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Oestrogen receptor ERα and ERβ agonists ameliorate oxidative brain injury and improve memory dysfunction in rats with an epileptic seizure

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Abstract

The susceptibility to epileptic seizures is dependent on sex as well as fluctuations in oestrogen levels, while exogenous oestrogen was shown to have no effect or to facilitate or to inhibit seizure activity. Oestrogen receptors (ERs) mediate antioxidant and anti-inflammatory actions in several inflammatory models, but the involvement of ERs in seizure-induced neuronal injury has not been evaluated previously. In order to assess the effects of resveratrol, progesterone, oestradiol (E2), an anti-testosterone (cyproterone acetate; CPA), a selective ER modulator (tamoxifen; TMX) and ERα/ERβ agonists (propyl pyrazole triol (PPT), diarylpropionitrile (DPN)) on oxidative brain damage and memory impairment due to epileptic seizure, male Wistar rats (n = 120) received one of the treatment choices either in drinking water or intraperitoneally for 31 days, and epileptic seizure was induced on the 28th day by injection of a single-dose of pentylenetetrazole (45 mg kg^{-1}). The results demonstrate that chronic pretreatment with resveratrol, progesterone, E2, CPA or TMX suppressed most of the inflammatory parameters indicative of oxidative neuronal injury, while treatment with the ER agonists DPN or PPT were found to be even more effective in limiting the oxidative damage. Treatment with DPN resulted in the up-regulation of cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) expression, while PPT up-regulated expression of CREB without affecting BDNF levels. Moreover, both ER agonists provided protection against seizure-induced memory loss with a concomitant increase in hippocampal GABA(A)α1-positive cells. In conclusion, ER agonists, and more specifically ERβ agonist, appear to provide maximum protection against seizure-induced oxidative brain injury and associated memory dysfunction by up-regulating the expression of CREB, BDNF and GABA(A)α1 receptors.

KEYWORDS
BDNF, GABA(A)α1 receptor, oxidative brain injury

1 | INTRODUCTION

Epidemiological studies have shown that the incidence of epilepsy is slightly less in women as compared to men (Christensen et al., 2007), while both the intensity and the frequency of seizures vary at different phases of the menstrual cycle (Herzog, 2008), during pregnancy (Battino et al., 2006; Sabers et al., 2010) and with treatments for assisted reproduction (Mostacci et al., 2018). In addition, frequencies of seizures, which are relatively increased in the perimenopausal period, are reduced during menopause (Harden, Pulver, Ravelin, & Jacobs, 1999). The change in the vulnerability of brain tissue to seizure generation during perimenarche and perimenopause was attributed to the variable ratio of oestrogen to progesterone (Bäckström, Zetterlund, Blom, & Romano, 1984; Frye, Manjarrez, & Camacho-Arroyo, 2000; Najafi et al., 2013). Although animal studies, similar to human data, have shown that females had higher seizure

thresholds than males (Reddy, 2009), several reports have also suggested that oestrogens could facilitate or inhibit or have no effect at all on seizure activity and seizure-induced damage (Velíšková, 2006, 2007; Velísková, Velísek, Galanopoulou, & Sperber, 2000).

In experimental models of epileptic seizure, it is well described that there is an accumulation of reactive oxygen species (ROS) during the acute phase of the initial insult, suggesting that oxidative stress has a crucial role in seizure-induced neurodegeneration and associated cognitive dysfunction (Martinc, Grabnar, & Vovk, 2014). Accordingly, the therapeutic potential of various antioxidants was evaluated in animal seizure models as well as in patients (Mehla, Reeta, Gupta, & Gupta, 2010; Militão, Ferreira, & de Freitas, 2010; Mohanan & Yamamoto, 2002). There is extensive evidence that oestrogens have neuroprotective effects (Ciriza, Carrero, Azcoitia, Lundeen, & Garcia-Segura, 2004; Melcangi & Panzica, 2006; Velíšková, 2010; Velísková et al., 2000) and alleviate oxidative stress in several other inflammation models (Kasimay et al., 2009; Şener, Arbak, Kurtaran, Gedik, & Yeğen, 2005). Although the involvement of oestrogen receptors (ERs) in the antioxidant and anti-inflammatory actions of oestrogens on many other tissues has been studied (Arabacı Tamer et al., 2019; Bulut et al., 2016; Kumral, Memi, Ercan, & Yeğen, 2014; Özdemir Kumral et al., 2016), the involvement of oestrogen receptors on seizure-induced neuronal injury has not been evaluated before.

ERs, which mediate a wide variety of complementary or antagonistic effects of oestrogens in various tissues, are classified as ERα and ERβ (Mueller & Korach, 2001), while the G protein-coupled ER (GPER) is a membrane receptor mediating rapid signalling in response to oestrogen (Revankar, 2005). Recordings in somatosensory cortex of female rats showed that oestrogen locally affects the excitability of neurons via the ERβ mechanism (Clemens et al., 2019). The activation of ERβ was suggested to be responsible for the anticonvulsant effects of oestrogen (Frye, Ryan, & Rhodes, 2009). On the other hand, the commonly prescribed antiepileptic drug phenytoin, behaving as a powerful antagonist of oestradiol but producing agonistic actions in the absence of oestradiol, was characterized as an ERα-selective ER modulator (SERM) (Fadiel et al., 2015), which explains the varying efficiency of the drug correlated with the changes in ovarian functions (Hines & Murphy, 2011; Juurlink, Mamdani, Kopp, Laupacis, & Redelmeier, 2003). The GPER-1 receptor was shown to mediate the epileptogenic effect of oestrogen by modulating the oxidative or anti-oxidative status of the brain tissue (Kurt, Bosnak, Inan, Celik, & Uremis, 2016), while pretreatment with a phyto-oestrogen has exhibited anticonvulsant and neuroprotective effects by improving oxidative stress and apoptosis (Amiri et al., 2017; Elsayed et al., 2018). However, none of these studies have investigated the involvement of the ERs in the seizure-induced neuronal injury and cognitive impairment.

Based on the aforementioned studies, the aim of the present study was to compare the effects of treatment with the anti-epileptic drug phenytoin, a non-selective ER agonist, a phyto-oestrogen, progesterone, a SERM and an anti-androgen drug with those of ERα and ERβ agonists on the occurrence of seizures, oxidative brain damage and memory impairment due to a single dose pentylenetetrazole (PTZ)-induced epileptic seizure in rats.

New Findings

- **What is the central question of this study?**
  Could different hormonally active substances, including oestrogen receptor (ER) agonists, protect against oxidative brain damage and memory impairment induced by a single epileptic seizure in rats? If so, which signalling mechanisms are involved in their anti-inflammatory effects?
- **What is the main finding and its importance?**
  Chronic administration of oestrogen, progesterone, ER modulators/agonists or blockade of testosterone exhibited anti-inflammatory and antioxidant actions on single seizure-induced neuronal injury, while ER agonists additionally improved memory function and up-regulated CREB signalling and hippocampal GABA(A)α1 receptor density, suggesting that ERα or ERβ receptor activation may be beneficial in protecting against seizure-related oxidative brain injury and cognitive dysfunction.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The present study was approved by the Marmara University (MU) Animal Care and Use Committee (approval code: 59.2015.mar; date: 14 July 2015) and the experiments were performed in compliance with the guidelines of the New York Academy of Sciences and the Turkish law on the use of animals in experiments. The ethical principles of the journal are understood, and the study complies with the checklist of animal ethics.

2.2 | Animals and drugs

Male Wistar (250–400 g) rats were obtained from the MU Animal Centre (DEHAMER) and housed in a temperature-controlled (22 ± 2°C) and air-conditioned room with a light schedule of 12 h light–12 h dark cycles. Rats were fed with a standard rat chow and water *ad libitum*.

Tamoxifen (AstraZeneca, UK), cyproterone acetate (Bayer İlaç Sanayii, Istanbul, Turkey), resveratrol (Solgar, Istanbul, Turkey), oestradiol (Bayer İlaç Sanayii) and phenytoin (Embil İlaç, Istanbul, Turkey) were dissolved in tap water. Propyl pyrazole triol (PPT; Tocris Bioscience, Bristol, UK), diarylpropionitrile (DPN; Tocris Bioscience) and progesterone (Sigma-Aldrich, St Louis, MO, USA) were dissolved in olive oil. Pentylenetetrazole (PTZ) was purchased from Sigma-Aldrich and dissolved in 0.9% NaCl.

