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ABSTRACT

Xeno-estrogens, a class of endocrine disrupting chemicals (EDCs), can disturb estrogen receptor-dependent pathways involved in differentiation, proliferation or protection. Multiple methods have been developed to characterize the disturbances induced by EDCs in different cells or organs. In this study we have developed a new tool for the assessment of estrogenic compounds on differentiation. For this purpose we used the global model of NGF-induced neurite outgrowth of a pseudoneuronal PC12 cell line stably transfected with estrogen receptor alpha (PC12 ER). This new test evidences a new selectivity in which estradiol, genistein and 4-hydroxytamoxifen increased the NGF-induced neurite outgrowth of PC12 ER cells in a dose-dependent manner. In contrast, the strong estrogen agonist 17α-ethynylestradiol, the strong antagonist raloxifene and the agonist bisphenol A were unable to modify the neuritogenesis of PC12 ER cells. Therefore, the analysis of neuritogenesis in PC12 ER cells constitutes a complementary tool for the characterization of xeno-estrogen activity and also serves as a basis for further studies focusing on the mechanisms of EDCs in a neuronal context. Moreover, this test constitutes an alternative to animal testing.

Key Words: Estrogen, endocrine-disrupting chemicals, PC12 cells, estrogen receptor, differentiation.

1. INTRODUCTION

It is known that estradiol (E2) acts on the reproductive system. However, it also has numerous actions on non-reproductive tissues such as bone, the cardiovascular system, the brain and the immune system (Turgeon et al., 2006). Several studies have reported that the actions of endogenous estrogens could be disrupted by pollutants, particularly xeno-estrogens, a family among endocrine-disrupting chemicals (EDCs) (Colborn et al., 1993; Guillette et al., 1994; Toppari et al., 1996). The primary environmental problem is that EDCs are widely dispersed in surface water (Campbell et al., 2006) throughout the world and the detected concentrations are mainly linked to waste water treatment plan that is not efficient enough to remove the overall compounds (Hamid and Eskicioglu, 2012). Their presence in environment constitutes a risk for Human and wildlife. In this context, the European Parliament has recently voted the inclusion in the list of priority substances of the Water Framework Directive, two well-known estrogenic compounds: E2 and 17α-ethynylestradiol (EE2), being used as pharmaceuticals (European Parliament News, 2012). These two compounds are present in environment at ng/L and ng/g in river and sediment (Huang et al., 2013; Leusch et al., 2013; Esteban et al., 2014). Therefore, several *in vivo* and *in vitro* methods have been developed to characterize the response of E2 as well as of other EDCs (Andersen et al., 1999; Kerdivel et al., 2013). *In vivo* methods utilize different species to study the different physiological functions of estrogens. For example, uterotrophic growth has been studied in rodents (Odum et al., 1997), vitellogenin expression has been investigated in rainbow trouthepatocytes (Flouriot et al., 1995; Sumpter and Jobling, 1995) or modification of life cycle was assessed in zebrafish (Micael et al., 2007). *In vitro* approaches were used to investigate the subcellular effects of E2 and EDCs that are mediated by estrogen receptor (ER)-dependent mechanisms, particularly transcriptional or membrane-initiated mechanisms (Mendelsohn, 2000; Edwards, 2005).

Transcriptional studies primarily utilize *in vitro* short-term assays with reporter genes under the control of consensus sequences in their promoters, such as the estrogen response element (ERE)(Petit et al., 1995; Arnold et al., 1996; Andersen et al., 1999; Balaguer et al., 1999), the AP1- or SP1-binding sites(Fujimoto et al., 2004; Schreihofer, 2005). In parallel, other *in vitro* approaches were used to study the global cellular effects of EDCs. For instance, using the E-Screen assay, studies report the proliferative effects of EDCs on ER positive breast cancer cells (Soto et al., 1992; Villalobos et al., 1995; Andersen et al., 1999). If xeno-estrogens have strong proliferative effects, they may also induce differentiating effects on certain tissues such as uterus, blood vessels, heart, bone and brain (Turgeon et al., 2006). With regard to the brain, many *in vivo* and *in vitro* studies have reported a role for E2 in brain protection and functioning (for review see Habauzit et al. 2011); however, there is no test currently available to assess the differentiating effects of EDCs in a neuronal context alternative to animal model.

The PC12 cell line is derived from rat pheochromocytoma, a tumor arising from the adrenal medulla(Greene and Tischler, 1976) and is highly regarded among the *in vitro* neuronal cell models. Thus many studies have investigated the mechanisms of neurodegeneration/neuroprotection, in relation notably with Parkinson's disease, by determination in PC12 cells of the biosynthesis and release of catecholamine (Kumar et al., 1998; Yoneda et al., 2003; Ando et al., 2013), the modulation of redox activity (Shearman et al., 1994; Vimard et al., 2011). Other studies have investigated the mechanisms of differentiation, using the capacity to extend neurites when treated with nerve growth factor NGF (Gollapudi and Oblinger, 2001). Moreover the ability of PC12 cells to take into account the dependence on the ER status has also been reported: we previously demonstrated that the stable transfection of PC12 cells with ERα was able to modify differentiation (neurite outgrowth) or survival in the presence or not of the natural hormone E2 (Merot et al., 2005; Merot et al., 2009; Ferriere et al., 2013).

