

# Sex Differences and Effects of Estrogenic Compounds on the Expression of Inflammatory Molecules by Astrocytes Exposed to the Insecticide Dimethoate

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**Abstract** A low dose of the organophosphorus insecticide dimethoate (DMT) produces oxidation of lipids and proteins and impairs mitochondrial function in the brain of male rats, together with a reduction of gonadal hormones in plasma. Here, we have assessed whether DMT affected the expression of inflammatory molecules, the production of reactive oxygen species (ROS), and the expression of steroidogenic proteins and estrogen receptors in cortical astrocyte-enriched cultures obtained separately from male and female CD1 mice pups. Furthermore, we have analyzed whether estradiol may counteract the effects of DMT. A dose of DMT (2 µg/mL) did not affect cell viability, increased interleukin (IL) 6, IL1β, tumor necrosis factor (TNF)α, interferon-γ-inducible protein 10 (IP10), ERβ, steroidogenic acute regulatory protein, and aromatase mRNA levels and ERα protein levels in male but not in female cultures. Estradiol decreased the mRNA levels of IL6, IP10, TNFα, and IL1β in male but not in female cultures treated with DMT. The effect of estradiol was prevented by the ER antagonist ICI 182,780, fully imitated by an ERβ agonist and partially imitated by an ERα agonist. Furthermore, DMT increased the production of ROS in male astrocytes while estradiol reduced ROS production to control levels. These findings indicate that a sublethal dose of DMT alters astrocyte function. The selective action of estradiol on male astrocytes and the sexually dimorphic action of DMT suggest that the pesticide may have different neurological outcomes in males and females.

**Keywords** Astrocytes · Dimethoate · Estradiol · Estrogen receptors · Pesticides · Sex differences

## Introduction

The presence of complex mixtures of toxic compounds in everyday residential and occupational environments is of concern for human health (Kapka-Skrzypczak et al. 2011; Astiz et al. 2011). Indeed, several epidemiological studies have linked the exposure to environmental pollutants with the incidence of neurological disorders (Ritz and Yu 2000; Di Monte 2003; Liu et al. 2003). Pesticides, which are required for global food production, are included among these pollutants. These molecules linger as residues in food from vegetable and animal origin, as well as in air and water.

The increasing use of organophosphorus pesticides (OPs) since the 50s has introduced a serious and novel hazard for humans and livestock animals. The first experimental evidence on the mechanism of action of OPs indicated that they inhibit acetylcholinesterase activity in peripheral and central nervous system (Koelle and Gilman 1949; for review, see Kwong 2002). Further studies revealed that OPs may cause disruption of a number of metabolic and cell signaling pathways that affect cell proliferation, differentiation, and survival (Rush et al. 2010; Hargreaves 2012).

Among the various molecular targets and mechanisms proposed to mediate OPs neurotoxicity (Casida and Quistad 2005; Soltaninejad and Abdollahi 2009; Banks and Lein 2012), the study of the pro-inflammatory effects has been of great interest. There is evidence suggesting that anti-inflammatory agents are neuroprotective following acute intoxications with OPs (Amitai et al. 2006).

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Moreover, some peripheral biomarkers of inflammation have been experimentally validated and correlate with neurobehavioral deficits (Mrak and Griffin 2005; Dziedzic 2006).

While microglia is the major immunocompetent cell within the central nervous system (CNS), astrocytes have the ability to produce pro-inflammatory mediators such as tumor necrosis factor (TNF) $\alpha$ , interleukin (IL) 1 $\beta$ , IL6, and interferon- $\gamma$ -inducible protein 10 (IP10), which has been shown in vitro (Cerciat et al. 2010; Bellini et al. 2011), and are implicated in a number of neurological diseases (Li et al. 2011; Sidoryk-Wegrzynowicz et al. 2011; Cambron et al. 2012; Losi et al. 2012; Parpura et al. 2012). Sustained inflammatory responses of astrocytes in chronic neurodegenerative diseases may enhance tissue damage through amplification of brain inflammation and consequent neuronal injury (Farina et al. 2007; Buchanan et al. 2010; Li et al. 2011).

Dimethoate (DMT) is an OP insecticide extensively used in horticulture for pest treatment in onions, tomatoes, and citric fruits and as an acaricide for treating gardens, vineyards, and field crops (CASAFE 2007). Previous studies have shown that the administration of DMT to male Wistar rats at a very low dose, during a subchronic period, results in alterations in the nervous system including high oxidation of lipids and proteins, reduction of the antioxidant defense system, and impairment of mitochondrial function in various brain regions (Astiz et al. 2009a,b). This treatment causes also a significant reduction in testosterone and estradiol levels in plasma with increased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels (Astiz et al. 2009c,d), suggesting that endocrine alterations may contribute to the neurotoxic effects (Bailek et al. 2004).

Estradiol is a neuroprotective hormone that exerts multiple regulatory actions in the nervous system, targeting neurons and glial cells, and promoting reorganization and repair of the damaged brain tissue (Azcoitia et al. 2011; Scott et al. 2012). Estradiol, among other actions, inhibits NF $\kappa$ B-mediated transcriptional regulation of numerous pro-inflammatory genes and stabilizes mitochondrial function, preventing the increase in harmful reactive oxygen species (ROS) (Bains et al. 2007; Arnold et al. 2012; Guo et al. 2012). Estradiol reduces astrocyte proliferation, activation, and their response under pathological conditions (Bruce-Keller et al. 2000; Dhandapani and Brann 2007; Lewis et al. 2008; Arevalo et al. 2010; Brown et al. 2009, 2010). Besides being a cellular target for the actions of estradiol in the brain, astrocytes produce estradiol under neurodegenerative conditions (Garcia-Segura 2008) and show sex differences in number, morphology, and in the response to pathological insults (Liu et al. 2007, Santos-Galindo et al. 2011). Sex differences in astrocytes have been suggested to

be involved in the sexual dimorphisms observed in the manifestation of brain pathological alterations (Liu et al. 2007; Santos-Galindo et al. 2011).

In this study, we have assessed whether a low concentration of DMT induces a modification in the expression profile of inflammatory molecules and in ROS production in astrocytes. Furthermore, we have analyzed whether estradiol may counteract the effect of DMT. These studies were conducted in astrocyte-enriched cultures obtained separately from male and female pups, in order to determine possible sex differences in the response of astrocytes to DMT.

## Materials and Methods

### Chemicals

The insecticide employed was purchased from local commercial sources, obtained as a gift from INTA (Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina), and was of analytical grade. The active ingredient was dissolved at 40 % (p/v) in aqueous solution of polyethyleneglycol-400 at 25 %. 17 $\beta$ -estradiol (E) and ICI 182,780 (ICI) were supplied by Sigma-Aldrich (St. Louis, MO, USA); the estrogen receptors' (ER) agonists 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) and 4,4',4''-(4-propyl-(1H)-pyrazol-1,3,5-triyl) trisphenol (PPT) were supplied by Tocris Bioscience (Bristol, UK).