2.3 | Experimental procedures

Male Wistar rats (n = 120) were randomly distributed to 10 groups with 12 rats in each group. Rats either received normal drinking water (control and PTZ-vehicle groups; n = 24) or were given a

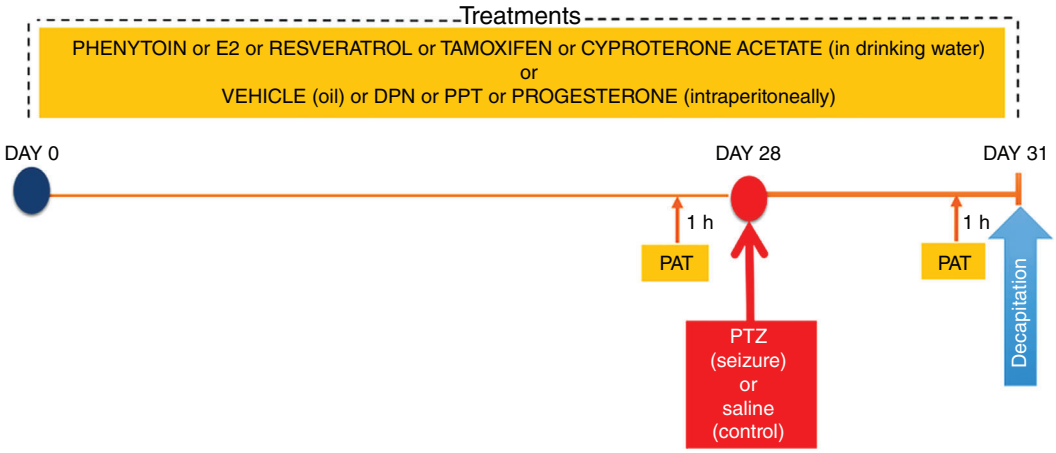

FIGURE 1 Design of the experiment. On the first day of the experimental protocol, male rats were randomly assigned to one of the treatments [17β-oestradiol (E2, 1 mg kg^{-1}), ERβ agonist (DPN, 1 mg kg^{-1}), ERα agonist (PPT, 1 mg kg^{-1}), resveratrol (40 mg kg^{-1}), progesterone (25 mg kg^{-1}), tamoxifen (TMX, 50 mg kg^{-1}), phenytoin (40 mg kg^{-1}) or anti-androgen (cyproterone acetate, 50 mg kg^{-1})], which were continued for 31 days. On the 28th day an epileptic seizure was induced by injecting pentylentetrazole (PTZ; 45 mg kg^{-1}), while the control rats were injected with saline. Rats were decapitated on the 31st day. A passive avoidance test (PAT) was performed at 1 h before seizure-induction (learning) and 1 h before decapitation (memory recall)

non-selective ER agonist 17β-oestradiol (E2; 1 mg kg^{-1}), phyto-oestrogen resveratrol (40 mg kg^{-1}), SERM tamoxifen (TMX; 40 mg kg^{-1}), anti-androgen cyproterone acetate (CPA; 50 mg kg^{-1}) or antiepileptic phenytoin (40 mg kg^{-1}) which were prepared as suspensions and given in their drinking water for 28 days (Figure 1). ERα agonist propylpyrazole-triol (PPT; 1 mg kg^{-1}), ERβ agonist diarylpropionitrile (DPN; 1 mg kg^{-1}), progesterone (25 mg kg^{-1}) or olive oil (PTZ-vehicle group) was intraperitoneally (I.P.) injected for 28 days. The doses of the treatments were based on previous studies reporting the antioxidant actions of the agents (Kumral et al., 2014; Reddy, 2009; Zheng et al., 2004; Wang et al., 2013; Gómez et al., 2009; Mansouri et al., 2013). Following the 28-day treatment period, epileptic seizure was induced by a single-dose of PTZ (45 mg kg^{-1}), while the tap water-given control group was injected with saline. Seizures were video-taped for Racine's scoring. Since the cessation of the treatments would result in acute rebound effects, treatments were continued in the following 3 days after PTZ injection, during which repetitive contractions, loss of free movement in the cage, or having difficulty in feeding or drinking were considered as humane endpoints for early killing. A passive avoidance test was performed on two occasions: before PTZ injection (acquisition phase) and immediately before they were decapitated at the 72nd hour of seizure induction (recall phase). Following the passive avoidance tests, all rats were then anaesthetized with ketamine and xylazine hydrochloride (100 and 10 mg kg^{-1}, I.P.). For histological analysis, four rats from each group were then perfused transcardially with paraformaldehyde and the brain tissues were removed. The eight non-perfused but anaesthetized rats from each group were decapitated and their trunk blood was collected to measure serum tumour necrosis factor alpha (TNF-α) and interleukin (IL)-6 levels. Biochemical measurements were performed to determine brain levels of malondialdehyde (MDA) and glutathione (GSH), myeloperoxidase (MPO) activity and chemiluminescence levels of luminol and lucigenin. Western blotting was applied to determine the expression of cAMP responsive element-binding protein (CREB), brain-derived neurotrophic factor (BDNF) and c-Fos proteins. For each of the measurements, a standardized part of the hemispheres was obtained.

2.4 | Seizure induction and evaluation

A single epileptic seizure was induced by PTZ (45 mg kg^{-1}, I.P.) injection and the rationale in selecting the intermediate convulsive dose of PTZ was based on our previous study (Koyuncuoğlu et al., 2017). Epileptic seizures of rats were recorded by a video camera for 30 min, while they were kept in Plexiglas observation boxes (38 × 30 × 25 cm). Severity of the recorded seizures was assessed by using the Racine's scoring system: 0: no behavioural changes; 1: facial movements with twitching of ears and whiskers; 2: myoclonic jerks without rearing; 3: myoclonic jerks with rearing; 4: clonic convulsions accompanied with posture loss; 5: generalized tonic–clonic seizures (Racine, Okujava, & Chipashvili, 1972).

2.5 | Assessment of memory function

In order to evaluate the effects of treatments on seizure-induced memory dysfunction, passive avoidance test (PAT) was performed. On the 28th day of the treatment period (before PTZ injection), animals were placed in the illuminated compartment of the passive avoidance apparatus with two built-in compartments (Northel, Istanbul; each 20 × 20 × 20 cm) for the acquisition trial of the PAT (Erşahin et al., 2010). When the rats instinctively moved from the illuminated compartment into the dark compartment, which is equipped with an electric grid floor, a guillotine door was closed and an electrical foot shock (0.3–0.6 mA) was applied for 5 s. Seventy-two hours after the training period, the rats were put again into the illuminated compartment and the time to enter the dark compartment was recorded to assess the memory recall. If a rat has avoided entering the dark chamber within 5 min (cut-off point), it was considered as a normal memory performance, but it was regarded as a memory dysfunction when

the dark compartment was entered with a shorter latency (Elrod & Buccafusco, 1988). During each session, both compartments were cleaned with 70% alcohol before the next animal was put in the apparatus. The rats were decapitated immediately after PAT to obtain blood and tissue samples.

2.6 | Measurement of serum TNF-α and IL-6 levels

After centrifugation of trunk blood samples at 4000 g and 4°C for 15 min, serum samples were stored at −80°C. According to the manufacturer's instructions and guidelines, TNF-α (cat no: 201-11-0765) and IL-6 (cat no: 201-11-0136) levels were detected by commercial enzyme-linked immunosorbent assay (ELISA) kits (SunRed; Shanghai Sunred Biological Technology Co., Ltd, China). The results were expressed as picograms per millilitre.

2.7 | Measurement of myeloperoxidase activity

Activity of MPO, which is an enzyme mostly located in the azurophilic granules of polymorphonuclear leukocytes, was assessed as an indicator of tissue neutrophil infiltration. Using a tissue homogenizer (Ultraturrax-T10; IKA, Germany), brain samples were homogenized with 50 mM potassium phosphate buffer (pH: 6.0) and centrifuged at 41,400 g for 10 min at 4°C. After removal of the supernatant, the pellets were mixed in 50 mM potassium phosphate buffer containing 1 g hexadecyltrimethylammonium bromide and 0.5 g EDTA. Then *o*-dianisidine-2HCl (50 mg) and H_2O_2 (20 mM) were added onto the pellet. It was kept at 37°C for 3 min and the reaction was stopped with 2% sodium azide. Using a spectrophotometer (T80+UV/VIS; spectrophotometer, PG Instruments Ltd., UK), absorbance was read at 460 nm and MPO activity was defined as U (g tissue)$^{-1}$ (Tuğtepe et al., 2007).

2.8 | Measurement of malondialdehyde and glutathione levels

The levels of MDA, an indicator of lipid peroxidation, and the antioxidant GSH were measured in brain tissues. Brain samples were homogenized in 10% trichloracetic acid solution and centrifuged at 4000 g for 15 min at 4°C. The supernatants were mixed with thiobarbituric acid in a 100°C water bath for 15 min. Lipid peroxidation was measured at 535 nm wavelength with a spectrophotometer and the results were given in nanomoles MDA per gram of tissue. For the GSH measurement, a modified Ellman protocol was used (Tuğtepe et al., 2007). Two hundred and fifty microlitres of supernatant was added to 1 ml of disodium hydrogen phosphate (0.3 M) solution. After Ellman's reagent (0.2 ml) was added, samples were kept at 37°C for 10 min in the dark room. The absorbance was measured at 412 nm and the amount of GSH was given as μmol (g tissue)$^{-1}$.