In this study, our objective was to develop a new tool for the evaluation of the EDC differentiating effect. In this way the EDC effect was evaluate in the context of undifferentiated / differentiated PC12 cells mainly by the evaluation of their ability to act on the classical estrogenic targets: transcription and differentiation. We used the following pharmaceutical products: 17α-ethynylestradiol (EE2), which is widely used for birth control (Lobo and Stanczyk, 1994); raloxifene (Ral) and 4-hydroxytamoxifen (4-OHT), which are two selective estrogen receptor modulators (SERMS) that display varying degrees of agonist or antagonist activities depending on the cellular context (Ho and Liao, 2002); the industrial product bisphenol A (BPA), which is a plasticizer and one of the most common EDCs (Willhite et al., 2008) and the clinically relevant phytoestrogen genistein (Gen), which is a plant-derived isoflavone (Polkowski and Mazurek, 2000). The concentrations of compounds used in this study were previously demonstrated to be near the physiologically active concentrations and near the concentration levels found in environment and/or plasma (Habauzit et al., 2011; Waye and Trudeau, 2011). Here, we report that the determination of neuritogenesis in PC12 ER positive cells constitutes a new tool for screening purposes as this technique provides information that are complimentary to other global bioassays based on cellular proliferation or to short-term subcellular analysis. The determination of neuritogenesis could also be a basis for further studies focusing on the mechanisms of EDCs in a neuronal context, which is currently poorly documented.

2. MATERIALS AND METHODS

2.1. Materials

The following reagents were purchased as powder from Sigma Aldrich (St. Louis, MO): E2, EE2, Gen, 4-OHT, Ral, BPA that were solubilized in ethanol or DMSO and then

prepared by serial dilutions. No differences were observed between EtOH and DMSO on nerve growth factor (NGF) induced PC12differentiation (data not shown). The final vehicle concentration was 0.1% in the culture medium. Recombinant NGF was prepared according supplier information (Sigma Aldrich, St. Louis, MO). The antibody raised against ERα C-terminal (HC-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody raised against β-actin (AC-15) was purchased from Sigma Aldrich (St. Louis, MO).

2.2. Plasmids

The ERα cDNA, ERE-TK-Luc and CMV-β-Gal reporter plasmids were previously described (Flouriot et al., 2000; Metivier et al., 2002). The SP1 reporter plasmids were obtained from Panomics (Panomics Inc., Fremont, CA).

2.3. Transcriptional activity of ERα in undifferentiated and differentiated PC12 cells

PC12 control and PC12 ER cells are naive PC12 cell lines that were transiently transfected respectively with the empty vector named pCR3.1 or with thepCR3.1 plasmid in which is integrated the coding sequence for full length Estrogen Receptor alpha (pCR3.1 ERα). These transient plasmid transfections were used in order to distinguish the ER dependent from ER independent EDCs transcriptional activities. These transcriptional activities were determined on two ER transcriptional pathways: - an ER direct interaction with DNA through ER/estrogen response element interaction (ERE consensus sequence integrated in plasmid containing luciferase as indicator of the compounds effects, ERE-TK-Luc) – an ER indirect interaction through the ER/specific factor 1 that binds with the SP1 consensus DNA sequence (SP1 consensus sequence integrated in plasmid containing luciferase as indicator of the compounds effects, SP1-Luc). The plasmid CMV-β-gal was used for the normalization of the efficiency of the transient transfection, and the cytomegalovirus

promoter led the compound independent expression of the β-galactosidase enzyme. Briefly, transient transfections of wild-type PC12 cells were performed in 24-well plates using the JetPEI transfection reagent (Polyplus transfection, Saint Quentin Yvelines France). One hour prior to transfection, the standard culture medium was replaced with phenol red-free DMEM-F12 containing 2% charcoal-stripped serum composed by 1.6% charcoal-stripped fetal calf serum and 0.4% charcoal-stripped horse serum. The cells were then transfected for 12 hours with a DNA mixture containing the expression vectors (100 ng per well) ERE-TK-Luc or SP1-Luc together with CMV-β-Gal as the internal control (100 ng per well) and either the expression vector pCR3.1 or pCR3.1 ERα (50 ng per well). The total plasmid amount was 250 ng/well. After washing with PBS, different estrogenic compounds and/or Nerve growth factor (NGF; that initiates the PC12 cells neurite outgrowth) were added to the cells for 30 hours in DMEM-F12 containing 2 % charcoal-stripped serum containing both fetal calf and horse serum. Cells were lysed with luciferase assay system with reporter lysis buffer (Promega, Madison). The reporter gene activity was quantified from the luciferase activity with Veritas Luminometer (Turner biosystems) and then the absorbance of the β-galactosidase activity was determined with iMark microplate reader (Biorad). The luciferase activity was then normalized by β-galactosidase activity. The fold of induction was finally determined by the normalization of tested compound on the PC12 control (+/- ER) with ethanol alone or with ethanol and NGF co-treatment, considered as reference compound (Flouriot et al., 2000).

2.4. Generation of stable PC12 ERα clones

Stable PC12 clones (PC12 control and PC12 ER) were previously obtained after transfection of wild-type PC12 cells with the expression vectors, empty pCR3.1 or pCR3.1 ERα containing the coding region cDNA of full length ERα. The stable transfections were

performed on PC12 cells, using the FuGENE6 reagent (Roche Diagnostics, Bâle, Switzerland). pCR3.1 plasmid also contains the resistance gene for geneticin antibiotics (G418, Invitrogen). The transfected cells, PC12 control clones (pCR3.1 empty) or PC12 ER (pCR3.1 ERα) clones were selected with 800 µg/ml of G418 for 1 month (Merot et al., 2005). The PC12 cells were then routinely cultured in phenol red-free DMEM/F12 medium (Sigma) containing 8% and 2% charcoal-stripped fetal calf and horse serum, respectively (FCS; Biowest and Life Technologies, Cergy Pontoise, France) with 400 µg/ml of G418 antibiotics treatment in order to keep stably transfected cell selection. The ERα expression was verified in the PC12 control and PC12 ER clones by western blot analysis (Fig. 3B).