### Animals

CD1 mice dams from Cajal Institute animal colony were kept on 12-hour light–dark schedule and received food and water ad libitum until birth. Male and female pups were distinguished by a larger genital papilla and longer anogenital distance in male than in female pups. Experimental procedures were approved by our Institutional Animal Use and Care Committee (Spanish National Research Council Animal Experimentation Committee). Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

### Cortical Astrocyte Cultures

Astrocytes were cultured from postnatal day 1 (PND1) male or female mice pups, separately. The brain was extracted, the meninges were removed, and the entire cortex was isolated under a dissecting microscope. Next, the cortex was dissociated mechanically, washed twice in Hank's balanced salt solution (Invitrogen, Paisley, UK), and filtered through a 40- $\mu$ m nylon cell strainer into

DMEM-F12 (Dulbecco's modified Eagle's medium) culture medium with phenol red (Invitrogen, Paisley, UK) containing 10 % fetal bovine serum (FBS; Invitrogen) and 1 % penicillin–streptomycin (Invitrogen). After centrifugation at 1,000 rpm for 10 min, cells were resuspended in the same medium and cultured in poly-L-lysine-coated 75 cm<sup>2</sup> tissue culture flasks at 37 °C and 5 % CO<sub>2</sub>. The medium was changed after the first day in vitro and subsequently two times per week until the cells reached confluence (~10 days). Next, enriched astrocyte cultures were obtained after overnight shaking at 37 °C and 280 rpm on a tabletop shaker (Thermo Forma, Marietta, OH, USA) to detach neurons, oligodendrocytes, and microglia. Astrocytes were washed and removed from the flask by incubation with 0.5 % trypsin (type II-S; Sigma-Aldrich, St Louis, MO, USA) and 0.04 % ethylenediaminetetraacetic acid (Sigma-Aldrich). The cell suspension was centrifuged and the pellet was resuspended and plated onto poly-L-lysine-coated 75 cm<sup>2</sup> tissue culture flask with phenol red-free DMEM-F12 with 10 % FBS and 1 % penicillin–streptomycin. When the cells reached confluence for the second time (~4 days), we repeated the subculture, but this time the cells were plated at a density of 40,000 cells/cm<sup>2</sup> onto poly-L-lysine-coated plates (6 or 96 wells), in phenol red-free DMEM-F12 with 10 % FBS and 1 % penicillin–streptomycin. Twenty-four hours after plating, the cultures were treated as indicated below in experimental treatment section. By this protocol, we obtained cultures with less than 4 % of Iba-1-positive cells, checked by double immunocytochemistry with anti-Iba I (microglial marker, 1:2000, Wako, Japan) and anti-GFAP antibodies (astroglia marker, 1:2000, Dako, Denmark). We did not detect fibroblast (checked with anti-CD90 (Thy-1), 1:500, Serotec). We did not observe differences in the microglial percentage between sexes (data not shown).

#### Cell Viability Assays

The non-cytotoxic dose of DMT used for all the experiments was set after the analyses of a dose–viability curve using DMT at final concentrations from 1 to 200 µg/mL in the culture. To assess cell viability, we performed the fluorescein diacetate (FDA)/propidium iodide (PI) assay. Cells from male and female pups were separately plated in 96-well microplates and treated for 24 h with DMT at concentrations of 1, 2, 10, 20, 100, or 200 µg/mL (in phenol red-free DMEM-F12 medium without FBS). Just before the end of the treatment, the cells were incubated for 50 min at 37 °C with FDA (100 µM) and PI (15 µM). After that, the cells were washed with culture medium and the number of FDA (alive) and PI (dead)-positive cells was determined using a fluorescence microscope (40×).

To assess the metabolic activity of the cells (mitochondrial respiration), the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay was performed with the DMT concentrations (1–20 µg/mL) that did not induce significant mortality according to the FDA/PI assay. Cells from male and female pups were separately plated in 96-well microplates and treated for 24 h with DMT at concentrations of 1, 2, 10, 15, or 20 µg/mL. After the addition of 20 µL of CellTiter 96 Aqueous One Solution (Promega, Madison, USA), the plates were incubated for 4 h at 37 °C and 5 % CO<sub>2</sub>. Absorbance at 490 nm wavelength was measured in a multiwell plate reader.

#### Cell Treatments

The following experiments were performed at a non-cytotoxic concentration of DMT (2 µg/mL). Cells from male and female pups were separately plated in 6-well plates (40,000 cells/cm<sup>2</sup>) in phenol red-free DMEM-F12 medium without FBS. Astrocytes were pre-treated for 4 h with 17β-estradiol (10<sup>-10</sup> M), the estrogen receptor (ER) antagonist ICI 182,780 (10<sup>-8</sup> M), the ERα agonist PPT (10<sup>-8</sup> M), the ERβ agonist DPN (10<sup>-8</sup> M) or vehicle, and then incubated for 24 h with DMT (2 µg/mL) or vehicle. Some cultures were incubated with lipopolysaccharide (LPS, from *Escherichia coli* 026:B6, Sigma; 500 ng/mL) for 24 h as a positive control of inflammatory response.

#### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After the treatment with DMT or DMT plus estrogenic compounds, culture medium was removed from 6-wells plates and the cells were lysed. Total RNA was extracted using illustra RNAspin Mini RNA isolation kit (GE Healthcare, Buckinghamshire, UK) to assess the mRNA expression levels of interleukin (IL) 6, IL1β, tumor necrosis factor (TNF)α, interferon-γ-induced protein (IP) 10, ERα, ERβ, steroidogenic acute regulatory protein (StAR), translocator protein of 18 kDa (TSPO), P450 cholesterol side-chain cleavage enzyme (P450scc), aromatase, and caspase-3.

First-strand cDNA was prepared from 2 µg RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. After reverse transcription, cDNA was diluted 1:4 and 1:8 for the target genes and 1:2,000 for the endogenous control (18S), and 5 µL of these cDNA solutions were amplified by real-time PCR in a 15 µL volume reaction using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7,500 Sequence Detection System (Applied Biosystems) with conventional Applied Biosystems cycling parameters (40 cycles of

changing temperatures, first at 95 °C for 15 s and then 60 °C for a minute). All the primer sequences were designed using Primer Express software (Applied Biosystems) and are shown in Table 1.

After amplification, a denaturing curve was performed to ensure the presence of unique amplification products. All samples were performed in duplicate. *Ct* (cycle threshold) values for all the genes analyzed ranged between 13 and 33. Data were represented using the comparative *Ct* method, and for a valid  $\Delta\Delta C_t$  value, we proved that the efficiency of amplification of the target and the reference gene is approximately equal (the absolute value of the slope of  $\Delta C_t$  vs. log relative concentration should be between  $-0.1$  and  $0.1$ ). The *Ct* was determined for each target gene in duplicate.  $\Delta C_t$  was calculated by the difference between the *Ct* of each target gene and the *Ct* of 18S (reference gene). Then,  $\Delta\Delta C_t$  was calculated by normalizing the  $\Delta C_t$  of each sample to the mean  $\Delta C_t$  value of the *C* (or DMT) group. Finally,  $2^{-\Delta\Delta C_t}$  was calculated and its mean value was represented as percentage of *C* (or DMT) values in the figures.

#### Immunoassay for Inflammatory Proteins

The protein levels of IL6, IL1 $\beta$ , TNF $\alpha$ , and IP10 in culture supernatants were analyzed with a designed on demand mouse cytokine/chemokine magnetic bead panel (Milliplex, Millipore Corp., St. Charles, MO), following the manufacturer's instructions.

#### Western Blotting

After treatments, cells were lysed and solubilized in 200  $\mu$ L of 25 mM Tris-HCl pH 6.8, containing 2 % (w/v) SDS, glycerol 10 %, dithiothreitol 100 mM and protease inhibitor cocktail, and finally boiled for 2 min. Solubilized

proteins (20  $\mu$ L) were resolved by 10 % SDS-PAGE at 100 V for 1 h at 25 °C and then electrophoretically transferred to nitrocellulose membrane for 1 h at 100 V and 4 °C. The nitrocellulose was treated with 5 % (w/v) BSA in 138 mM NaCl, 25 mM Tris, pH 8.0, and 0.1 % (w/v) Tween-20 at 25 °C for 1 h, and then probed overnight at 4 °C with either anti-ER $\alpha$  (MC-20, Santa Cruz Biotechnology, Santa Cruz, CA; final dilution 1:1,000) or anti-ER $\beta$  (H-150, Santa Cruz Biotechnology; final dilution 1:1,000). The nitrocellulose was then probed with mouse monoclonal anti- $\beta$ -actin (clone AC-74; Sigma-Aldrich; final dilution 1:4,000) to normalize total lysate. Antibody reaction was visualized by ECL chemiluminescence. Densitometric analyses were performed by ImageJ software for Microsoft Windows (NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>).