2.9 | Western blot assays

The frozen brain tissues were weighed and homogenized in ice-cold 10 mM Tris–HCl (pH 7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} leupeptin, 1 μM pepsitatine, 10 μg ml^{-1} soybean trypsin inhibitors). Whole homogenates were used in western blots and the protein contents of the whole homogenates were determined with the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Mixed with the loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), samples containing 50 μg protein were denaturated at 100°C for 3–5 min and electrophoretically transferred onto nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Dassel, Germany) for 120 min at 80 V. The membranes were blocked with Tris-buffered saline containing 1% bovine serum albumin and 0.05% Tween-20 at room temperature for 60 min and incubated overnight at 4°C with antibodies against BDNF (1:1000), CREB (1:1000), c-Fos (1:1000) and β-actin (1:1000). β-Actin was used as an internal control. The BDNF, CREB, c-Fos and β-actin specific antibodies were supplied from Abcam (ab32515, ab108319; Cambridge, MA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA; sc 7202, sc130657).

The blots were washed three times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20 °C). The antibody–antigen complex was detected with nitro blue tetrazolium (NBT)–5-bromo-4-chloro-3-indolyl phosphate (BCIP). The apparent molecular masses of BDNF, CREB and c-Fos are 32, 40 and 15 kDa, respectively. The densitometric analyses were carried out with Image Studio Lite ver 5.2 software (Cambridge, UK).

2.10 | Chemiluminescence assays

The amount of generated ROS in the brain tissue samples was measured by the chemiluminescence method using specific enhancers. Tissue samples were put into tubes containing 500 mM phosphate-buffered saline (PBS) + 20 mM HEPES, pH 7.8. After addition of 0.2 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or lucigenin (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate) enhancers, measurements were performed using a luminometer (Berthold, LB9509, Bad Wildbad, Germany) at room temperature for 5 min with 1 min intervals. Luminol detects a group of ROS, including $^{\bullet}OH$, H_2O_2 and HOCl radicals, while lucigenin is specific for $O_2^{\bullet-}$ production. Then, the area under the curve was calculated and corrected for wet weights of the tissues. Results are given as relative light unit per mg of tissue.

2.11 | Histopathological preparation and analyses

Four rats in each group were anaesthetized with ketamine (100 mg kg^{-1}) and xylazine hydrochloride (10 mg kg^{-1}) and perfused transcardially with 4% paraformaldehyde (dissolved in 0.1 M PBS; pH 7.4). The brain tissues were removed, fixed in the same fixative overnight, and submerged in ascending sucrose solutions (10%, 20% and 30%) until they sank. Later, they were quick-frozen in dry ice and were subsequently cut with a cryomicrotome (Leica, Nussloch, Germany; CM1950) into 6 μm-thick coronal sections. Brain sections were then stained with cresyl violet to evaluate the degree of neuronal damage.

TABLE 1 Seizure scores and passive avoidance test results in all PTZ-injected groups

	Seizures following PTZ injection				Passive avoidance test	
	No seizure (*n* (%))	Stages 2–3 (*n* (%))	Stages 4–5 (*n* (%))	Average of stage scores (1–5)	Initial latency (s)	Retention latency (s)
Vehicle	0	4/12 (33.3)	8/12 (66.7)	4 ± 0.3	11.8 ± 3.3	17.7 ± 4.4 (12/12)
Phenytoin	4/12 (33.3)	6/12 (50)	2/12 (16.7)	2.5 ± 0.5*	16.4 ± 4.4	208.0 ± 4.0** (3/12)
Oestradiol	0	4/12 (33.3)	8/12 (66.7)	4.3 ± 0.4	16.0 ± 4.0	94.3 ± 36.2 (8/12)
Resveratrol	1/12 (8.3)	5/12 (41.7)	6/12 (50)	3.5 ± 0.5	17.6 ± 4.2	178.8 ± 43.3 (5/12)
DPN	0	4/12 (33.3)	8/12 (66.7)	4.6 ± 0.2	23.5 ± 4.4	199.0 ± 42.0* (5/12)
PPT	1/12 (8.3)	2/12 (16.7)	9/12 (75)	4.3 ± 0.3	23.5 ± 6.2	222.3 ± 40.1** (3/11)
Tamoxifen	2/12 (16.7)	1/12 (8.3)	9/12 (75)	4.1 ± 0.5	10.0 ± 1.8	111.3 ± 38.3 (9/12)
Progesterone	0	0	12/12 (100)	4.8 ± 0.1	25.2 ± 5.3	142.3 ± 40.4 (7/12)
CPA	0	5/12 (41.7)	7/12 (58.3)	3.6 ± 0.4	17.2 ± 6.5	127.0 ± 38.6 (8/12)

*$P < 0.05$, **$P < 0.01$ compared to vehicle-treated group. CPA, cyproterone acetate, anti-androgen; DPN, ERβ agonist; PPT, ERα agonist.

For immunohistochemistry, sections were rinsed in PBS (pH 7.4). Endogenous peroxidase activity was suppressed by immersing the sections in 3% hydrogen peroxide (in methanol) for 10 min. Following washing in PBS, all slides were treated with Super block solution (SensiTek anti-polyvalent HRP kit, ScyTek Laboratories, Logan, UT, USA). Later, slides were incubated with the rabbit anti-GABA(A)α1 (G4416, Sigma-Aldrich, 1:500) primary antibody for 1 h at 37°C. Later, the sections were washed twice in PBS and incubated in biotinylated secondary antibody for 20 min (SensiTek anti-polyvalent HRP kit, ScyTek Laboratories). After washing with PBS, the slides were incubated with streptavidin peroxidase (SensiTek anti-polyvalent HRP kit) for 20 min. After washed with PBS, a 3,3-diaminobenzidine tetrahydrochloride dihydrate (DAB) substrate kit (ab64238, Abcam) was applied. Slides were stained with Mayer haematoxylin and finally mounted with Entellan. Negative controls were also generated by omission of the primary antibody. Sections were analysed with a ×40 objective under a light microscope (Olympus BX51, Tokyo, Japan) and GABA(A)α1-positive cells were counted in the photomicrographs of cortex and dentate gyrus and CA3 regions of the hippocampi. In scoring of each of the regions, three photomicrographs were taken to calculate the average score for that animal.

2.12 | Statistics

Statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA). All data are expressed as means ± standard deviation (SD). Difference between groups was analysed by one-way ANOVA followed by a *post hoc* Tukey test. Student's *t* test and the Mann-Whitney *U* test were used to compare two groups. $P < 0.05$ was considered as statistically significant.

3 | RESULTS

3.1 | PTZ seizure scores

Following the injection of PTZ, the rats had generalized seizures that continued for at most 10 min. All the PTZ-injected rats survived throughout the experimental procedure. None of the rats had uncontrollable or non-stopping seizures that required termination of the experiment for an early killing. After PTZ injection, seizures were observed in all the rats pretreated with vehicle, 17β-oestradiol (E2), DPN, progesterone or anti-androgen, while one-third of the phenytoin-pretreated rats and only one to two animals out of dozens of resveratrol-, PPT- or TMX-pretreated groups had no seizures (Table 1). Except for the phenytoin-pretreated group, more than 50% of the rats in each group had stage 4 or 5 seizures. Accordingly, the average stage score was significantly lower in the phenytoin-pretreated PTZ group ($P < 0.05$) with respect to that of the vehicle-pretreated rats, while the averages of the stage scores reached in all the other treatment groups were similar, suggesting that none of the treatments had a potent anti-convulsive effect that could be comparable to that of the phenytoin treatment.

3.2 | Cognitive functions evaluated by the passive avoidance test

In the passive avoidance test, no significant differences were observed among the initial latency periods of rats recorded at their acquisition trials (Table 1). At the recall phase of the test that was performed at 72 h after PTZ seizure, all of the rats in the vehicle-treated group had entered the electric shock-giving dark chamber within a very short period of time, indicating memory dysfunction. On the other hand, the latency to enter the dark chamber was prolonged in all the treatment groups, but statistically significant differences were present only in phenytoin- ($P < 0.01$), DPN- ($P < 0.05$) or PPT- ($P < 0.01$) pretreated rats, and the majority of the animals in these groups had avoided entering the dark chamber within the cut-off period, suggesting an improved memory function.

3.3 | Effects of treatments on oxidative brain injury parameters, BDNF, CREB and c-Fos expression

When the impact of pre-PTZ treatments on oxidative brain injury was investigated, initially the comparisons were made between E2 and