2.5. Determination of ERα -dependent proliferation of PC12 clones

PC12 control clones and PC12 ERα clones were plated at 10^4 cells per well on 24-wells plate in phenol red-free DMEM/F12 medium (Gibco, Life Technologies, Cergy Pontoise, France) containing 5% charcoal-stripped fetal calf (Biowest, Nuaille, France). 24 hours later, ethanol or different concentrations of E2 were added. The medium was renewed every 2 days. The proliferation test was stopped at day 5 by trypsination and then 100 µl of fresh medium were added. Cells were then counted with malassez counting chamber. In order to verify our result and to assess the molecules effects a second proliferation test was performed. Cells were seeded onto 96-well plates at $2x10^3$ cells per well in phenol red-free DMEM/F12 containing 10% of serum (Horse and calf). Twenty-four hours later, cells were transferred into phenol red-free DMEM/F12 containing either 5% of charcoal stripped FCS (Sigma-Aldrich) for 24 hours. Then cells were treated with EtOH, E2 and others molecules for 7 days. The treatment was renewed every two days. At the end of the experiment, relative cell number was then assessed by quantification of cellular ATP content (ViaLight HS kit, Lonza).

190

191 *2.6. Determination of ERα-dependent neurite outgrowth of PC12 cells*

192 PC12 control and PC12 ER clones, plated at a density of 4 x 10^4 cells/well in 12-well
193 plates, were transferred to phenol red-free DMEM/F12 medium containing 2 % charcoal-
194 stripped serum (1.6 % charcoal-stripped fetal calf serum and 0.4 % charcoal-stripped horse
195 serum) and then treated with 5 ng/mL NGF and/or EtOH, E2, EE2, Gen, 4-OHT, Ral and
196 BPA at different concentrations. Sixty hours later, the differentiation of PC12 cells was
197 scored as previously described (Merot et al., 2005). Briefly, the cells with at least one neurite
198 with a length greater than one cell body were scored on at least 30 fields (10 fields x 3
199 separate experiments) under light microscopy. Results were expressed as the mean ratio of
200 differentiating / total cells for each field. Secondly, the ratio adherents' cells / total cells was
201 also calculated for each experimental condition.

202

203 *2.7. Statistical analysis*

204 The statistical analyses were performed by an analysis of variance followed by
205 analysis of individual group differences using the Statview 5.0 software (SAS Institute Inc.,
206 Cary, NC). One-way analysis of variance (ANOVA) was used to determine the effects of
207 estrogenic compounds on transcription (for more details see figure 1) or on neuritogenesis
208 (for more details, see figure 4). Moreover a two-way analysis (two-AOV) was used to
209 determine possible interactions between 2 variables: 1) interaction between effects of NGF
210 and effects of estrogenic compounds on transcription.

211

212 **3. RESULTS**

213

214 *3.1. Transcriptional activity of EDCs in undifferentiated and differentiated PC12 cells*

We evaluated the estrogenic activities of classical estrogenic compounds on the transcriptional activity through ERE and SP1 reporter assay in a context of undifferentiated or differentiated cells. In this way these activities were evaluated in PC12 cells treated or not with NGF. As expected, in the PC12 control cells (ER negative), the ERE-TK activity was not modified by either E2 or by the EDCs in the presence or in the absence of NGF treatment (Fig. 1A). By contrast, in the PC12 cells transiently transfected with ERα, the compounds E2, Gen, EE2 and BPA increased the ERE-TK transcriptional activity, whereas 4-OHT and Ral decreased it whatever the NGF status. Both of these effects occurred in a dose-dependent manner (Fig. 1B). In the ER negative cells, higher concentrations of BPA and 4-OHT slightly decreased the SP1-Luc activity (Fig. 2A). SP1 luciferase activity was regulated in ER dependent manner by the estrogenic compounds. Indeed, E2, Gen, EE2, 4-OHT and BPA upregulated the SP1 reporter gene transcriptional activity in the PC12 ER cells in the presence or not of NGF (Fig. 2B). No significant interaction was identified following a 2-way ANOVA analysis between the treatments with EDCs and NGF for both ERE- and SP1- dependent transcription.

3.2. E2 and EDC promote a new selectivity on NGF-induced neurite outgrowth of PC12 ER clones

The dose-related neuritogenic effects of estradiol or different EDCs were determined on PC12 cells that were stably transfected with either pCR3.1 ERαor the empty vector, pCR3.1. Treatment with NGF (5 ng/mL) for 60 h provoked the neurite outgrowth of PC12 control and PC12 ER clones (Fig. 3A). The expression of ER in PC12 ER cells was confirmed by Western-Blot (Fig. 3B).

E2 (10^{-9} and 10^{-8} M) significantly increased the NGF-inducedneurite extension of the PC12 ER clones but not the PC12control clones (Fig. 4A). Gen (concentrations from 10^{-8} to