#### Reactive Oxygen Species (ROS) Assessment

In order to analyze the production of ROS, we used dihydroethidium (DHE, Invitrogen, USA), at a final concentration of 3.2  $\mu$ M. This dye is oxidized specifically by superoxide anion ( $O^{\cdot-}$ ) generating red fluorescence ( $\lambda$  606 nm). Cells were plated in glass coverslips (pre-coated with poly-L-lysine) and at the end of the treatment with DMT or DMT plus 17 $\beta$ -estradiol, as described above, cells were incubated for 10 min at 37 °C and 5 % of CO<sub>2</sub> with DHE. Then, cells were fixed for 20 min with 4 % paraformaldehyde and mounted with vectashield-DAPI.

Identical acquisition and exposure time conditions were used to capture the images using a fluorescence microscope (Leica DMI6000 microscope equipped with a Leica DFC350 FX camera) at 40 $\times$  magnification. Red fluorescence intensity per unit area was quantified using the ImageJ software (<http://rsbweb.nih.gov/ij/>). To this aim, we first delineated the outline of each cell in the microphotograph using the freeform selection tool. Then, we selected

**Table 1** Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Forward primer	Reverse primer
IL 6	5'-GAAACCGCTATGAAGTTCCTCTCTG-3'	5'-TGTTGGGAGTGGTATCCTCTGTGA-3'
IL 1 beta	5'-CGACAAAATACCTGTGGCCT-3'	5'-TTCTTTGGGTATTGCTTGGG-3'
TNF alpha	5'-GAAAAGCAAGCAGGCAACCA-3'	5'-CGGATCATGCTTTCTGTGCTC-3'
IP 10	5'-CAGGAGAATGAGGGCCATAGG-3'	5'-CGGATTCAGACATCTCTGCTCAT-3'
ER alpha	5'-GATCCCACCATGCACAGTGA-3'	5'-GGAGCATCTACAGGAACACAGGTA-3'
ER beta	5'-CCTGGTCTGGGTGATTTCTCA-3'	5'-ACTGATGTGCCTGACATGAGAAAG-3'
TSPO	5'-TGCAGAAACCTCTTGGCATC-3'	5'-TGAAACCTCCCAGCTCTTTCC-3'
StAR	5'-GTCCTCCAAGACTAAACTCACTTG-3'	5'-GGTGGTTGGCGAACTCTATCTG-3'
P450sc	5'-GGATGCGTCGATACTCTTCTCA-3'	5'-CCCAATGGGCCTCTGATAATAC-3'
Aromatase	5'-GGATGTGTTGACCCTCATGAGAC-3'	5'-GATGTTTGGTTGATGAGGAGAGC-3'
Caspase-3	5'-GGTTCATCCAGTCCCTTGC-3'	5'-AATTCCGTTGCCACCTTCTC-3'

“area” and “integrated density” under the option “set measurements” in the analyze menu, and red fluorescence intensity and area were acquired using the “measure” command. To set the background, for each individual cell, we selected a neighboring extracellular region in the picture, where red fluorescence intensity per unit area was acquired as described before. The background value was then subtracted from the value of the cell. At least 50 cells were assessed for each experimental treatment. Each experiment was repeated 6 times. *N* for statistical analysis was 6.

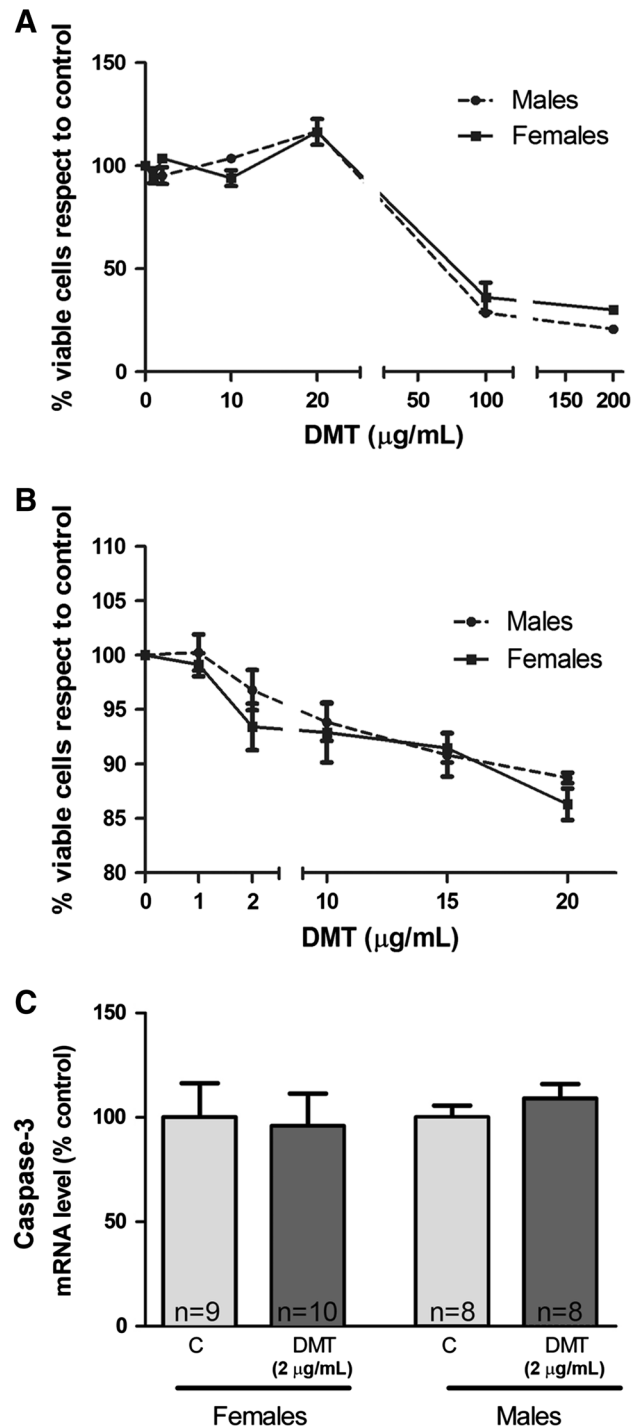
### Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics 1.9 software (Chicago, IL, USA). We first assessed the normal distribution of data by Kolmogorov–Smirnov test and homoscedasticity by Levene’s test. Data analysis was assessed by two-factor ANOVA, considering DMT treatment and sex as fixed factors, followed by Bonferroni post hoc test. In the case of the data represented in Figs. 5, 6, the statistical significance was assessed by one-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at  $p \leq 0.05$ . Data are presented as mean + SEM and expressed as percentage of control values in each sex, except in Figs. 2, 3, and 4, where the results were expressed as percentage of control female values to determine possible sex differences. The *n* for statistical analysis ( $n = 4–12$ ) was the number of independent culture preparations and it is indicated in the figures. Within each culture preparation, the assays were conducted in duplicate.

## Results

### Effects of DMT on Cell Viability

In order to select a concentration of DMT without cytotoxic effect, we performed a dose–viability curve. We first analyzed the effect of DMT in the FDA/PI assay. DMT was studied at final concentrations in the culture medium ranging from 1 to 200  $\mu\text{g}/\text{mL}$ . FDA-stained (alive) cells and PI-stained (dead) cells were counted. Cell viability was calculated as the ratio between the number of live cells and the number of total cells and expressed as the percentage of control (vehicle) values (Fig. 1a). The results indicated that DMT concentrations over 20  $\mu\text{g}/\text{mL}$  caused more than 10 % of cell death. Thus, DMT concentrations ranging from 1 to 20  $\mu\text{g}/\text{mL}$  were selected for the next viability test, the MTS assay. The results of the MTS assay (Fig. 1b) indicated that the metabolic activity was decreased more