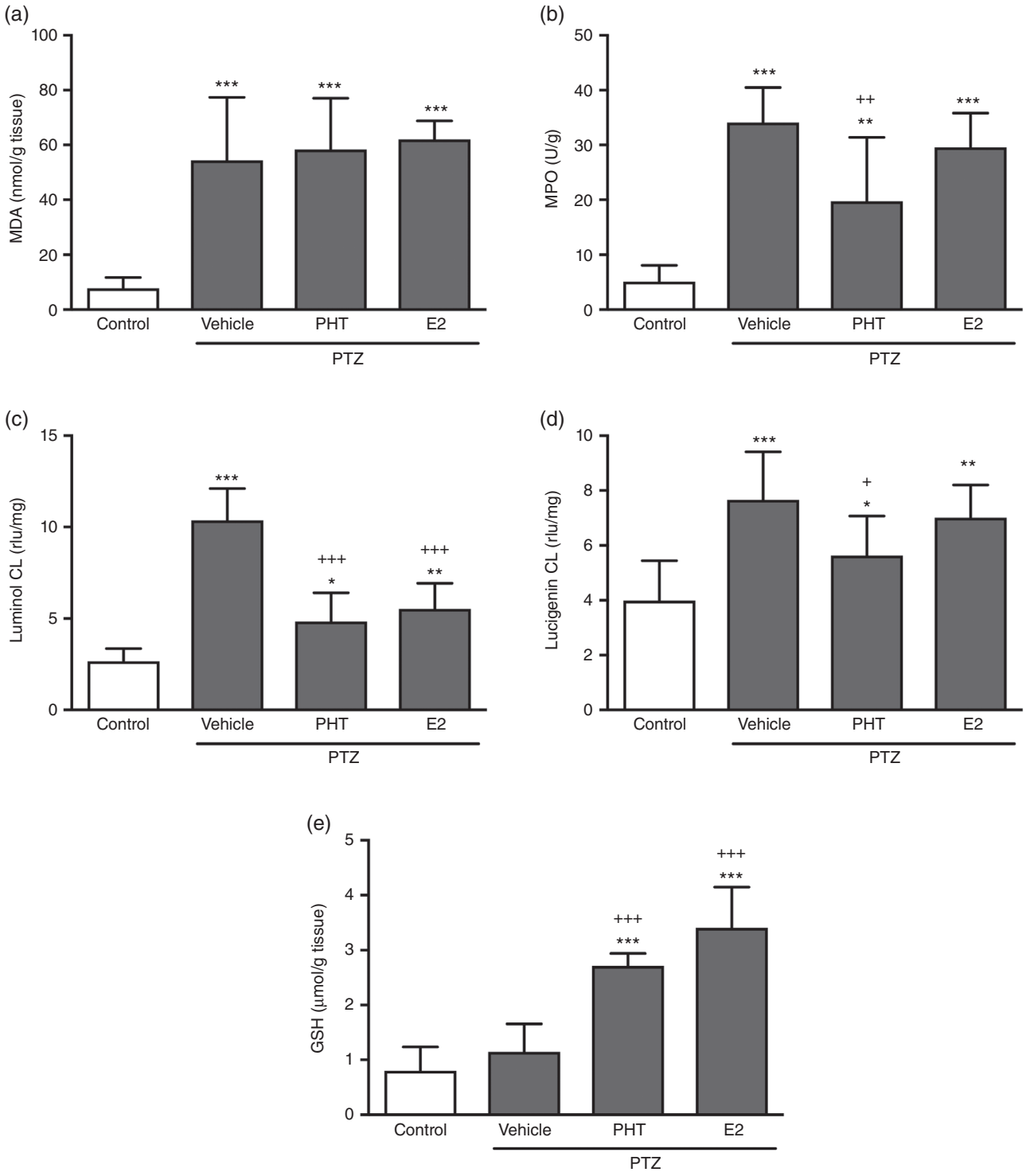

FIGURE 2 Malondialdehyde (MDA) level (a), myeloperoxidase (MPO) activity (b), luminol- (c) or lucigenin (d)-enhanced chemiluminescence (CL), and glutathione (GSH) content (e) in the brain tissues of seizure (pentylentetrazole, PTZ)-induced rats treated with phenytoin (PHT), 17β-oestradiol (E2) or vehicle with respect to control rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared to vehicle-treated PTZ group

the conventional anticonvulsant drug phenytoin. Compared with the no-seizure control group, brain MDA levels, indicative of enhanced lipid peroxidation, were increased in the vehicle-pretreated PTZ rats ($P < 0.001$; Figure 2). However, treatment with neither phenytoin nor E2 altered seizure-induced lipid peroxidation. MPO activity and luminol- and lucigenin-enhanced chemiluminescence level were all elevated in the brain tissues of vehicle-treated PTZ-rats with respect to those of the control group ($P < 0.001$; Figure 2), showing enhanced generation of ROS and facilitated neutrophil infiltration following a single seizure. Treatment with phenytoin reduced PTZ-induced

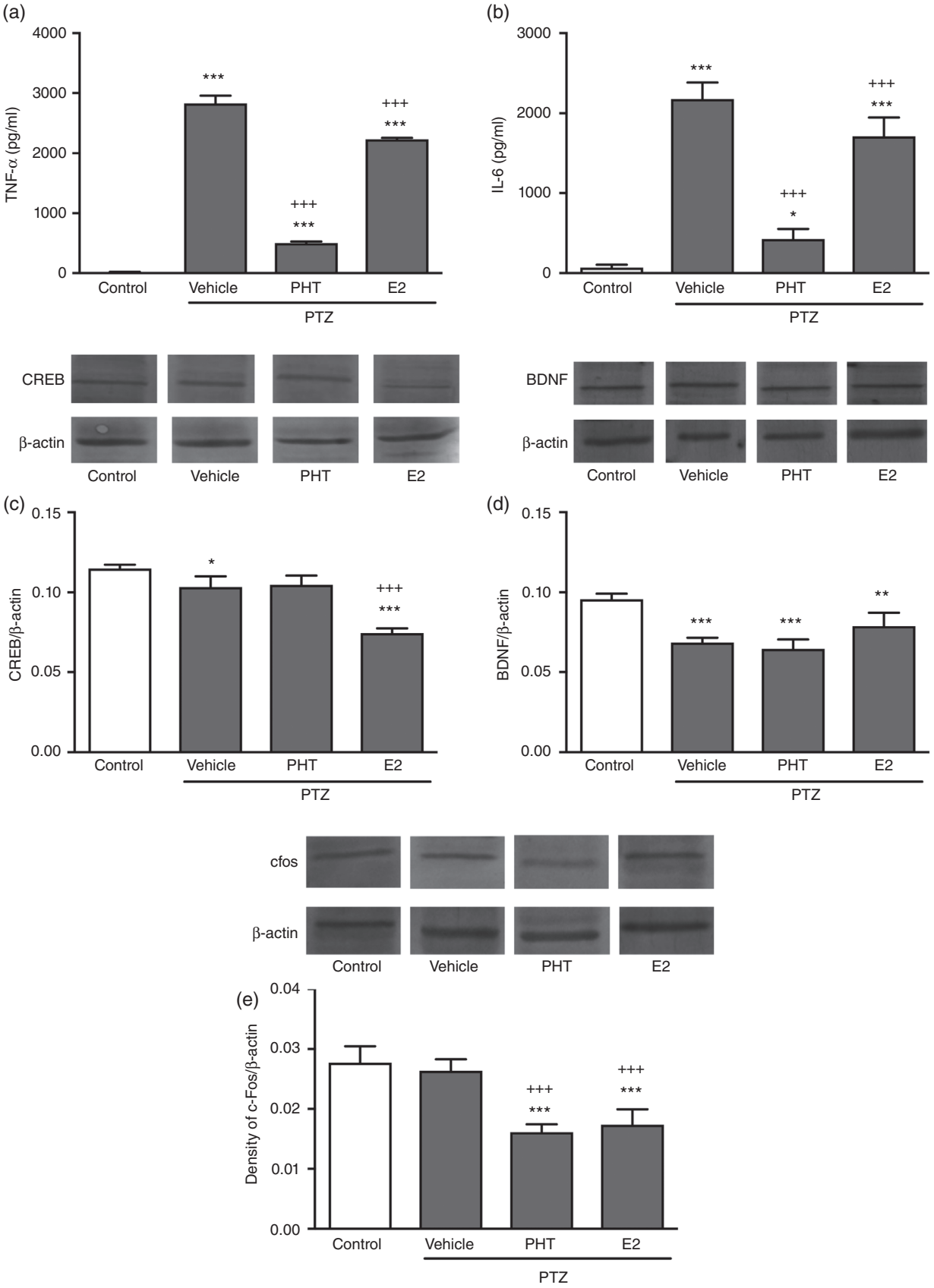

FIGURE 3 Level of tumor necrosis factor α (TNF-α) (a) and interleukin-6 (IL-6) (b) and expression of cAMP response element binding protein (CREB) (c), brain-derived neurotrophic factor (BDNF) (d) and c-Fos (e) in the brain tissues of seizure (pentylentetrazole, PTZ)-induced rats treated with phenytoin (PHT), 17β-oestradiol (E2) or vehicle with respect to control rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control group; $^{+++}P < 0.001$ compared to vehicle-treated PTZ group

elevation in MPO activity and ROS release ($P < 0.05$–0.001). However, E2 treatment only suppressed PTZ-induced elevation in luminol-detected ROS levels ($P < 0.001$), but had no effect on $O_2^{\bullet -}$ production detected by the lucigenin probe or on MPO activity. Despite the seizure induction having not affected brain GSH content significantly, either phenytoin or E2 treatment increased the antioxidant levels significantly ($P < 0.001$; Figure 2). In accordance with the observed oxidative injury, serum levels of pro-inflammatory cytokines (TNF-α and IL-6) were still elevated on the third day of the epileptic seizure in the vehicle-treated rats ($P < 0.001$; Figure 3), while both phenytoin and oestradiol treatments significantly depressed these levels ($P < 0.001$). Expression of CREB and BDNF, as determined by western blotting in the brain tissues of seizure-induced rats, was significantly depressed with respect to that of the control group ($P < 0.05$ and $P < 0.001$; Figure 3). Although phenytoin treatment had no impact on the depressed expression of CREB and BDNF, E2 further suppressed CREB expression ($P < 0.001$). Similarly, treatment with phenytoin or oestradiol significantly down-regulated the expression of c-Fos, which was not altered by a single seizure in the vehicle-treated group ($P < 0.001$).