240 10^{-6} M) and 4-OHT (concentrations from 10^{-8} to 10^{-6} M) also increased the NGF-
241 inducedneuritogenesis *via* an ERα-dependent mechanism (Fig. 4B-C). By contrast, EE2, Ral
242 and BPA did not have significant effects on PC12 ER clones treated with NGF (Fig. 4D-F). In
243 the absence of NGF, the compounds E2, EE2, Gen, 4-OHT, Ral and BPA did not modify
244 neurite outgrowth of both PC12 ER and PC12 control clones (data not shown). Only the
245 highest concentration of Gen (10^{-5} M) inhibited the neurite outgrowth in both the control and
246 PC12 ER clones treated with NGF (Fig. 4B) and also decreased the ratio adherent cells / total
247 cells (respectively 32 ± 8.5 % and 33.5 ± 12.5 % for PC12 ER and PC12 control clones, when
248 this ratio was near 1 for other conditions (data not shown)).
249
250 **4. DISCUSSION**
251 Many studies reported that estrogens display neuroprotective effects (for review, see
252 Habauzit et al, 2011) in physiopathological context of brain ischemia or Alzheimer's disease.
253 For instance E2 reduces in ER-dependent manner brain damage induced by cerebral occlusion
254 (Dubal et al., 2001) or prevents hippocampal neurons (Miller et al., 2005) or PC12 cells
255 (Ferriere et al., 2013) from apoptosis. Another target of estradiol in brain is neuronal
256 extension (for review see Toran-Allerand et al., 1999). For instance, estradiol enhanced
257 neurite proliferation in organotypic cultures of the newborn mouse forebrain (Toran-Allerand,
258 1980) and in preoptic area grafted into adult rat brain(Matsumoto et al., 1988). In cell lineage,
259 it was recently shown that E2 and diethylstilbestrol (DES) were able to increase the NGF
260 induced neurite outgrowth in PC12 cells (Merot et al., 2005; Merot et al., 2009; Habauzit et
261 al., 2011). But there are lacks in the evaluation of classical estrogenic compounds effect on
262 cell differentiation and there is no available simple and fast cellular method for the estrogenic
263 compounds differentiating effect evaluation other than those requiring animal testing.

Several *in vitro* assays have been developed to characterize the global and subcellular effects of EDCs (Andersen et al., 1999). Like E2, the effects of EDCs can be mediated by classical transcriptional mechanisms through ERs (Fujimoto et al., 2004), and a large number of *in vitro*short-term tests were developed for this screening purpose. The majority of these tests were based on EDC-induced transcriptional activity in different cells, such as yeast (Petit et al., 1995) or mammalian cells, that were transiently transfected with reporter plasmids containing ERE (Andersen et al., 1999), SP1 or AP1 binding sites in their promoters (Safe, 2001; Fujimoto et al., 2004; Schreihofer, 2005). These ER-dependent transcriptional effects had not yet been extensively studied in the context of pseudoneuronal PC12 cells, which can be present at undifferentiated or differentiated states. We found that E2, Gen, EE2 and BPA increase the ERE-dependent transcriptional activity of both undifferentiated and differentiated PC12 cells, whereas 4-OHT and Ral decreased this activity. These respective full agonistic and repressive effects were previously reported for other cell lines (Petit et al., 1995; Andersen et al., 1999; Paris et al., 2002; Fujimoto et al., 2004; Schreihofer, 2005). The 4-OHT and Ral activity in PC12 cells may be dependent upon the cellular context of these cells and changes in the conformation of the ligand binding domain affecting the interaction of ER with coactivators or repressors (Smith and O'Malley, 2004). The ERE-mediated transcriptional effects of 4-OHT and Ral, which were observed in PC12 cells and MCF7 cells, were undetectable in neuro2A cells (Schreihofer, 2005). These differences underline the SERMs activities of these compounds. We clearly distinguish the activity of E2 from those of SERMs such as 4-OHT to modulate ERE-dependent genes/promoter construct. That was not true when we studied the ability of ER/ SP1 complexe to modulate transcription. Indeed, E2, 4-OHT and BPA increased SP1-dependent transcriptional activity in PC12 cells transfected with ERα, as previously reported in breast cancer cells cotransfected with a SP1 construct and wild-type ERα(Kim et al., 2003; Safe and Kim, 2008; Wu et al., 2008).Gen, EE2 also

289 increased SP1-dependent transcription effects. Only Ral had no effects. Interestingly, profiles
290 were similarin the context of undifferentiated or differentiated pseudoneuronal PC12 ER cells.
291 The global effects of EDCs are demonstrated primarily by the proliferation of ER-
292 positive breast cancer cells, particularly MCF-7 (E-SCREEN) and T47D cell lines treated for
293 4 to 6 days(Soto et al., 1992; Villalobos et al., 1995; Andersen et al., 1999; Fujimoto et al.,
294 2004; Habauzit et al., 2010). Indeed, if E2 effectively induces the proliferation of MCF7 and
295 T47D cells at 10^{-12} M - 10^{-9} M, E2 had no effect on the proliferation of PC12 cells when it is
296 tested in the same range of concentration in the present study (data not shown). Thus, the
297 proliferation of PC12 cells cannot be considered as a valid model for the evaluation of the
298 estrogenic effects of EDCs.
299 Xeno-estrogens can also mediate differentiation processes either *in vivo* or *in vitro* in
300 tissues such as the reproductive tract (Steinmetz et al., 1998; Svechnikov et al., 2010) or the
301 brain (Panzica et al., 2009; Habauzit et al., 2011). These effects are not well documented. The
302 global model of PC12 cell neuritogenesis can provide interesting specific information in the
303 context of neuronal differentiation. A dose-dependent and ERα-dependent increase of the
304 NGF-inducedneuritogenesis is observed in PC12 cells treated with E2. As previously reported
305 (Gollapudi and Oblinger, 2001; Merot et al., 2005; Merot et al., 2009), E2 (10^{-9} and 10^{-8} M)
306 significantly increased the NGF-inducedneurite extension of the PC12 ER clones but not the
307 PC12control ones (Fig. 4A).We find that among the EDCs, Gen and 4-OHT also display
308 estrogenic effects in PC12 ER clones in a concentration range of 10^{-8} M to10^{-6} M. By contrast,
309 10^{-5} M Gen decreases neuritogenesis, as previously reported for high concentrations of this
310 compound (Bouron et al., 1999). This inhibition appears to be ER-independent and could be
311 explained by a toxic effect of Gen at this concentration. Indeed, we observed a decrease of
312 more than 60% of adherent cells treated with 10^{-5} M Gen that could support this hypothesis.
313 This effect has been already observed in rat primary cortical neurons (Linford et al., 2001).