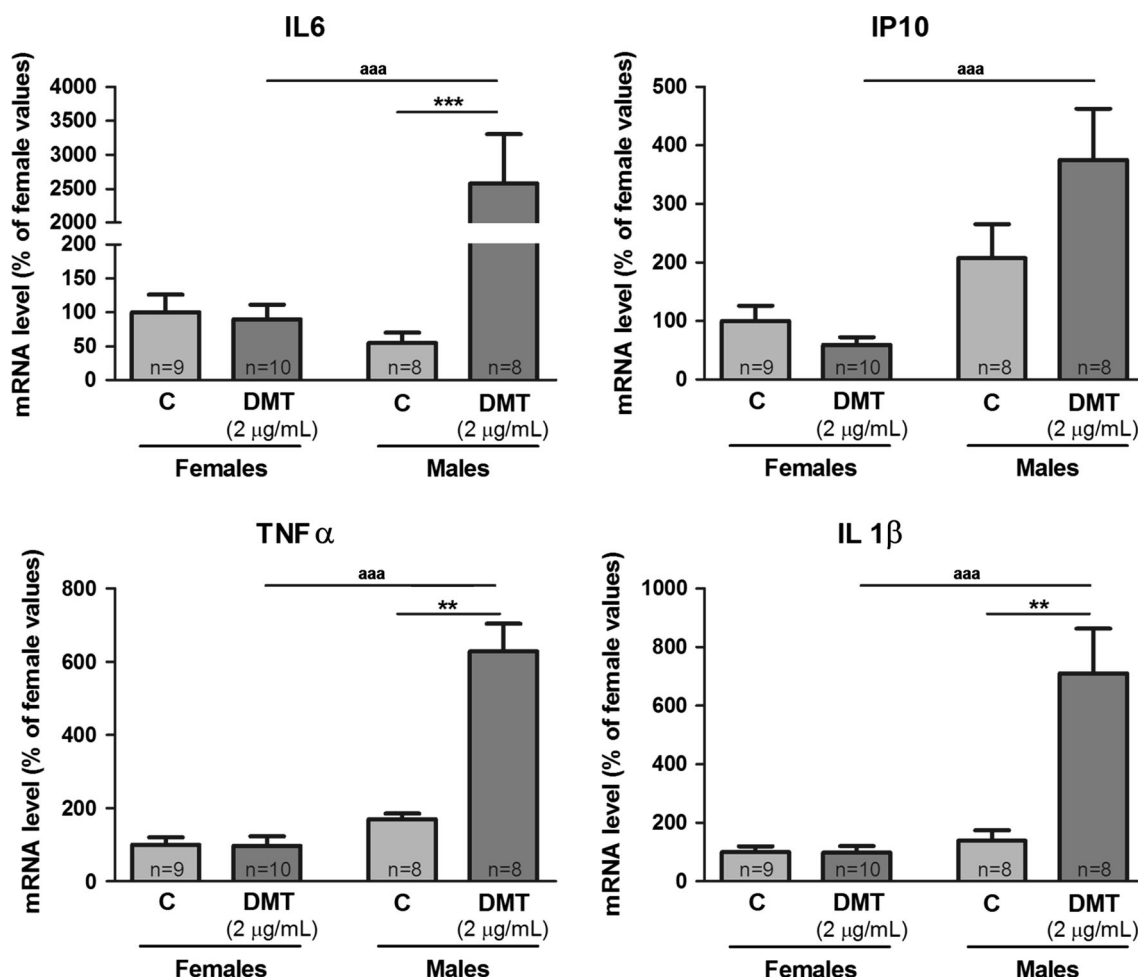


**Fig. 1** Results of the cell viability assays in astrocyte cultures from male and female pups. **a** Effect of different doses of DMT on cell viability assessed by the FDA/PI assay. **b** Effect of different doses of DMT on metabolic activity assessed by MTS assay. Four independent cultures were used for viability tests. **c** Effect of DMT (2  $\mu\text{g}/\text{mL}$ ) on caspase-3 mRNA levels. The number of independent cultures (*n*) for each experimental group is indicated within the bars of the histogram. Data are represented as mean + SEM. Male and female astrocytes showed similar changes in cell viability in response to DMT. No significant differences were detected in caspase-3 mRNA levels

than 5 % at concentrations higher than 10  $\mu\text{g/mL}$ . Male and female astrocytes showed similar changes in cell viability in response to DMT. We selected the two lower DMT concentrations tested (1 and 2  $\mu\text{g/mL}$ ) for a preliminary study in which the mRNA levels of IL1 $\beta$  were assessed in male and female astrocyte cultures. The lower dose of DMT did not significantly affect IL1 $\beta$  mRNA levels but a significant effect was detected with the dose of 2  $\mu\text{g/mL}$  in male astrocytes. Thus, we selected the concentration of 2  $\mu\text{g/mL}$  DMT for further studies. This concentration did not increase caspase-3 mRNA levels (Fig. 1c).

### Sex Differences in the Expression of Inflammatory Mediators in Response to DMT

The mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  were assessed in male and female cultures under basal conditions and 24 h after DMT (2  $\mu\text{g/mL}$ ) treatment (Fig. 2). DMT treatment resulted in a significant increase in IL6, TNF $\alpha$ , and IL1 $\beta$  mRNA levels, but only in astrocytic cultures from males. In consequence, there were significant differences in IL6, TNF $\alpha$ , and IL1 $\beta$  mRNA levels on IL6 expression between female and male cultures after DMT



**Fig. 2** Expression of inflammatory mediators on astrocyte cultures from male and female pups under basal conditions (C) and after treatment with DMT (2  $\mu\text{g/mL}$ ). IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  mRNA levels are expressed as percentage of female control values and are represented as mean + SEM. The number of independent cultures ( $n$ ) for each experimental group is indicated within the bars of the histogram. Statistical significance was assessed using a two-factor ANOVA, with sex and DMT as fixed factors, followed by Bonferroni post hoc test. ANOVA revealed a significant interaction between sex and DMT treatment on the mRNA levels of IL6 [ $F_{(1,31)} = 36.070$ ,  $p < 0.001$ ], IP10 [ $F_{(1,31)} = 4.582$ ,  $p = 0.04$ ], TNF $\alpha$  [ $F_{(1,31)} = 9.148$ ,

$p = 0.005$ ], and IL1 $\beta$  [ $F_{(1,31)} = 9.506$ ,  $p = 0.04$ ]. DMT showed a significant effect on the mRNA levels of IL6 [ $F_{(1,31)} = 31.055$ ,  $p < 0.001$ ], TNF $\alpha$  [ $F_{(1,31)} = 4.750$ ,  $p = 0.037$ ], and IL1 $\beta$  [ $F_{(1,31)} = 10.532$ ,  $p = 0.03$ ]. In addition, there was a sex effect on the mRNA levels of IL6 [ $F_{(1,31)} = 16.689$ ,  $p < 0.001$ ], IP10 [ $F_{(1,31)} = 21.876$ ,  $p < 0.001$ ], TNF $\alpha$  [ $F_{(1,31)} = 35.789$ ,  $p < 0.001$ ], and IL1 $\beta$  [ $F_{(1,31)} = 18.043$ ,  $p < 0.001$ ]. Results of the post hoc analysis: <sup>aaa</sup>significant sex differences (<sup>aaa</sup> $p < 0.001$ ) between the groups identified; \*\*,\*\*\*significant treatment differences (\*\* $p < 0.01$  and \*\*\* $p < 0.001$ , respectively) between the groups identified

treatment. In addition, there was a significant difference in IP10 mRNA levels between female and male cultures after DMT treatment.

Protein levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  in the medium from male cell cultures were determined using a 4-plex assay. In both control and DMT-treated cultures, the levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  were under the detection limit. As a positive control, we quantify these proteins in the medium from male astrocytes treated for 24 h with LPS (500 ng/mL). In this case, levels of IP10 ( $7,840 \pm 143$  pg/mL;  $p < 0.05$  from control), IL6 ( $40,689 \pm 261$  pg/mL;  $p < 0.05$  from control), TNF $\alpha$  ( $636 \pm 7$  pg/mL;  $p < 0.05$  from control), and IL1 $\beta$  ( $366 \pm 45$  pg/mL;  $p < 0.05$  from control) were detectable (all the results are expressed as mean  $\pm$  SEM of 4 independent experiments, statistically analyzed by Mann–Whitney test). It should be noted that LPS (at the same time/dose conditions) strongly induced the mRNA expression of IP10 (90-fold), IL6 (660-fold), TNF $\alpha$  (38-fold), and IL1 $\beta$  (60-fold), while DMT increased the expression by 1.8-, 47-, 3.7-, and 5.1-fold, respectively. Thus, we may conclude that DMT at 2  $\mu$ g/mL induced a moderate induction in the expression of pro-inflammatory mediators in astrocytes from male pups.