Similar to the effects of E2 on a single seizure-induced brain injury, ERα and ERβ agonists (PPT and DPN) as well as progesterone and anti-androgen CPA suppressed ROS generation (both luminol- and lucigenin-enhanced) and elevated brain GSH content ($P < 0.05$–0.001; Figure 4). Moreover, DPN, PPT and progesterone, but not CPA, also depressed MDA level and MPO activity, which were not altered by E2 treatment ($P < 0.01$–0.001). Similar to all the other treatments, the phyto-oestrogen resveratrol also supported the brain GSH content and suppressed the release of superoxide radicals ($P < 0.001$), while the SERM TMX exerted an inhibitory effect on MDA level and increased antioxidant capacity of the seizure-injured brain ($P < 0.001$). That is, similar to E2, resveratrol, TMX or CPA demonstrated somewhat limited protection against oxidative brain injury, while DPN, PPT and progesterone were efficient in reversing the PTZ-induced alterations in oxidant–antioxidant status of the brain tissue. In parallel with these results, serum levels of both pro-inflammatory cytokines were depressed in all the PTZ-induced rats that received a hormonal treatment instead of the vehicle ($P < 0.05$–0.001; Figure 5). When compared with E2, resveratrol, DPN, PPT and TMX exerted an even greater inhibitory effect on the cytokines ($P < 0.001$).

Expression of the transcription factor CREB and one of its target genes, BDNF, which was reduced in the vehicle- or E2-pretreated rats as compared to the control rats, was also at a low level in the brain tissues of resveratrol-, TMX- or CPA-pretreated rats induced with seizures ($P < 0.001$; Figure 5). However, expression of CREB was up-regulated in DPN-, PPT- or progesterone-pretreated groups, and was not different from control level, but significantly higher than that of the E2-treated group ($P < 0.001$). Except for the progesterone-pretreated rats, c-Fos expression was lower in all the pretreated rats with seizure. On the other hand, expression of BDNF was up-regulated back to control level in only the DPN-treated rats and was increased nearly twofold with respect to that of E2-treated rats ($P < 0.001$).

3.4 | Histopathological assessment of neuronal injury and immunostaining scores for GABA(A)α1-positive cells

Cresyl violet staining revealed increased neuronal degeneration in the dentate gyrus (DG) of the vehicle-treated PTZ group as compared to DG of control rats (Figure 6). Reduced neuronal degeneration was observed in the groups treated with phenytoin, E2, TMX, progesterone, resveratrol or DPN. Regular organization of the DG granular cell layer with less degenerated neurons was more evident in the PPT-administered groups.

The immunostaining score for GABA(A)α1-positive cells was significantly reduced ($P < 0.05$) in the DG of vehicle-treated PTZ rats (Figures 7 and 8). Despite the scores of GABA(A)$\alpha 1^+$ cells in the other groups with any of the treatments not being different from that of the control group, scores of only E2-, DPN- or PPT-treated groups were significantly increased as compared to vehicle-treated rats ($P < 0.05$). Although a similar trend was observed in the immunostaining of both CA3 and cortex regions, differences were not statistically significant (data not shown).

4 | DISCUSSION

The results demonstrate that chronic pretreatment with E2, resveratrol, TMX, progesterone or anti-testosterone, which were continued following a single epileptic seizure, could suppress most of the inflammatory parameters indicative of oxidative neuronal injury, while treatment with ER agonists DPN or PPT was found to be even more effective in limiting the oxidative damage. Treatment with the ERβ agonist DPN resulted in the up-regulation of CREB and BDNF expression, while PPT up-regulated expression of CREB without affecting BDNF levels. Moreover, both ER agonists provided protection against seizure-induced memory loss. In accordance with that, the depressed number of GABA(A)α1-positive cells in the DG of the non-treated PTZ group was significantly increased in the DPN- and PPT-pretreated rats. Thus, among the tested oestrogen-mimicking or testosterone-blocking agents, ER agonists and specifically ERβ agonist DPN provided the maximum protection against seizure-induced oxidative brain injury and associated memory dysfunction by up-regulating the expression of CREB, BDNF and GABA(A)α1 receptors (Figure 9).

In the present study, the severity of PTZ-induced seizures was not changed by any of the treatment modalities, which were more or less effective in ameliorating the oxidative injury or in improving memory dysfunction. It is well known that overactivity of neurons, as occurs in generalized tonic–clonic motor convulsions, triggers immediate-expression genes, including c-Fos. In the brains of rats, c-Fos reaches its maximum expression level within the first 2 h after seizure induction and returns to baseline values at 6 h (Barros et al., 2015). Thus, in the current study, c-Fos protein expression in the vehicle-treated rats, measured at 72 h of PTZ seizure, was not different from that of the control group. On the other hand,

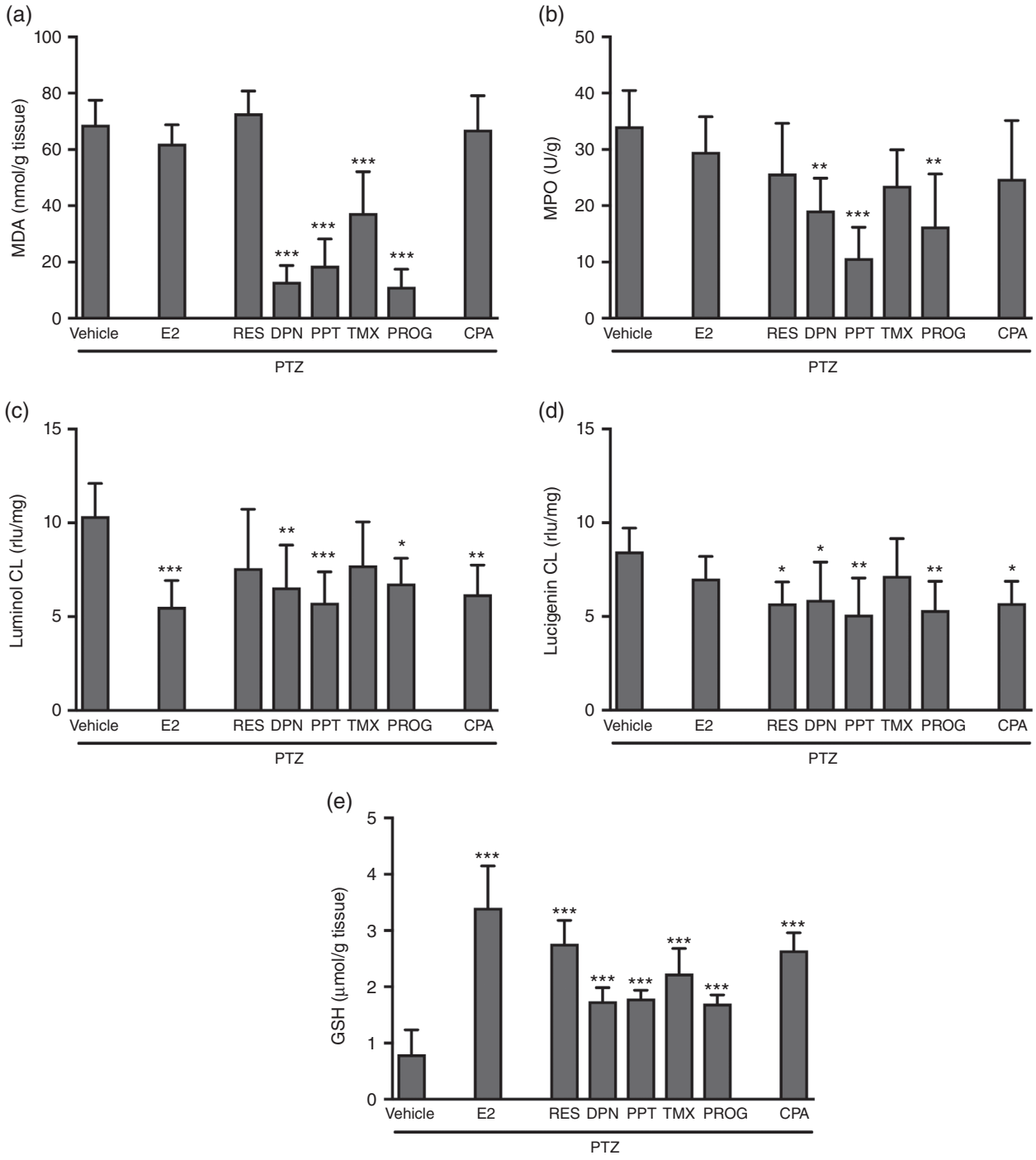

FIGURE 4 Malondialdehyde (MDA) level (a), myeloperoxidase (MPO) activity (b), luminol- (c) or lucigenin (d)-enhanced chemiluminescence (CL) level and glutathione (GSH) content (e) in the brain tissues of seizure (pentylentetrazole, PTZ)-induced rats treated with 17β-oestradiol (E2), resveratrol (RES), oestrogen receptor-β agonist (DPN), oestrogen receptor-α agonist (PPT), selective oestrogen receptor modulator (tamoxifen; TMX), progesterone (PROG) or anti-androgen (cyproterone acetate; CPA). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to vehicle-treated PTZ group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared to E2-treated PTZ group

the anticonvulsant phenytoin and all the treatments, except for progesterone, down-regulated the expression of c-Fos, suggesting an inhibition of post-seizure neuronal activity. In parallel with our findings, there are indications that the neuroprotective effect of oestradiol against ischaemic injury is associated with attenuation of c-Fos induction (Wise, Dubal, Rau, Brown, & Suzuki, 2005). Both clinical and experimental studies have demonstrated that an epileptic seizure triggers the generation of inflammatory mediators, which play an important role in the pathophysiology of epileptogenesis. In epileptic patients, the plasma levels of the pro-inflammatory cytokine IL-6 were higher in comparison with healthy humans and were further elevated following seizures (Liimatainen, Fallah, Kharazmi,