The selective activation of ER by ligands that is dependent upon the tissue environment is true for the SERMs, 4-OHT and Ral. For instance, both Ral and 4-OHT displayed an anti-estrogenic activity in a proliferation assay of T47D cells(Habauzit et al., 2010), whereas the ER could not discriminate among chemicals with estrogenic or anti-estrogenic activitiesin the context of yeast (Petit et al., 1995).*In vivo*, both Ral and 4-OHT displayed cell-specific estrogenic agonist activity in the skeleton and antagonist activity in the breast (Cauley et al., 2001; Smith and O'Malley, 2004). However, Ral may lack the uterotrophic activity associated with tamoxifen (Delmas et al., 1997; Anthony et al., 2001). Like in the uterus, we found that Ral lacks in PC12 cells a neuritogenic activity compared with 4-OHT. However, Nilsen *et al*. demonstrated that Ral induced neurotrophic effects in PC12 ER positive cells (Nilsen et al., 1998). The opposite results could be explained by the experimental procedures and by the levels of ERα expressed in those cells. Indeed, Nilsen *et al.* induced ERα expression with a 2-week NGF treatment, whereas we used the stable transfection of PC12 cells. BPA and EE2 are generally defined as estrogenic compounds. Concentration of BPA was found in several biological samples such as blood, milk and urine (Lee et al., 2013). BPA mimics E2 in numerous *in vivo* and *in vitro* studies in a concentration range of 10^{-9} M to 10^{-5} M. For example, BPA induced cell proliferation in the uterus of ovariectomized rats *in vivo,*and its effects were nearly identical to those induced by E2 (Steinmetz et al., 1998). BPA did not modify neuritogenesis of PC12 ER clones, even at the highest concentrations used (10^{-7} M and 10^{-6} M), whereas E2 and the well-established estrogenic compound diethylstilbestrol increased neuritogenesis at a concentration of 10^{-9} M (Merot et al., 2009). In PC12 cells, only concentrations of BPA higher than 50 μM had been reported to decrease viability and neurite extension *via* an ER-dependent mechanism(Lee et al., 2007). EE2 is a clinically relevant estrogenic compound that is more potent than E2 and is widely used for birth control (Lobo and Stanczyk, 1994). With regard to the subcellular or global cellular assays of estrogenicity,

339 10^{-10} M to 10^{-9} M EE2 can display strong estrogenic activity. It is verified using the ERE-
340 dependent transcriptional activity in PC12 cells (present study) or in the global model of
341 proliferation in T47D or MCF7 cells (Andersen et al., 1999; Habauzit et al., 2010). By
342 contrast, EE2 did not modify PC12 neuritogenesis in the concentration range of 10^{-10} M to 10^{-7}
343 M. Distinct biological cell-specific potencies of E2 and EE2 that are mediated by the ER are
344 not usually reported. However, E2, but not EE2, induced ER-dependent NO synthesis and
345 protection against oxidative stress in endothelial cell cultures (Andozia et al., 2010).

346 The effects of EDCs on ERE-, SP1-mediated transcription in undifferentiated and
347 differentiated PC12 cells gave profiles that have been classically reported for other cell lines,
348 especially for breast cancer cells. The global analysis of neuritogenesis in PC12 cells stably
349 transfected with ERα has demonstrated a selectivity of the EDCs that is distinct from that
350 observed for the subcellular bioassay of transcription. The effects of estrogens can be the
351 result in neuronal cells of a tissue-specific complex interplay of the activation/inhibition of
352 DNA binding-dependent, DNA binding-independent gene transcription but also membrane-
353 initiated mechanisms (Habauzit et al. 2011). The pathways involved in the EDC-induced
354 neuritogenesis in the PC12 ER clones could be different from those involved in the
355 differentiation and proliferation of other cell lines and could be linked to the specific cellular
356 context, especially the presence of specific transcription factors, coactivators and corepressors
357 (Smith and O'Malley, 2004).

358 In the future, investigations into the mechanisms that sustain the effects of EDCs
359 should integrate different approaches and interassay comparisons. As a result, the
360 determination of neurite outgrowth of PC12 ER clones constitutes an original and
361 complementary global bioassay for the characterization of xeno-estrogens. PC12 ER clones
362 and cells expressing different deleted or mutated forms of ERα have been previously used to
363 investigate subcellular ER-dependent mechanisms sustaining E2 activity (Merot et al., 2009).

Thus, neuritogenic effects of E2 were suppressed in PC12 cells stably transfected with a DNA binding domain deleted ERα (Merot et al., 2009). These different PC12 clones could also be used to characterize the ER domains that are involved in the effects of EDCs. Moreover, these PC12 clones provide the basis for further studies focusing on the effects of EDCs in the specific context of brain development or diseases, which remain poorly documented.