#### Sex Differences in the Expression of Estrogen Receptors and Steroidogenic Proteins in Response to DMT

The mRNA levels of ER $\alpha$ , ER $\beta$ , StAR, aromatase, P450scc, and TSPO were assessed in male and female cultures under basal conditions and 24 h after DMT treatment (Fig. 3). The mRNA levels of ER $\alpha$ , ER $\beta$ , StAR, aromatase (Fig. 3), P450scc, and TSPO (data not shown) were not significantly different between males and females, under basal conditions. In addition, the mRNA levels of ER $\alpha$  (Fig. 3), P450scc, and TSPO (data not shown) were not significantly affected by DMT treatment. In contrast, there was a significant increase in the mRNA levels of ER $\beta$ , StAR, and aromatase after DMT treatment, but only in astrocytic cultures from males. In addition a significant difference in StAR and aromatase mRNA levels between female and male cultures was detected after DMT treatment (Fig. 3).

Since several studies have shown that ER $\alpha$  and ER $\beta$  mRNA levels do not necessarily correlate with ER $\alpha$  and ER $\beta$  protein levels (Chu et al. 2007; Al-Bader et al. 2008; Fox et al. 2008), we assessed protein levels by Western blot in male and female cultures (Fig. 4). The treatment with DMT resulted in a significant increase in ER $\alpha$  protein levels, but only in astrocytic cultures from males. In consequence, there was a significant difference in ER $\alpha$  levels after DMT treatment between female and male cultures. The levels of ER $\beta$  were neither affected by the treatment

with DMT nor by the sex. No significant differences in ER $\alpha$  and ER $\beta$  protein levels between male and female cultures were detected under basal conditions.

#### 17 $\beta$ -Estradiol Reduced the Effect of DMT on Inflammatory Molecules in Male Cultures by a Mechanism Involving Classical Estrogen Receptors

The effect of 17 $\beta$ -estradiol ( $10^{-10}$  M final concentration) was analyzed in male cultures treated with DMT (Fig. 5). The hormone significantly decreased the mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  (Fig. 5). In contrast, 17 $\beta$ -estradiol did not significantly affect the mRNA levels of these inflammatory markers in DMT-treated female cultures (data not shown). The effect of 17 $\beta$ -estradiol on IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  mRNA levels in DMT-treated cultures was counteracted by the ER antagonist ICI 182,780 (Fig. 5).

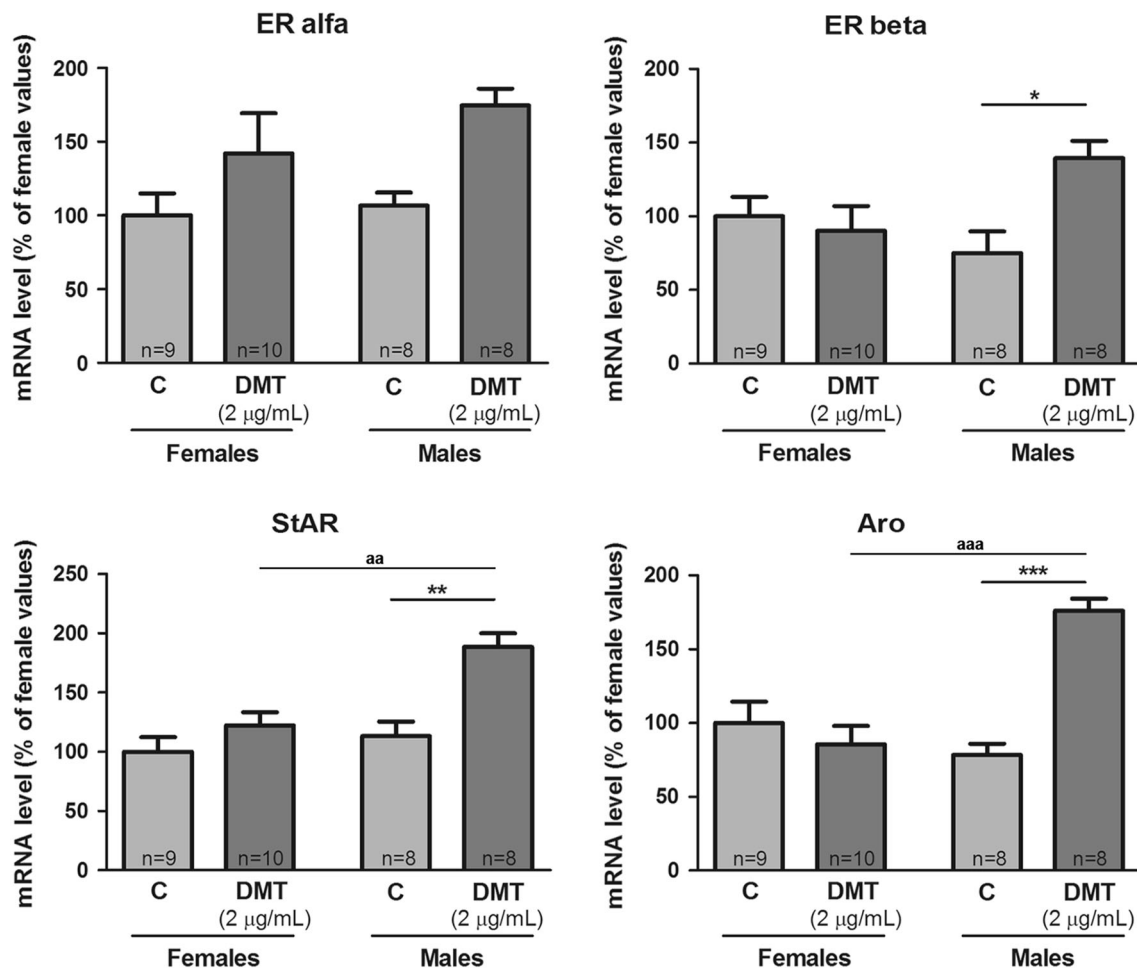
The ER $\beta$  agonist DPN imitated the effect of 17 $\beta$ -estradiol on the mRNA levels of IL6, IL1 $\beta$ , IP10, and TNF $\alpha$  (DMT vs. DMT + DPN, statistic tendency  $p = 0.06$ ). The ER $\alpha$  agonist PPT imitated the effect of 17 $\beta$ -estradiol only for IL1 $\beta$  mRNA levels. 17 $\beta$ -estradiol did not significantly affect the mRNA levels of ER $\alpha$ , ER $\beta$ , StAR, and aromatase in DMT-treated male and female cultures (data not shown).

#### 17 $\beta$ -Estradiol Reduced the Production of ROS in Male Astrocytes Exposed to DMT

Taking into account previous results regarding the production of reactive oxygen species by DMT in vitro (Gargouri et al. 2011; Ben Abdallah et al. 2012), we analyzed the possible antioxidant effect of 17 $\beta$ -estradiol in DMT-treated cultures using the fluorescent dye DHE, which detect superoxide anion production. DMT increased the fluorescence intensity in astrocytes while 17 $\beta$ -estradiol reduced the fluorescence intensity to control levels (Fig. 6).