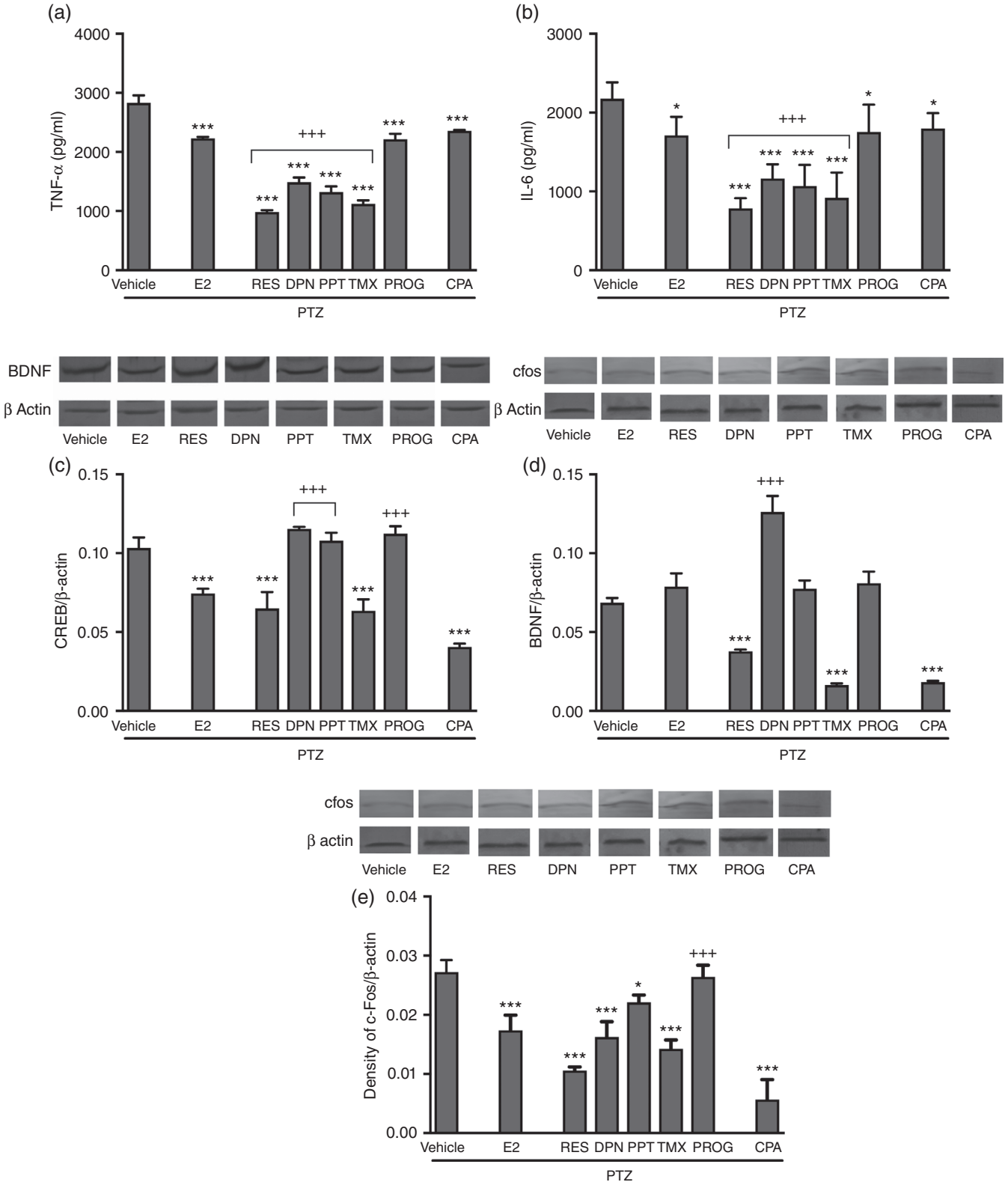

FIGURE 5 Level of tumor necrosis factor α (TNF-α) (a) and interleukin-6 (IL-6) (b) and expression of cAMP response element binding protein (CREB) (c), brain-derived neurotrophic factor (BDNF) (d) and c-Fos (e) in the brain tissues of seizure (pentylentetrazole, PTZ)-induced rats treated with 17 beta- oestradiol (E2), resveratrol (RES), oestrogen receptor-β agonist (DPN), oestrogen receptor-α agonist (PPT), selective oestrogen receptor modulator (tamoxifen; TMX), progesterone (PROG) or anti-androgen (cyproterone acetate; CPA). $^{*}P < 0.05$, $^{***}P < 0.001$, compared to vehicle-treated PTZ group; $^{+++}P < 0.001$ compared to E2-treated PTZ group

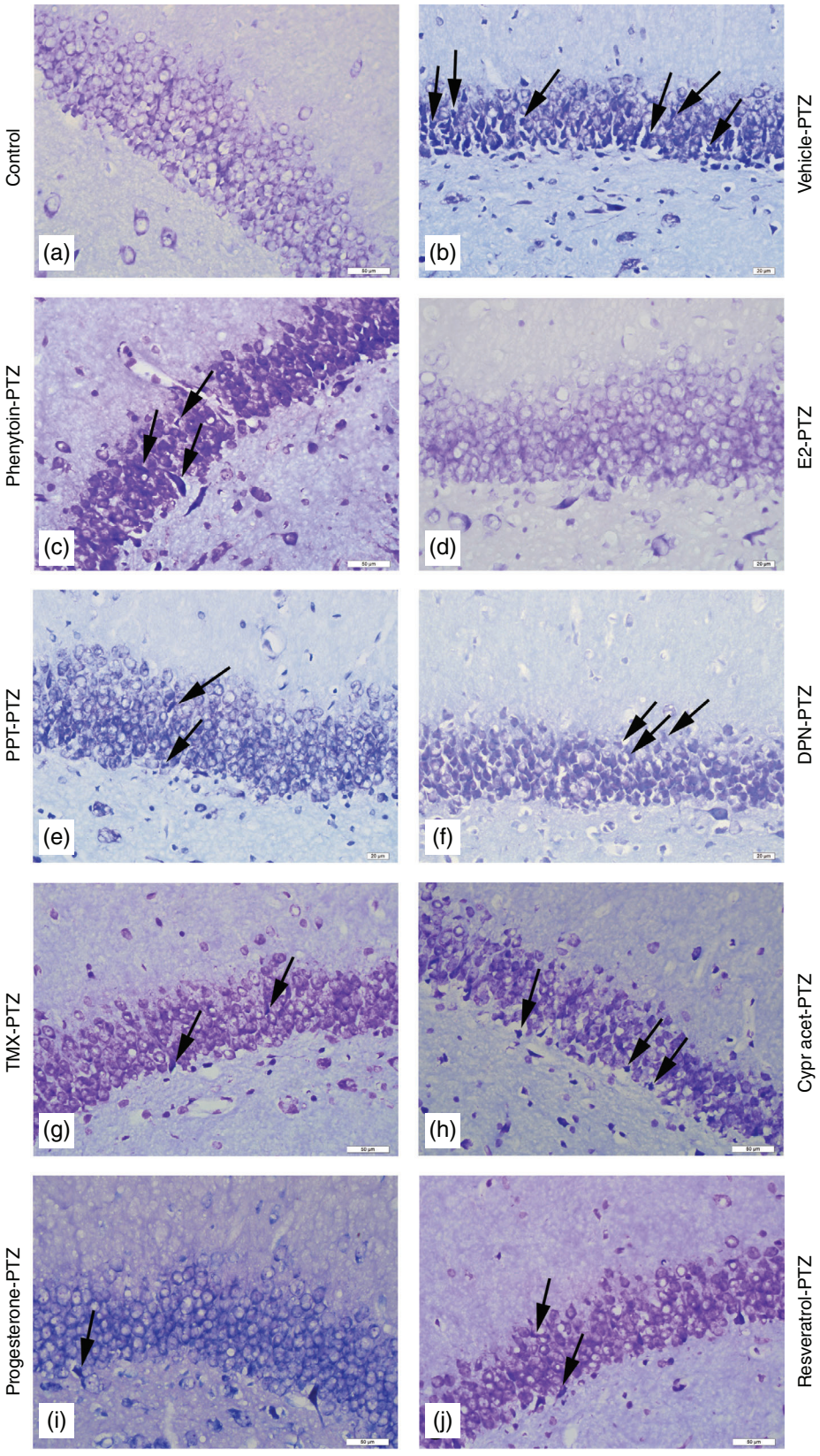

FIGURE 6 Representative micrographs of the hippocampal DG region of the seizure (pentylentetrazole, PTZ)-induced rats treated with pheytoin, 17β-oestradiol (E2), resveratrol (RES), oestrogen receptor-β agonist (DPN), oestrogen receptor-α agonist (PPT), selective oestrogen receptor modulator (tamoxifen; TMX), progesterone (PROG) or anti-androgen (cyproterone acetate; Cypr acet). (a) Control, (b) Vehicle–PTZ, (c) phenytoin–PTZ, (d) E2–PTZ, (e) PPT–PTZ, (f) DPN–PTZ, (g) TMX–PTZ, (h) Cypr acet–PTZ, (i) progesterone–PTZ, (j) resveratrol–PTZ. Arrows indicate degenerated neurons. Staining with cresyl violet. Scale bars: (a,c,g–j): 50 μm; (b,d–f): 20 μm