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562 **Figure Legends**

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564 Fig. 1. The transcriptional effects of estrogenic compounds measured by the ERE-TK-Luc
565 reporter assay in undifferentiated or differentiated PC12 cells. PC12 cells were transiently
566 transfected with ERE-TK-Luc reporter gene together with CMV-β-Gal and the empty pCR3.1
567 plasmid (A) or the pCR3.1 plasmid encoding ERα (B). Twelve hours after transfection, the
568 cells were differentiated or not with NGF (5 ng/mL) (respectively right and left panels) and
569 treated with vehicle control ethanol (EtOH) or 17 β estradiol (E2: 10^{-9} M), genistein (Gen: 10^{-9} M and 10^{-7} M), 17 α ethynylestradiol (EE2: 10^{-9} M and 10^{-7} M), 4-hydroxytamoxifen (4-
571 OHT: 10^{-9} M and 10^{-6} M), raloxifen (Ral: 10^{-7} M), bisphenol A (BPA: 10^{-9} M and 10^{-7} M) for
572 30 h. The luciferase activities after E2 and EDCs treatments were expressed in reference (fold
573 induction) to the luciferase activity measured in cells treated with EtOH (A and B). The data
574 are the mean ± SEM of four experiments. No significant interaction was identified following a
575 2-way ANOVA analysis of the EDC and NGF treatments. *, $P < 0.05$; **, $P < 0.01$ and ***,
576 $P < 0.001$: for the significant effects of the estrogenic compounds compared to the respective
577 controls, which are the cells treated with EtOH +/- NGF.

578

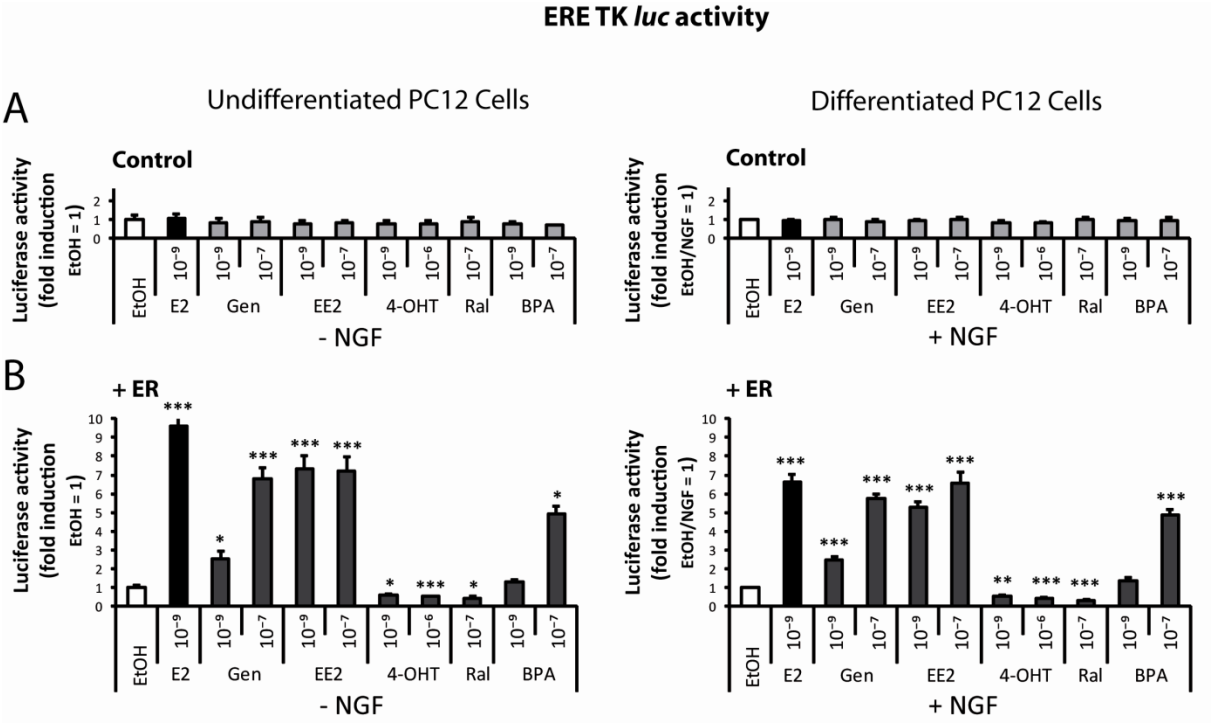
579 Fig. 2. The transcriptional effects of estrogenic compounds measured by the SP1-luc reporter
580 assay in undifferentiated or differentiated PC12 cells. PC12 cells were transiently transfected
581 with SP1-Luc reporter gene together with CMV-β-Gal and the empty pCR3.1 plasmid (A) or
582 the pCR3.1 plasmid encoding ERα (B). Cells were then differentiated or not with NGF and
583 treated with control EtOH or E2, Gen, EE2, 4-OHT, Ral, BPA for 30 h (for more details, see
584 Figure 1).

585

Fig. 3. PC12 clones obtained after stable transfection. (A) PC12 cells stably transfected with pCR3.1 plasmid encoding ERα (PC12 ER clones) or the empty pCR3.1 plasmid (PC12 Control clones). The NGF treatment induces neurite outgrowth of both PC12 clones (lower panel). (B) ERα expression was controlled by western blot in PC12 clones that stably express ERα (PC12 ER) or that do not express ERα (PC12 control), and the expression of β-Actin was used as internal control.

Fig. 4. Neuritogenic effects of estrogenic compounds in PC12 clones. Neurite outgrowth was induced by treatment with NGF (5 ng/ml) for 2 days in the PC12 clones that stably express ERα (PC12 ER) or not (PC12 control). Simultaneously with NGF, clones were treated with different concentrations of 17 β estradiol (A) and EDCs (B-F). The neurite outgrowth was quantified by scoring the ratio differentiated cells/undifferentiated cells for each microscopy field. Differentiated cells were ones that have at least one neurite that the length is greater than one cell body. For both PC12 control and PC12 ER clones, results were expressed in reference (fold induction) to the neurite outgrowth of differentiated cells treated with EtOH. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$: significant effects of the different concentrations of EDCs in reference with EtOH. The data are the mean ± SEM of 30 to110 light microscopy fields.