## Discussion

Our findings indicate that a sublethal concentration of the organophosphorus pesticide DMT is able to induce an increase in the mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  in primary astrocytic cultures derived from CD1 male mice. Since strain differences in the neuroinflammatory responses have been described (Nikodemova and Watters 2011), further studies should determine whether DMT has similar effects in other mouse strains. In addition, it should be mentioned that the experiments were performed with neonatal cells, which may not necessarily reflect the response of adult cells to DMT. However, the study of the



**Fig. 3** mRNA levels of ERs and steroidogenic proteins in astrocyte cultures from male and female pups under basal conditions (C) and after treatment with DMT (2 µg/mL). ER $\alpha$ , ER $\beta$ , StAR, and aromatase mRNA levels are expressed as percentage of female control values and are represented as mean + SEM. The number of independent cultures (*n*) for each experimental group is indicated within the bars of the histogram. Data were analyzed using a two-factor ANOVA, with sex and DMT as fixed factors, followed by Bonferroni post hoc test. ANOVA revealed a significant interaction between sex and DMT on the mRNA levels of ER $\beta$  [ $F_{(1,31)} = 6.399$ ,

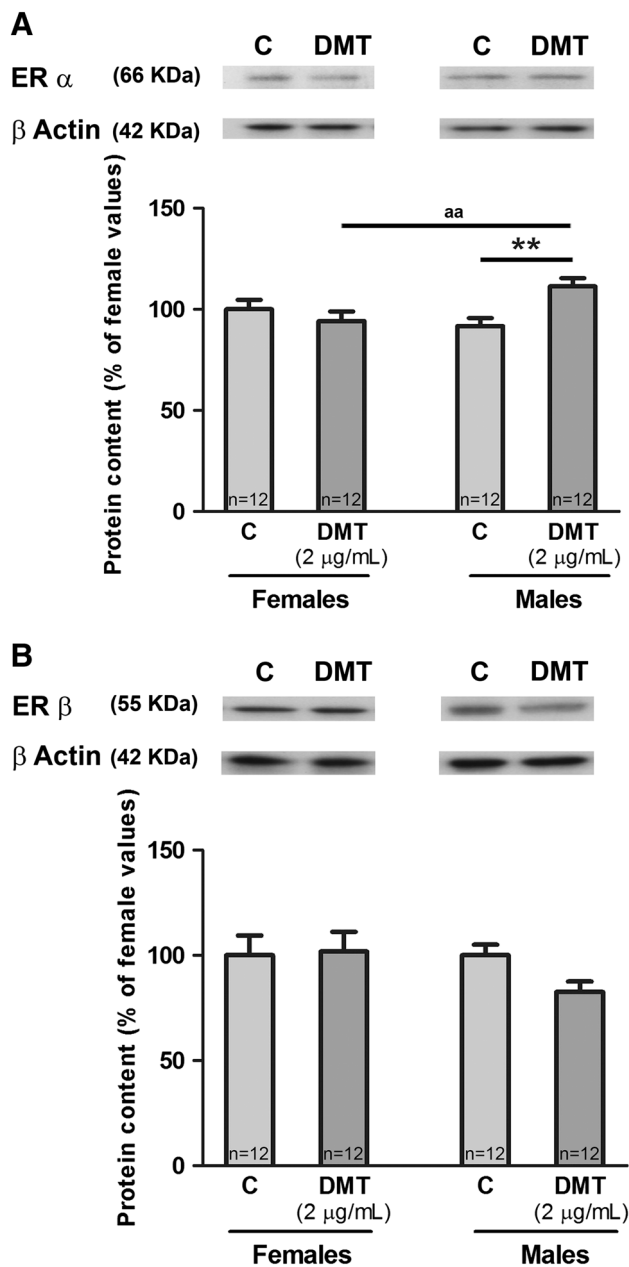
$p = 0.017$ ], StAR [ $F_{(1,31)} = 4.958$ ,  $p = 0.033$ ], and aromatase [ $F_{(1,31)} = 23.682$ ,  $p < 0.001$ ]. In addition, there was a significant effect of sex and a significant effect of DMT treatment on the mRNA levels of StAR [Sex,  $F_{(1,31)} = 11.233$ ,  $p = 0.002$ ; DMT,  $F_{(1,31)} = 16.973$ ,  $p < 0.001$ ] and aromatase [Sex,  $F_{(1,31)} = 8.966$ ,  $p = 0.005$ ; DMT,  $F_{(1,31)} = 13.178$ ,  $p = 0.001$ ]. Results of the post hoc analysis: <sup>aaa</sup>significant sex differences (<sup>aa</sup> $p < 0.01$  and <sup>aaa</sup> $p < 0.001$ , respectively) between the groups identified; \*, \*\*, \*\*\*significant treatment differences ( $*p < 0.05$ ;  $**p < 0.01$ , and  $***p < 0.001$ , respectively) between the groups identified

effects of DMT on developing astrocytes is of relevance, since the vulnerability for the cognitive, affective, behavioral, and neuroanatomical alterations caused by OPs is higher during the developmental period (Levin et al. 2010; Venerosi et al. 2010; Horton et al. 2012; Rauh et al. 2012; Venerosi et al. 2012).

The increase in the mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  induced by DMT in astrocytes, which to our knowledge has not been previously reported (Zurich et al. 2004; Banks and Lein 2012), may have multiple functional consequences. Cytokines such as IL6, IL1 $\beta$ , and TNF $\alpha$  have different physiological functions, including regulation of neuronal development, ionic homeostasis, neuropeptide release, and synaptic plasticity (Raber et al. 1998; Albensi

and Mattson 2000; Skoff et al. 2009; Park and Bowers 2010). In addition, astrocyte-driven production of IL6 in vivo increases chronic inflammatory damage (Campbell et al. 1993) but protects the central nervous system against an acute focal injury (Swartz et al. 2001; Penkowa et al. 2003; Quintana et al. 2008). IL1 $\beta$  participates in brain repair after injury (Rothwell 2003) and in the remyelination of multiple sclerosis lesions (Mason et al. 2001). TNF $\alpha$  regulates microglia reactivity (Syed et al. 2007), the function of blood–brain barrier (Worrall et al. 1997), and the survival of oligodendrocyte precursors (Kim et al. 2011; Su et al. 2011). IP10 is involved in the recruitment of T lymphocytes, natural killer cells, and monocytes into the central nervous system (Farber 1997; Dufour et al. 2002).