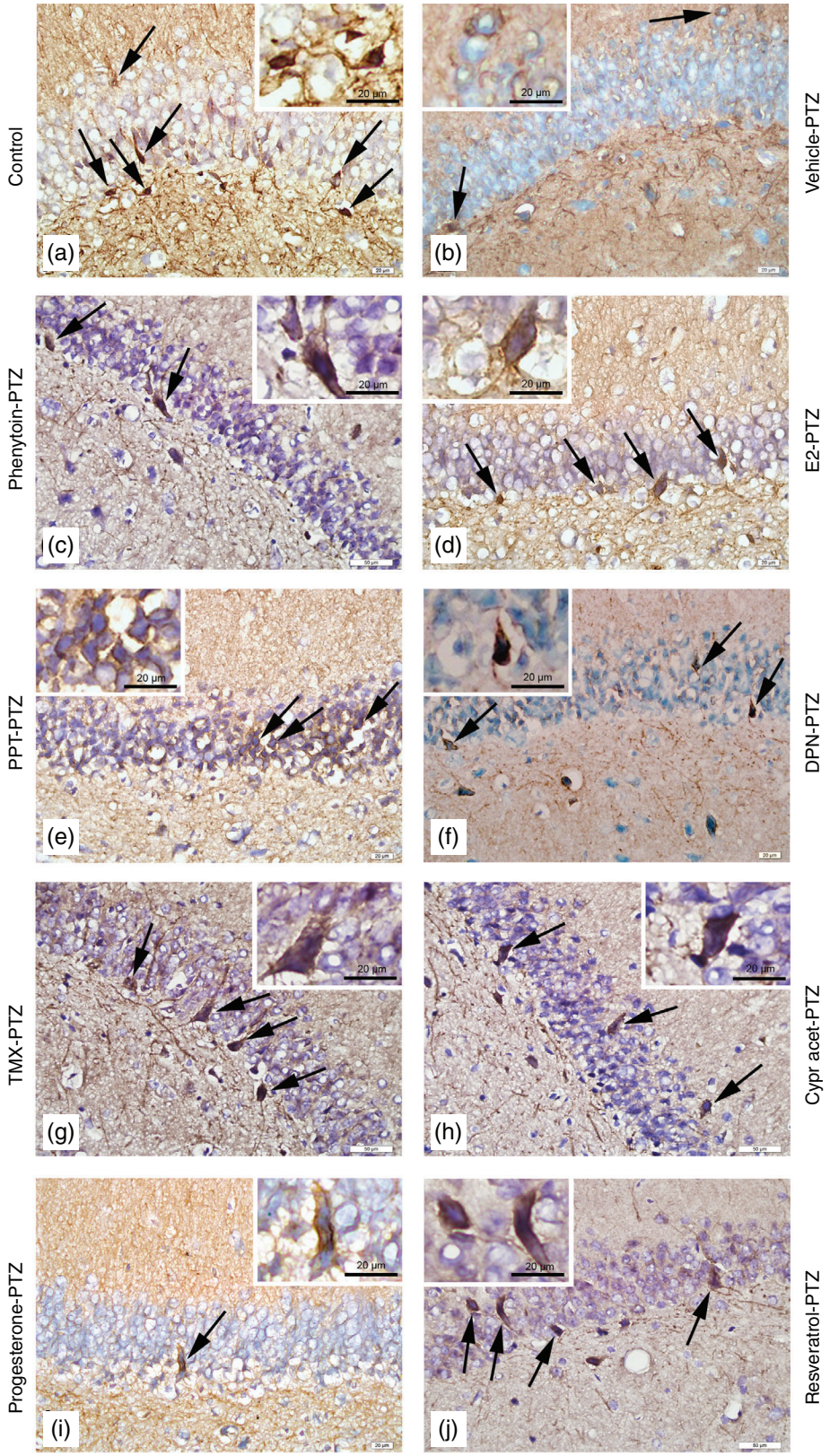

FIGURE 7 Representative micrographs of the GABA(A)α1 receptor-IR-positive neurons in the hippocampal DG region of the seizure (pentylentetrazole, PTZ)-induced rats treated with pheytoin, 17β-oestradiol (E2), resveratrol (RES), oestrogen receptor-β agonist (DPN), oestrogen receptor-α agonist (PPT), selective oestrogen receptor modulator (tamoxifen; TMX), progesterone (PROG) or anti-androgen (cyproterone acetate; Cypr acet). (a) Control, (b) vehicle–PTZ, (c) phenytoin–PTZ, (d) E2-oestradiol–PTZ, (e) PPT–PTZ, (f) DPN–PTZ, (g) TMX–PTZ, (h) Cypr acet–PTZ, (i) progesterone–PTZ, (j) resveratrol-PTZ. Arrows indicate immunopositive neurons. Scale bars: (a,b,d–f,i): 20 µm; (c,g,h,j): 50 µm; all insets: 20 µm

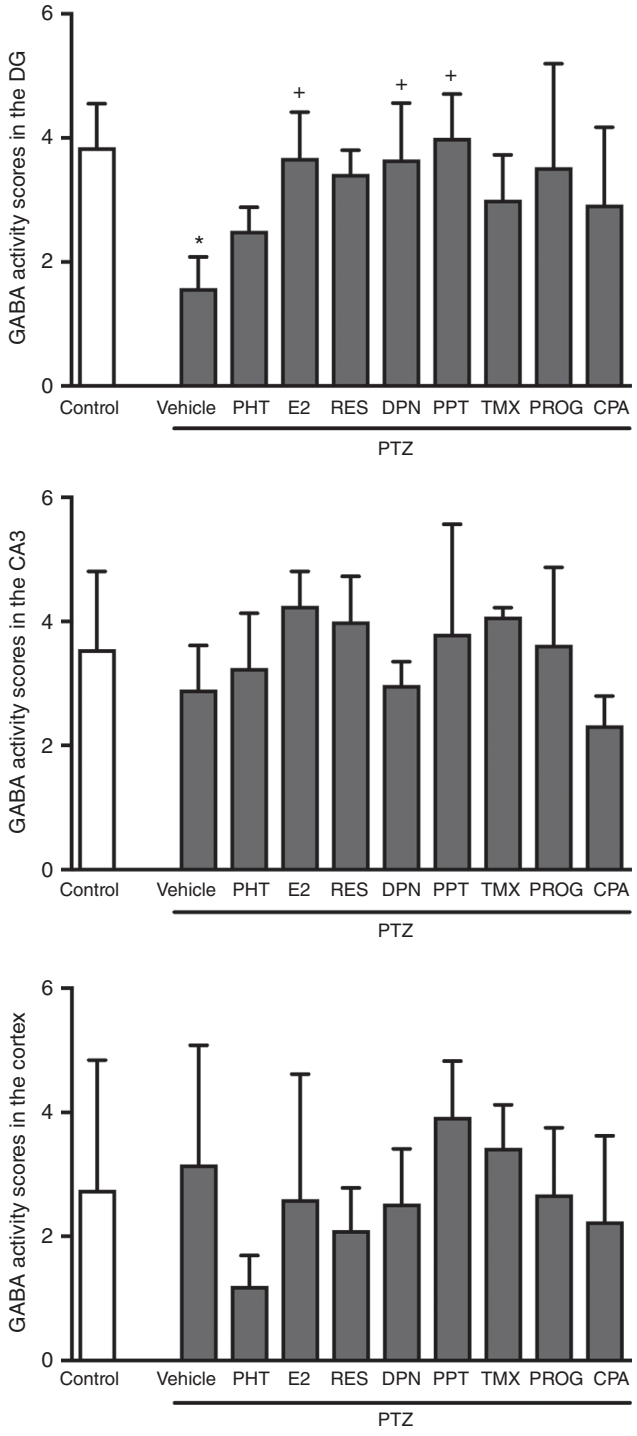

FIGURE 8 GABA immunoreactivity scores in the hippocampal dentate gyrus, hippocampal CA3 areas and cortex in the brain tissues of seizure (pentylentetrazole, PTZ)-induced rats treated with vehicle, 17β-oestradiol (E2), resveratrol (RES), oestrogen receptor-β agonist (DPN), oestrogen receptor-α agonist (PPT), selective oestrogen receptor modulator (tamoxifen; TMX), progesterone (PROG), anti-androgen (cyproterone acetate; CPA). $^{*}P < 0.05$, compared to control group; $^{+}P < 0.05$, compared to vehicle-treated PTZ group