605 **Fig 1**



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608 Fig.2

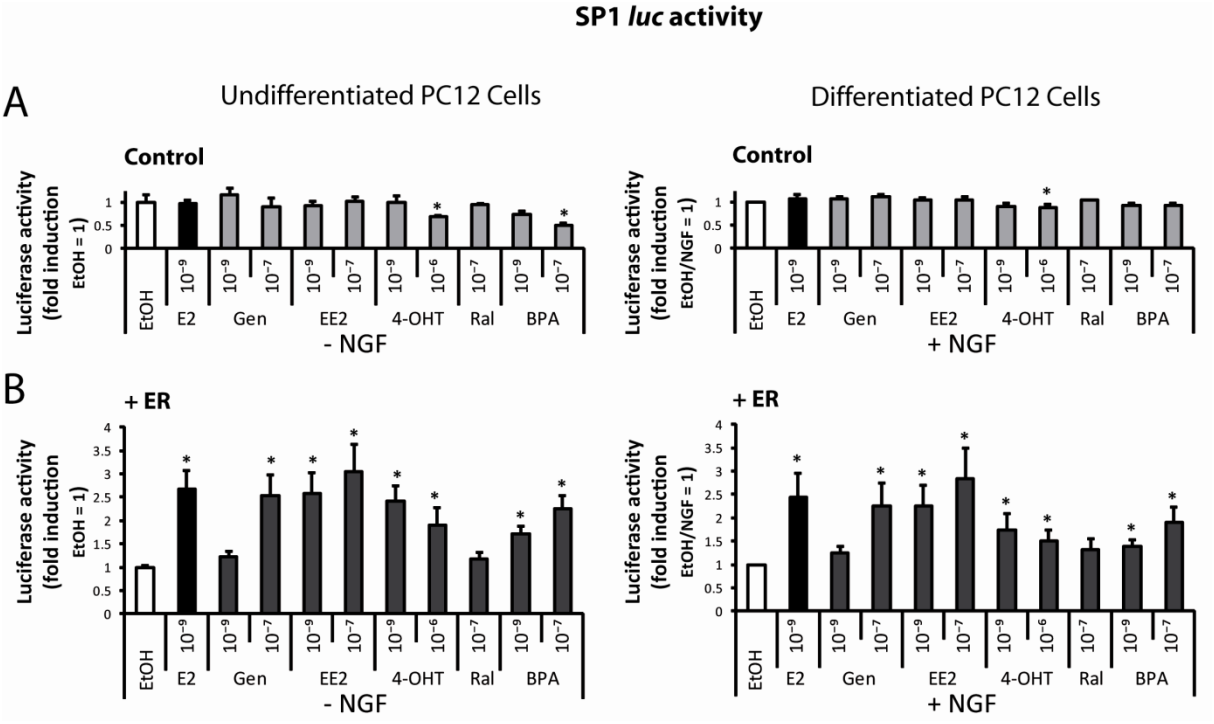

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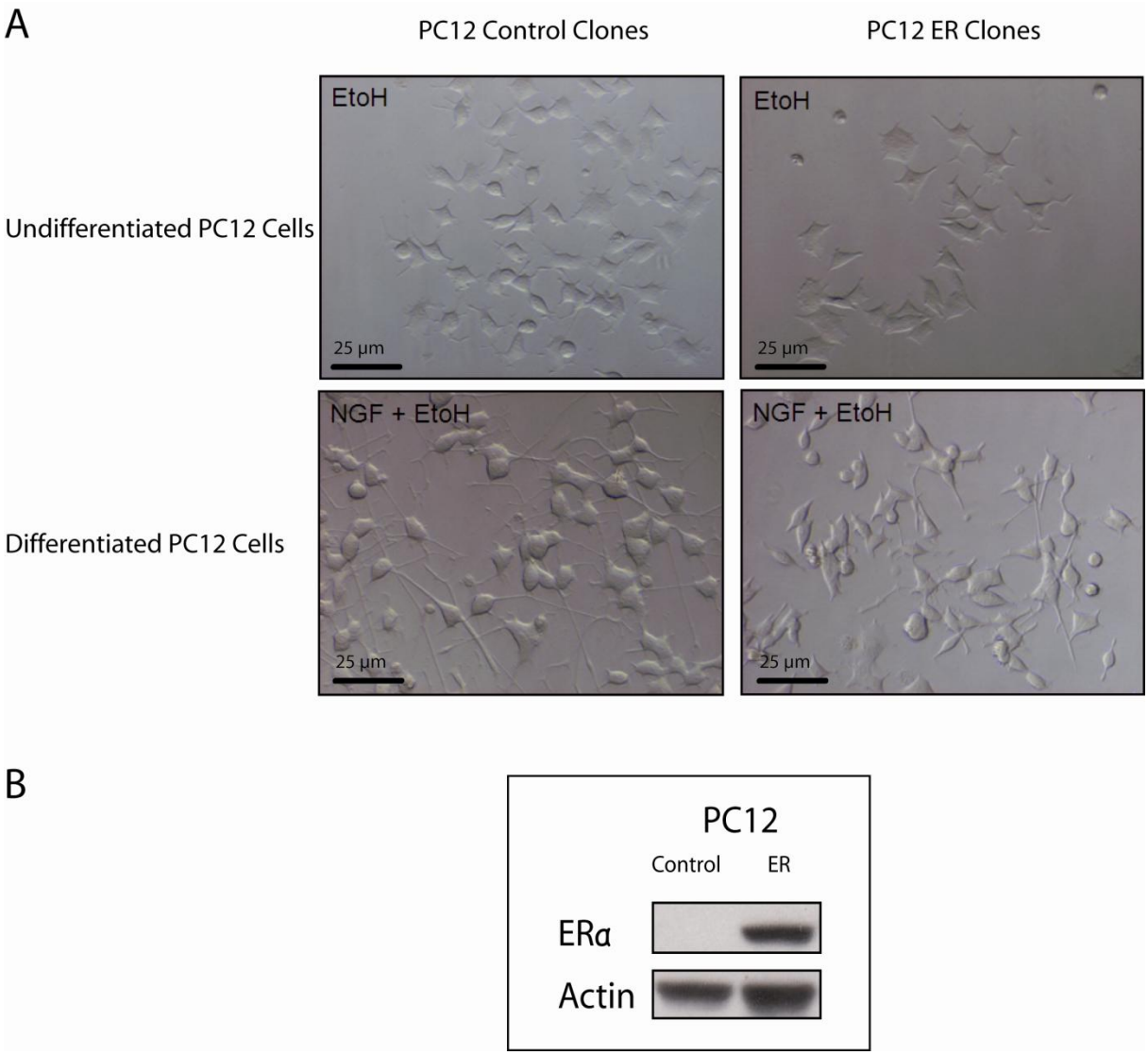
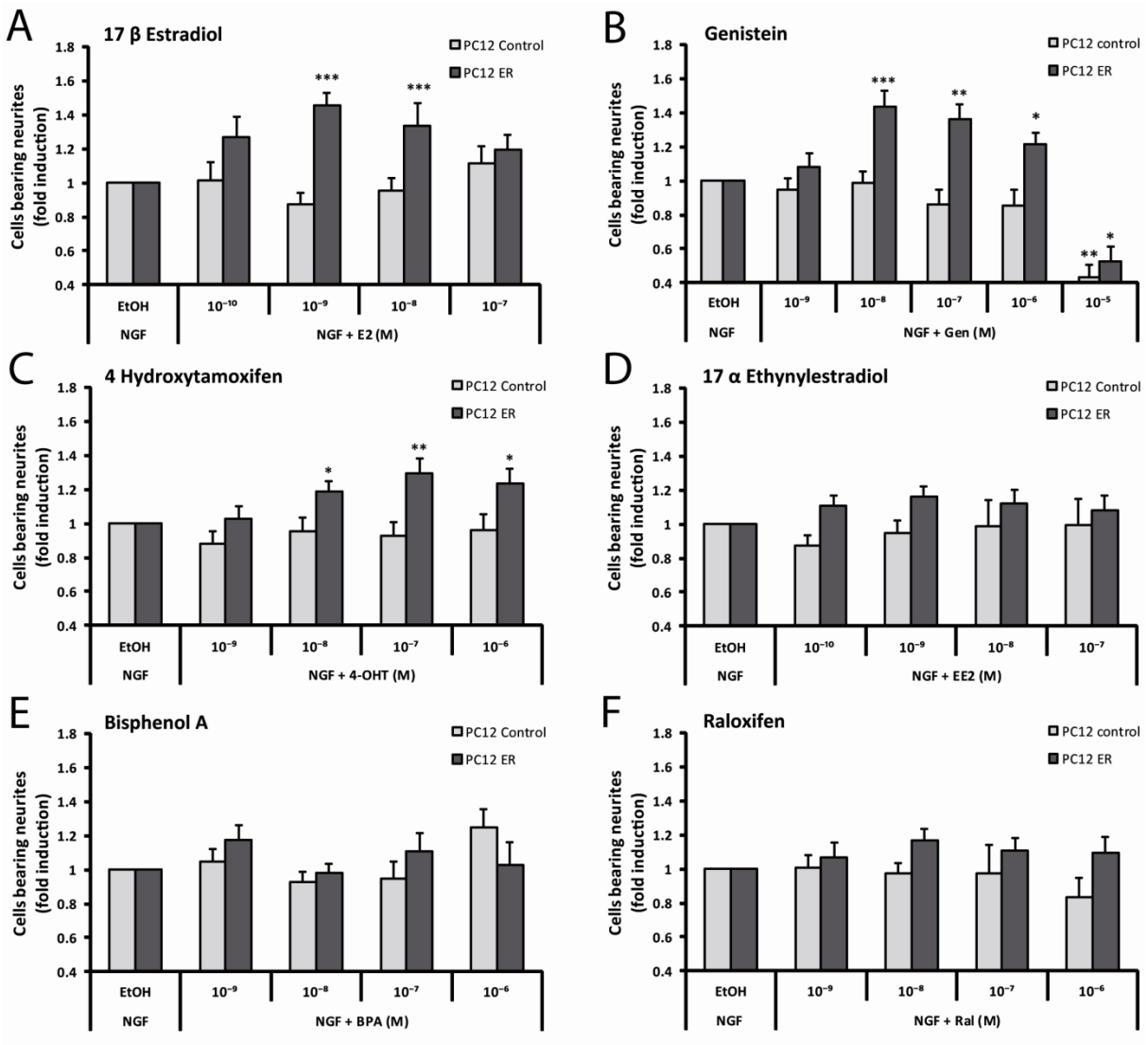
Fig. 3.


A
PC12 Control Clones
PC12 ER Clones
Undifferentiated PC12 Cells
Differentiated PC12 Cells
EtoH
EtoH
NGF + EtoH
NGF + EtoH
25 µm
25 µm
25 µm
25 µm
B
PC12
Control
ER
ERα
Actin

618 Fig. 4.



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