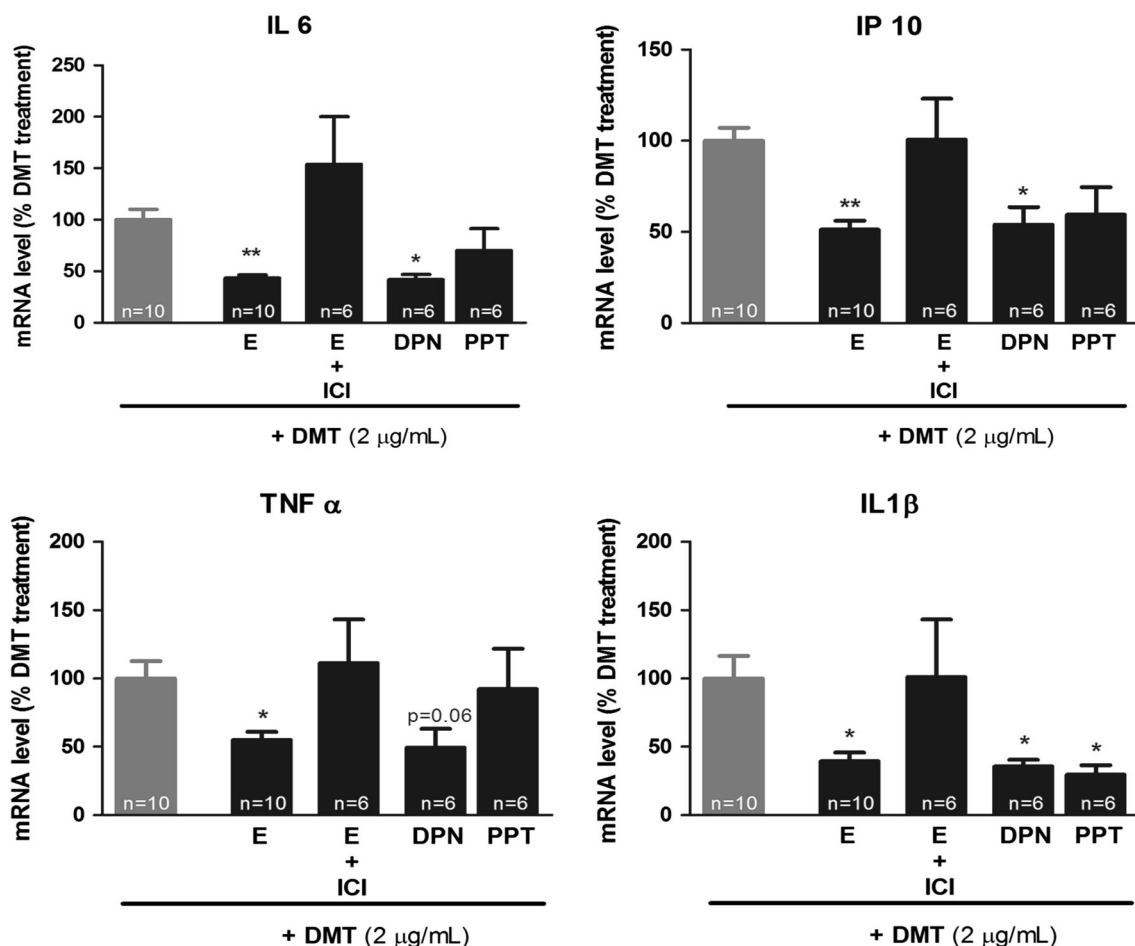
**Fig. 4** Protein levels of ER $\alpha$  (a) and ER $\beta$  (b) in astrocyte cultures from male and female pups under basal conditions (control, C) and after treatment with DMT (2  $\mu$ g/mL). ER $\alpha$  and ER $\beta$  protein levels were normalized to  $\beta$ -actin and expressed as the percentage of female control values. Data are represented as mean + SEM. The number of independent cultures was 12 for each experimental group. Data were analyzed using a two-factor ANOVA, with sex and DMT as fixed factors, followed by Bonferroni post hoc test. ANOVA revealed a significant interaction between sex and DMT on ER $\alpha$  protein levels [ $F_{(1,48)} = 11.779$ ,  $p = 0.001$ ]. Results of the post hoc analysis: <sup>aa</sup>significant sex differences (<sup>aa</sup> $p < 0.01$ ) between the groups identified; <sup>\*\*</sup>significant treatment differences (<sup>\*\*</sup> $p < 0.01$ ) between the groups identified

Changes in the expression of inflammatory molecules may also be involved in the induction of oxidative stress by the pesticide (Hald and Lotharius 2005). Therefore, the DMT-

induced increase in the mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  in astrocytes may affect numerous physiological processes and the response of neural tissue to pathological insults. Furthermore, the fact that DMT induces a significant increase in the mRNA levels of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  at a sublethal concentration indicates that this molecule may alter cell function even at concentrations that do not compromise cell viability.

Our present findings, showing an effect of DMT on the immune response of astrocytes, extend the results of a recent study *in vivo* showing that a low dose of the pesticide increases the proportion of microglia with reactive phenotype in the dentate gyrus and the striatum (Astiz et al. 2013). This activation of microglia *in vivo* in the animals treated with DMT may be a direct effect of the pesticide on this cell type or be the consequence of the activation of other cell types, including astrocytes. Further studies are therefore necessary to determine whether DMT may also directly affect the inflammatory response of microglia. Nevertheless, a direct effect on purified microglia *in vitro* has been shown for Dichlorvos, another OP (Sunkaria et al. 2012).

An important finding in our study is that male and female astrocytes differ in their response to DMT. DMT induces an upregulation of IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  in male astrocytes, but does not have a significant effect on female cells at the assayed dose. This suggests that female astrocytes are less sensitive than male astrocytes to the effects of DMT on the expression of inflammatory molecules. Other studies have reported sex differences in the immune response of astrocytes and in their vulnerability to oxidative damage (Guevara et al. 2011; Santos-Galindo et al. 2011; Sundar Boyalla et al. 2011; Loram et al. 2012; Schwarz and Bilbo 2012). Liu et al (2007) observed that female astrocytes are more resistant than male astrocytes to oxygen and glucose deprivation and to cell death induced by oxidants. Sundar Boyalla et al (2011) have shown that male mesencephalic astrocytes are more sensitive to the toxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), showing higher ROS levels than female astrocytes. Santos-Galindo et al (2011) detected a higher LPS-induced inflammatory response in male astrocytes, compared to female astrocytes. They attributed the difference to the effect of testosterone peak during the perinatal period in males, because male astrocytes showed similar responses to LPS than astrocytes from androgenized female pups (Santos-Galindo et al. 2011). Our present findings extend these previous observations to the response of astrocytes to a pesticide, pointing to the need of considering sex dimorphisms when analyzing the toxic effects of these molecules *in vitro*. Based on the previous findings (Garcia-Segura et al. 1988, 1995; Mong et al. 1999; Conejo et al. 2005; Santos-Galindo et al. 2011), it is plausible that



**Fig. 5** Effect of 17 $\beta$ -estradiol (E,  $10^{-10}$  M), ICI 182,780 (ICI,  $10^{-8}$  M), DPN ( $10^{-8}$  M), and PPT ( $10^{-8}$  M) on the expression of inflammatory modulators in DMT (2  $\mu$ g/mL)-treated astrocyte cultures derived from male pups. IL6, IP10, TNF $\alpha$ , and IL1 $\beta$  mRNA levels are expressed as percentages of the values in male cultures treated with DMT alone and are represented as mean + SEM. The

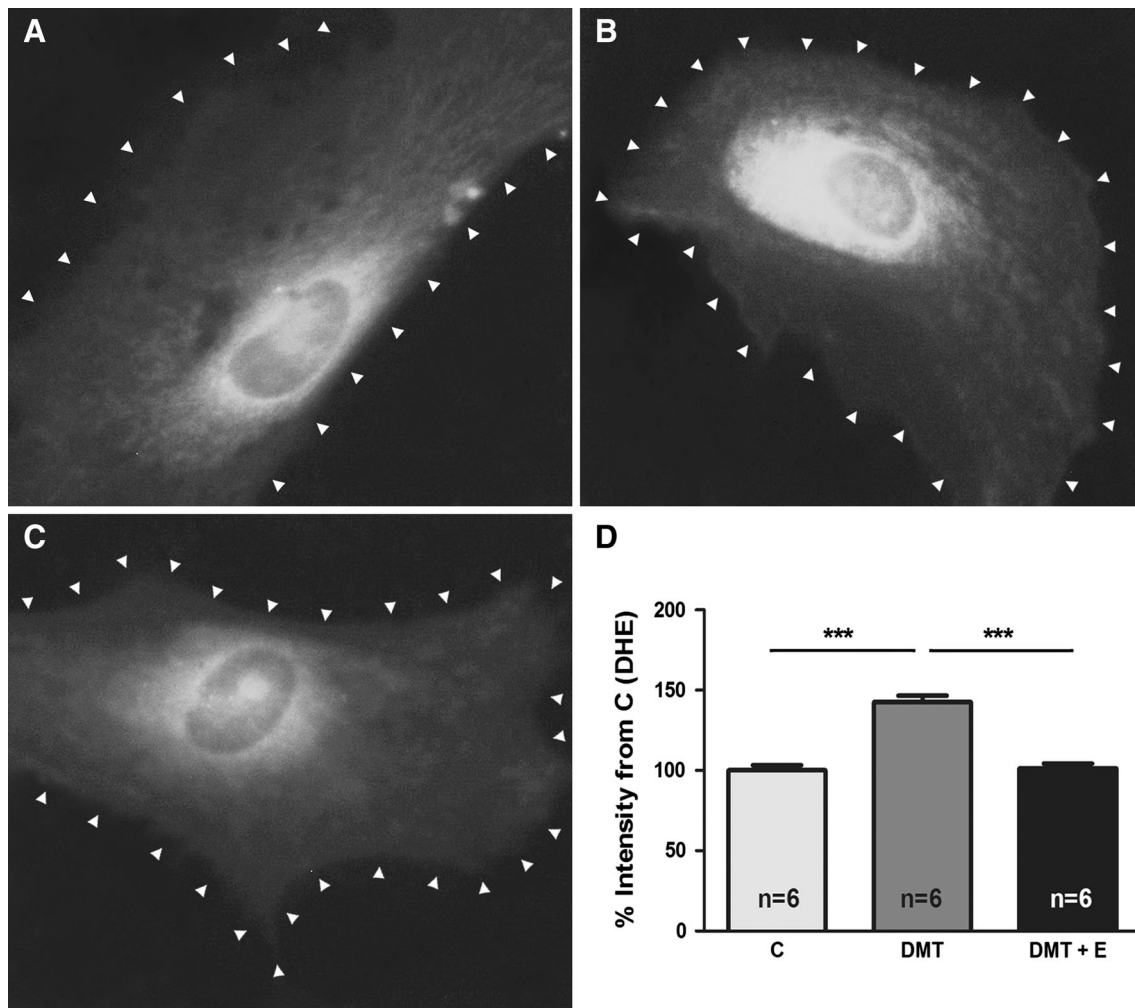
number of independent cultures ( $n$ ) for each experimental group is indicated within the *bars* of the histogram. Statistical significance was assessed using one-way ANOVA followed by Bonferroni post hoc test. Significant differences, \* $p < 0.05$  and \*\* $p < 0.01$ , versus DMT alone

the origin of such differences is the perinatal androgen peak and the consecutive metabolism of testosterone to estradiol within the brain. However, sex chromosome genes may also potentially contribute to the different responses of male and female astrocytes (Arnold 2009). Therefore, further studies are necessary to determine the causes for the sex difference in the response of astrocytes to DMT.

The consequences of the different reaction of male and female astrocytes to DMT remain elusive, since no differences in viability after DMT exposure were detected. However, an enhanced production of inflammatory molecules by astrocytes may compromise the viability of other cell types in the brain, such as neurons and oligodendrocytes (Pannu et al. 2005; Moreno et al. 2009; Steele and Robinson 2012). Thus, the different response of male and female astrocytes to DMT may contribute to the generation

of sex differences in the neural outcome of DMT intoxication. Although to our knowledge possible sex differences in the effects of DMT have not been explored, it is well known that other OPs cause sex-specific cognitive, affective, behavioral, and neuroanatomical alterations in rodents and humans (Levin et al. 2010; Venerosi et al. 2010; Horton et al. 2012; Rauh et al. 2012; Venerosi et al. 2012). These alterations, which may be mediated in part by astrocytes, affect in general more to males than to females.

Although sex differences in the effects of DMT have not been previously explored, it is known that this OP may act as an endocrine disruptor, decreasing steroidogenesis in isolated Leydig cells and in rat testis in vivo (Walsh et al. 2000; Astiz et al. 2009d). We therefore analyzed in the present study whether DMT altered the expression of molecules involved in steroid synthesis and steroid signaling in astrocytes. Our findings indicate that the basal



**Fig. 6** Effect of 17 $\beta$ -estradiol on the production of reactive oxygen species (ROS) in astrocytes obtained from male pups and treated with vehicle, DMT (2  $\mu$ g/mL) or DMT (2  $\mu$ g/mL) + 17 $\beta$ -estradiol (E, 10<sup>-10</sup> M). **a–c** Representative examples of cells treated with vehicle (**a**), DMT (**b**), and DMT + E (**c**). **d** Results of the quantitative analysis. DHE fluorescence intensity per unit area is expressed as the

percentage of control values (C) and is represented as mean + SEM. The number of independent cultures (*n*) for each experimental group is indicated within the *bars* of the histogram. The outline of the cells is indicated with *white arrowheads*. Statistical significance was assessed using one-way ANOVA followed by Bonferroni post hoc test. \*\*\*Significant differences ( $p < 0.001$ )

levels of expression of ER $\alpha$ , ER $\beta$ , StAR, and aromatase did not differ between male and female cultures, suggesting that differences in steroid synthesis and/or estrogen signaling are not involved in the different response of male and female astrocytes to DMT.

Dimethoate induced a significant increase in the mRNA levels of ER $\beta$ , StAR, and aromatase, but only in male cultures. In addition, ER $\alpha$  protein levels were increased by DMT in male but not in female cultures. In contrast, DMT did not affect ER $\beta$  protein levels. It is well known that the expression of StAR, aromatase, and ERs increases in the brain under neurodegenerative conditions that are associated with neuroinflammatory damage, such as stroke, excitotoxicity, and traumatic injury (Garcia-Ovejero et al. 2002; Carswell et al. 2005; Dubal et al. 2006; Lavaque

et al. 2006; Garcia-Segura 2008; Saldanha et al. 2009). The increased expression of ER $\alpha$ , ER $\beta$ , StAR, and aromatase is thought to represent an adaptive response to cope with neurodegenerative insults by increasing estradiol synthesis and neuroprotective estradiol signaling (Garcia-Ovejero et al. 2005; Liu et al. 2007; Carbonaro et al. 2009). Therefore, the increased expression of StAR, aromatase, and ERs in male astrocytes after DMT treatment may be the consequence, rather than one of the causes, of the sex-dimorphic inflammatory response.

Since estradiol is known to decrease the inflammatory response of astrocytes (Bruce-Keller et al. 2000; Dhandapani and Brann 2007; Lewis et al. 2008; Arevalo et al. 2010; Brown et al. 2010), we explored whether the hormone may counteract the effects of DMT on these cells.

Our findings indicate that estradiol reduces the inflammatory response of male astrocytes treated with DMT. This effect is mainly mediated by ER $\beta$ , because the ER $\beta$  agonist DPN imitated the action of exogenous estradiol on the levels of expression of all the inflammatory markers studied (IL6, IP10, TNF $\alpha$ , and IL1 $\beta$ ). ER $\alpha$  may also contribute to this regulation, since the ER $\alpha$  agonist PPT imitated the action of exogenous estradiol on IL1 $\beta$  mRNA levels.

Oxidative stress underlies the pathogenesis of a broad range of human diseases, particularly at neurodegenerative disorders (Halliwell and Gutteridge 1999). Within the brain, neurons are the most vulnerable cells to oxygen species and their survival relies on the antioxidant protection promoted by neighboring astrocytes (Fernandez-Fernandez et al. 2012). In addition to the increased expression of inflammatory mediators, DMT increased ROS formation (at least superoxide anion) in astrocytes from males. This effect of the insecticide was previously reported in vivo and in vitro but in other cell types (Astiz et al. 2009a; Gargouri et al. 2011; Ben Abdallah et al. 2012). Moreover, DMT at sublethal doses is able to alter the mitochondrial membrane potential in the brain (Astiz et al. 2009b). Thus, the action of estradiol, reducing ROS formation in DMT-treated astrocytes, could be due to an increased expression of antioxidant enzymes and also to the mitoprotective effects previously described for the hormone (Razmara et al. 2007; Ritz and Hausmann 2008; Simpkins et al. 2010). Nowadays, it is proposed that the therapeutic strategies directed to control glial activation and the excessive production of pro-inflammatory and pro-oxidant factors may be valuable to control neurodegeneration (Agostinho et al. 2010).

In conclusion, our findings indicate that a sublethal dose of DMT that does not compromise cell viability is able to alter cell function, increasing the production of inflammatory molecules and ROS formation in male astrocyte cultures. The action of DMT on astrocytes may be relevant for the neurotoxic effects of the pesticide, given the essential role of this cell type in the maintenance of brain homeostasis. Our results also indicate that estradiol counteracts the effects of DMT, reducing the expression of inflammatory molecules through classical estrogen receptors (mainly ER $\beta$ ) and reducing ROS production. Finally, the sexually dimorphic response of astrocytes to DMT suggests that the exposure to the pesticide may have different neurological outcomes in males and females.

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**Conflict of interests** The authors declare that they have no conflict of interest.

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