Peltola, & Peltola, 2009; Peltola, Hurme, Miettinen, & Keränen, 1998). Similarly, the concentrations or expressions of nuclear factor κB (NF-κB), IL-6, IL-1β and TNF-α have been elevated in the hippocampi of seizure-induced rats (Mansoor et al., 2018; Singh et al., 2019). Although the effect of sex steroids on the seizure-induced cytokine response was not studied before, oestradiol treatment in mice was shown to suppress the pro-inflammatory transcription factor NF-κB-induced genes and the levels of cytokines and chemokines, while anti-oestrogen treatment inhibited these reductions (Evans, Eckert, Lai, Adelman, & Harnish, 2001). Similarly, higher levels of IL-1 and TNF-α were found in patients with surgical menopause as compared to patients with intact ovaries, and the elevated levels of these pro-inflammatory cytokines were returned to pre-surgical levels by a 4-week oestrogen treatment (Pacifici et al., 1991). In accordance with these findings, the present results revealed that serum IL-6 and TNF-α levels were elevated at 72 h of PTZ-seizure, indicating that a single seizure results in a sustaining systemic inflammation. Treatment with an oestrogen-mimicking or an anti-testosterone agent was significantly effective in reducing the levels of the cytokines, but these reductions, as could be expected, were relatively less than those observed in the anticonvulsant phenytoin-pretreated group. In support of that, pretreatment with ERα or ERβ agonists was reported to attenuate the cytokine responses in astrocyte cultures induced by lipopolysaccharide (LPS) (Lewis, Johnson, Stohlgren, & Simpson, 2009). Since ERs are present in microglia, which are the resident immune cells responsible for the first line of protection against noxious insults in the brain, it is possible that microglia represent a target for the neuroprotective actions of oestrogens in preventing and controlling the inflammatory response (Villa, Vegeto, Poletti, & Maggi, 2016).

Several lines of evidence based on a large number of clinical trials and preclinical research reports suggest that oestrogens protect neuronal cells against injurious (glucose deprivation, oxidative insult, glutamate toxicity, etc.) and neurodegenerative diseases by diminishing cell death and supporting survival and integrity of neurons (Goodman, Bruce, Cheng, & Mattson, 2010; Siddiqui et al., 2016; Villa et al., 2016; Weaver, Park-Chung, Gibbs, & Farb, 1997; Wise et al., 2005). Pretreatment with selective ER modulators, which can bind to both ERα and ERβ receptors, was shown to protect neural tissue against various oxidative challenges (Arevalo, Santos-Galindo, Lagunas, Azcoitia, & Garcia-Segura, 2011; Obata & Kubota, 2001; Rossberg, Murphy, Traystman, & Hurn, 2000), including kainic acid-induced seizures (Ciriza et al., 2004; Reibel et al., 2000; Yalcin et al., 2005). In PTZ-induced seizures, the phyto-oestrogen genistein was shown to exert neuroprotective activity by improving oxidative stress and apoptosis (Elsayed et al., 2018). It was demonstrated that a single dose of E2 or a chronic pretreatment with E2 before ischaemia exerted a neuroprotective effect and prevented hippocampal cell loss, while this protective effect on neuronal apoptosis was also observed with ERβ agonist pretreatment (Ma et al., 2016; Wappler et al., 2010), but ERα agonist PPT had no effect on the severity of neuronal damage (Carswell, Macrae, Gallagher, Harrop, & Horsburgh, 2004). Moreover, LPS-induced augmentation of the expression of the pro-inflammatory cytokines in the microglial cultures was inhibited by the ERβ agonist

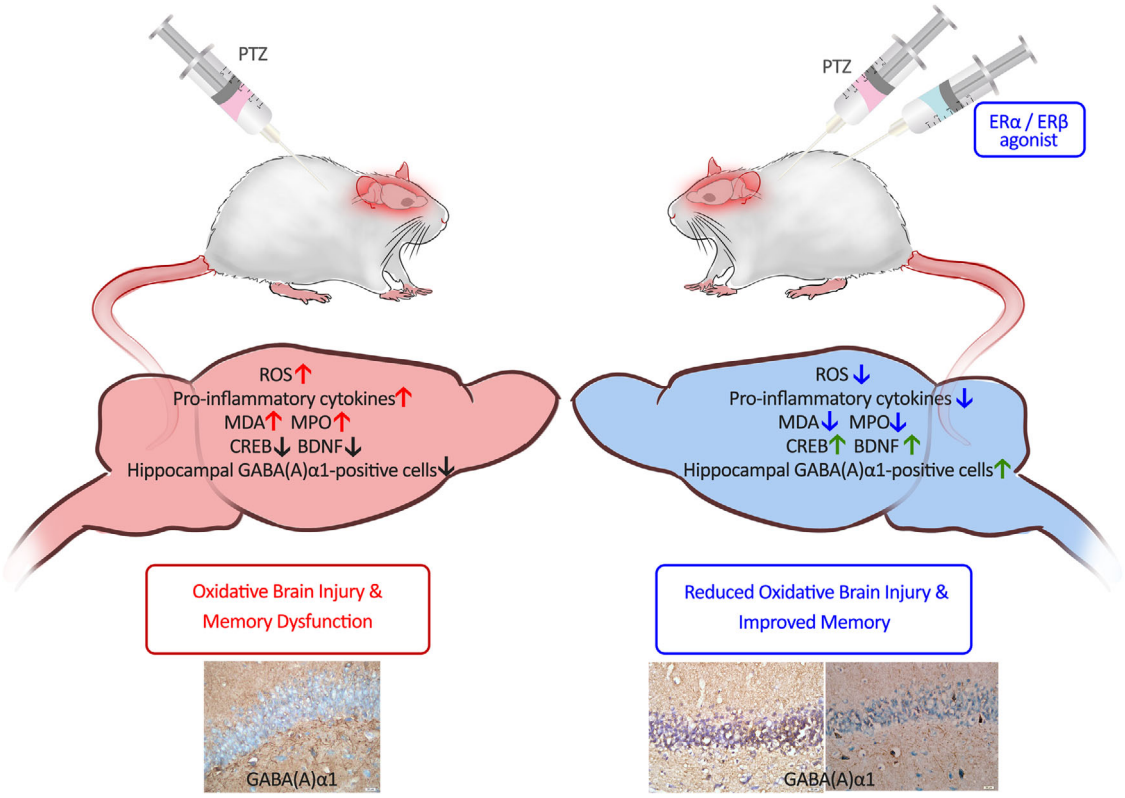

FIGURE 9 Summary of the protective effects of ERα and ERβ on experimentally induced epileptic seizure

DPN, but not by PPT (Lewis et al., 2009). On the contrary, the neuroprotective action of oestradiol against ischaemia-induced cell death was abolished in ERα knockout mice, while oestradiol continued to be protective in the absence of ERβ (Wise et al., 2005). Apart from the aforementioned reports that have examined the neuroprotective effects of ER agonists in ischaemia models and a single article that has suggested a controversial epileptogenic effect of GPER in a PTZ-kindling model (Kurt et al., 2016), the present study is the first to demonstrate the differential neuroprotective effects of ERα and ERβ on seizure-induced neuronal injury. Elevated levels of MDA and MPO and enhanced generation of ROS in the brain tissue, which were elicited by PTZ administration, were suppressed by both DPN and PPT treatments, while both agonists replenished the antioxidant GSH content. It was already documented that PTZ-induced seizures result in cognitive impairment and oxidative stress with the contribution of ROS (Goel & Saxena, 2018; Xie et al., 2012), but the inhibitory effects of ER agonists, progesterone or CPA on the generation of ROS have not been demonstrated before. Although all the tested agents in the study were efficient in supporting the antioxidant GSH stores, PTZ-induced memory impairment was attenuated only in the DPN- or PPT-treated groups, which also had alleviation of all the other inflammatory parameters. Thus, the more powerful antioxidant and anti-inflammatory effects of ERα and ERβ on PTZ-induced neuronal damage may contribute to the improvement of memory function. Nevertheless, both ERα and ERβ seem to be involved in the neuroprotective actions by the activation of different regulatory mechanisms that control different aspects of neuroinflammation (Azcoitia, Arevalo, De Nicola, & Garcia-Segura, 2011).

Immunohistochemical and autoradiographic reports on murine brain have indicated that both ERα and ERβ mRNA are expressed in several hypothalamic and amygdaloid nuclei, while ERβ mRNA is also found in the olfactory bulbs, the bed nucleus of the stria terminalis, the hippocampus, the cerebral cortex, the cerebellum, the midbrain raphe and the basal forebrain (Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998; Shughrue, Lane, & Merchenthaler, 1997; Shughrue, Lane, & Merchenthaler, 1999; Shughrue, Lubahn, Negro-Vilar, