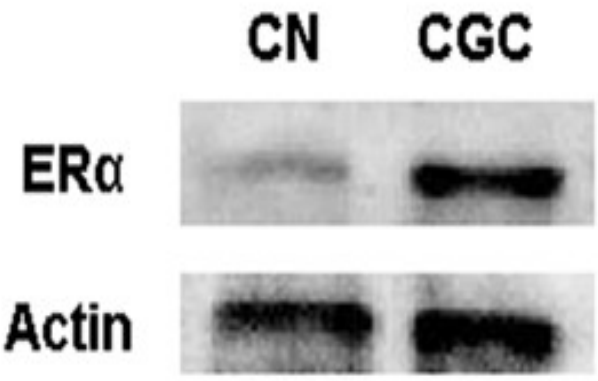


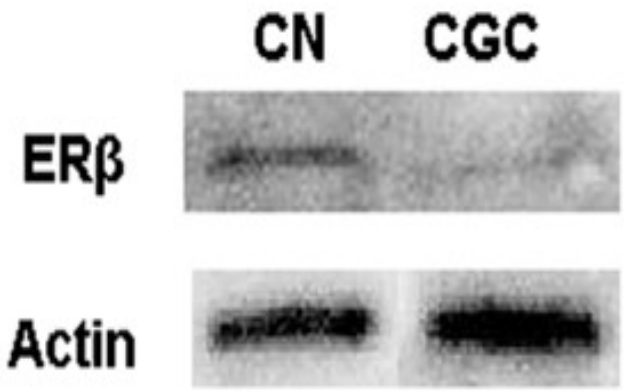


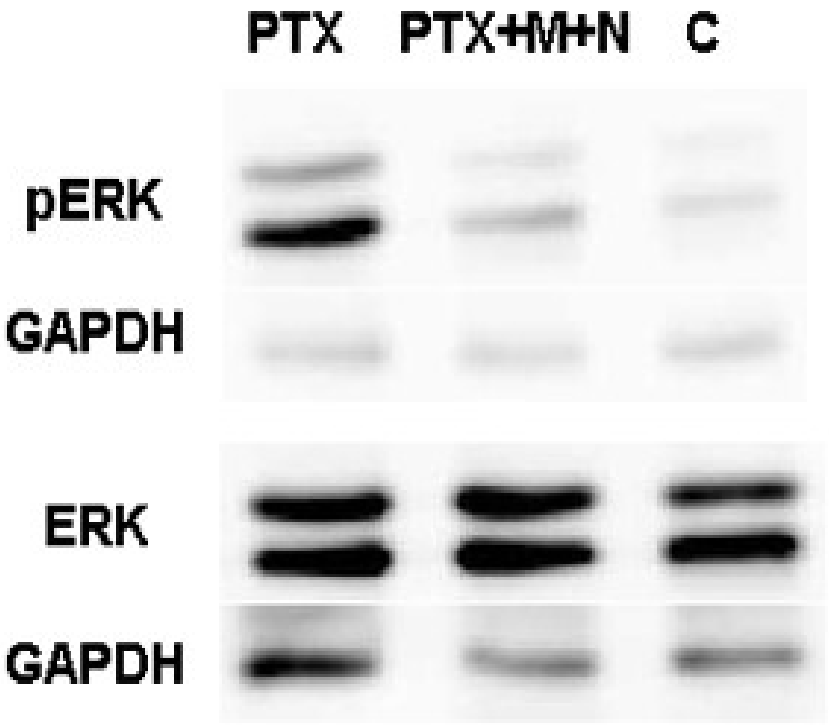


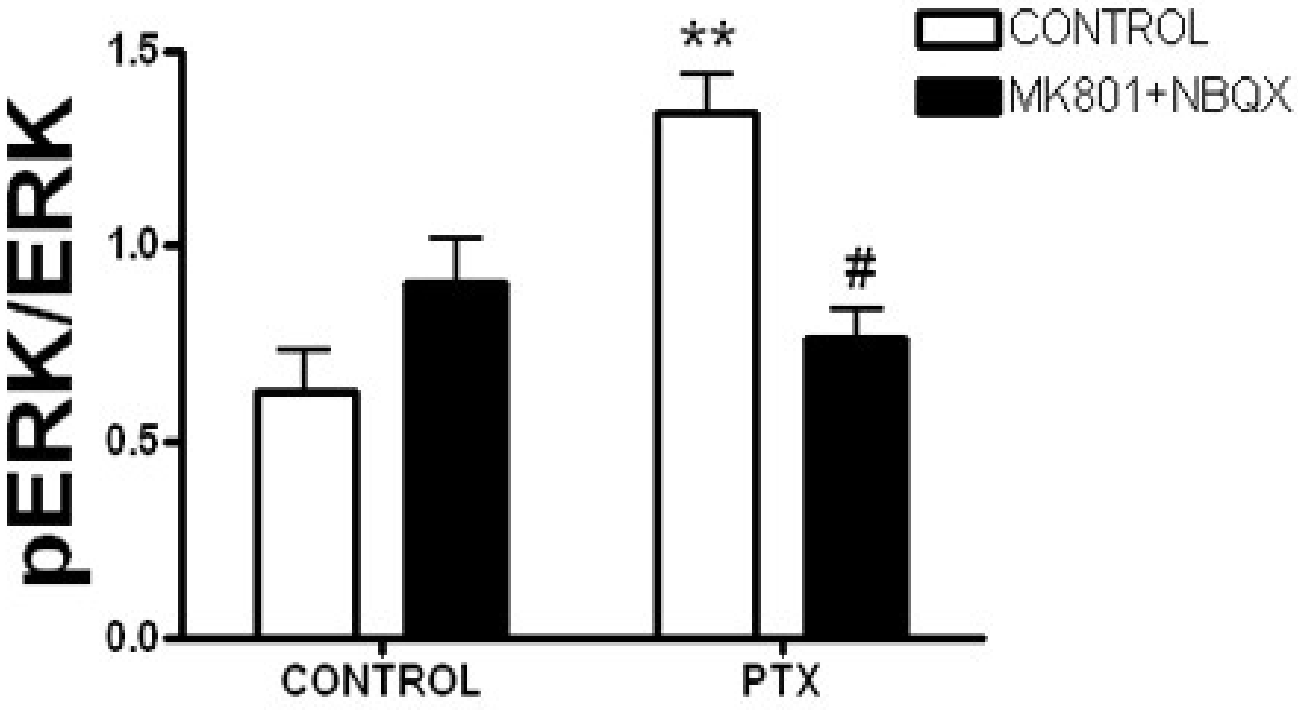


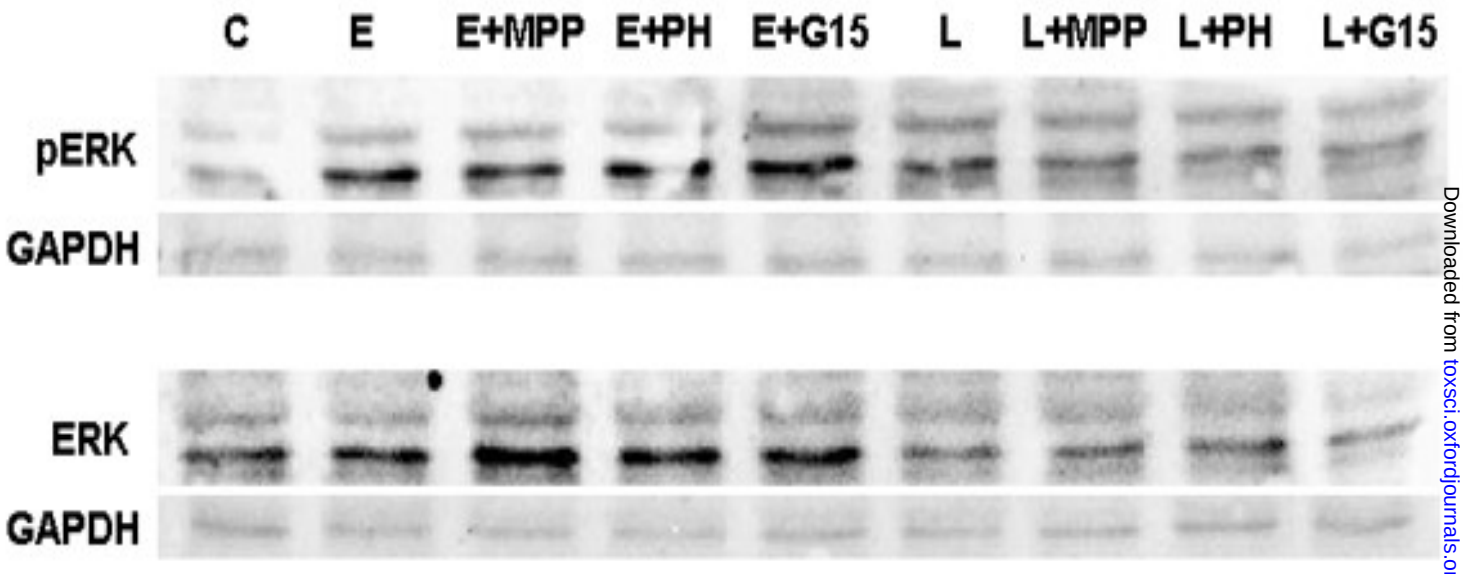


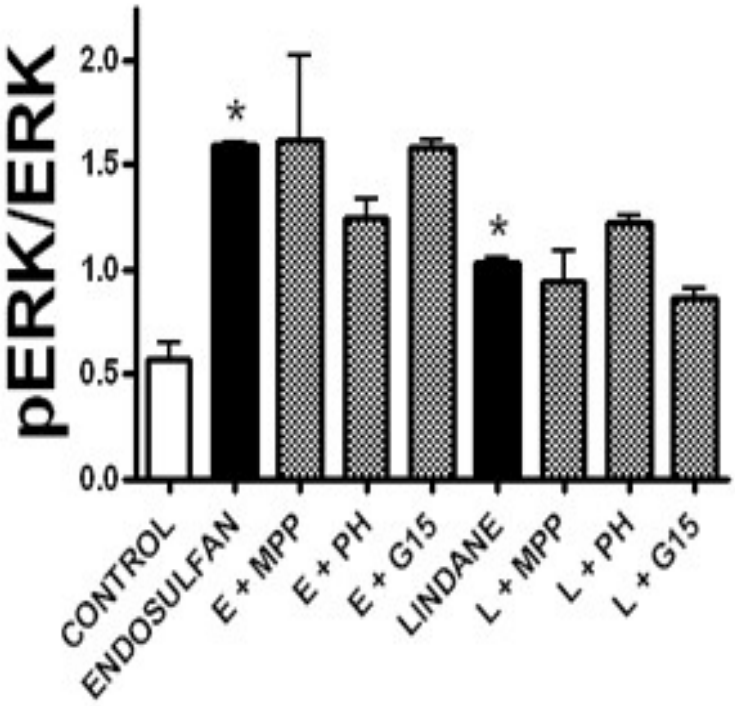


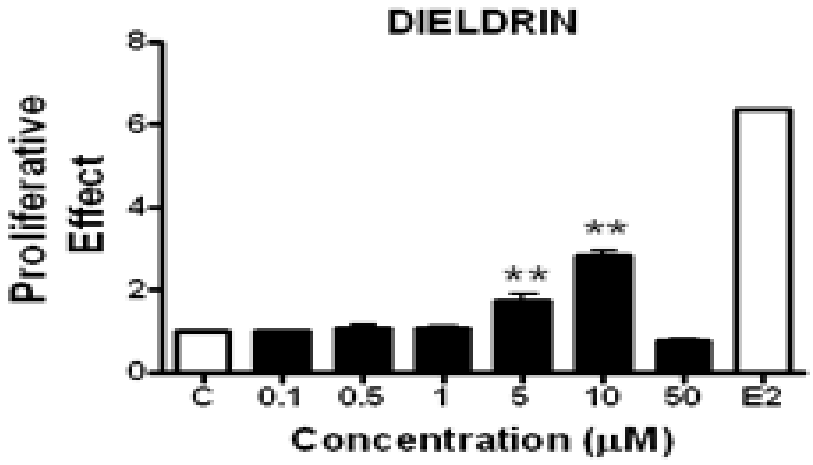


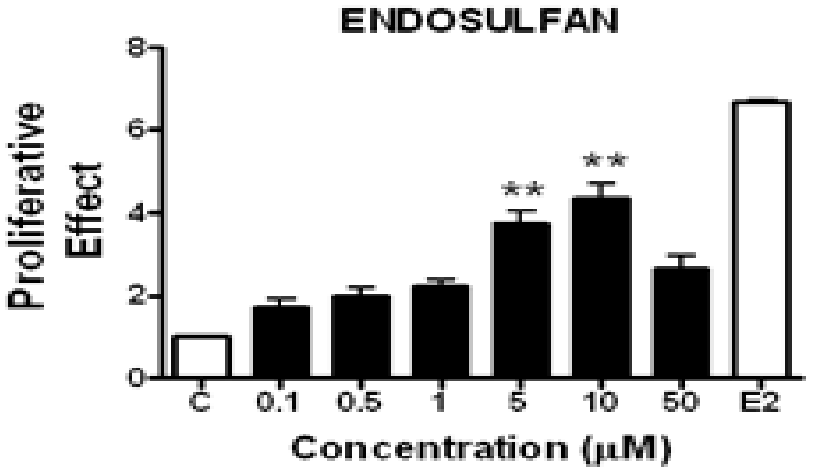


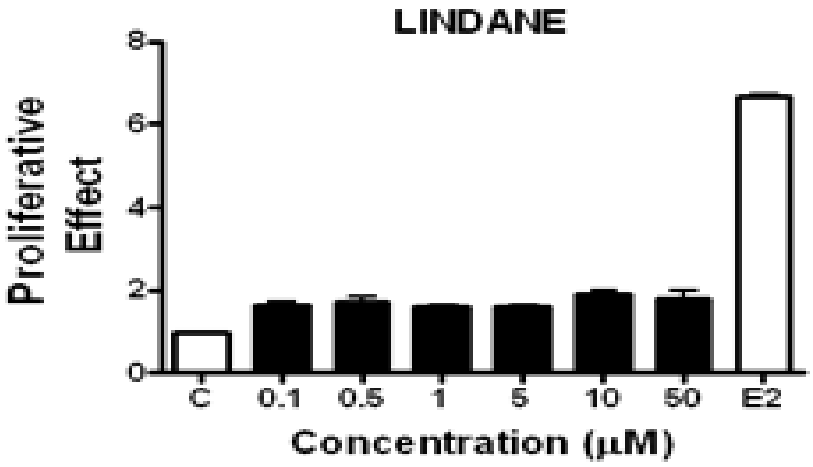












SUPPLEMENTARY DATA

Supplementary figure legends

Supplementary Fig. 1. Differential expression of ERα and ERβ in primary cultures of cerebellar granule cells (CGC) and of cortical neurons (CN). After 7-8 DIV, neuronal cultures were subjected to Western Blot for the detection of ERα (66 KDa) and ERβ (56 KDa). Immunoblots for the indicated proteins are representative of 3 independent experiments.

Supplementary Fig. 2. Glutamate receptor antagonists inhibit picrotoxinin (PTX)-induced ERK1/2 phosphorylation in cortical neurons. Cells were treated with water (Control or C) or 100μM PTX for 5 h. both in the absence and presence of the glutamate receptor antagonists MK-801 and NBQX (M+N, both at 10μM). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of two independent experiments. Statistical comparisons were made by Two-way ANOVA: ** $p < 0.01$ versus control; $^{\#}$ $p < 0.05$ PTX-treated cells.

Supplementary Fig. 3. ER antagonists failed to reverse endosulfan- and lindane-induced ERK1/2 activation in CN. Cells were treated with DMSO (Control or C), 10μM endosulfan (E) or 30μM lindane (L) for 5 h. both in the absence and presence of the ER antagonists MPP, PHTPP (PH) and G-15 (all at 1μM). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of two independent experiments. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$ versus control.

Supplementary Fig. 4. Concentration-dependent proliferative effect of dieldrin, endosulfan and lindane in MCF-7 cells. Cells were incubated for 6 DIV at 37 °C in the presence of OCPs at the indicated concentrations. Results are expressed as proliferative effect (calculated as the ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Data are mean ± SE of 3 independent experiments, each one performed in triplicates. Statistical comparisons were made by One-way ANOVA: ** $p < 0.01$ *vs*. control.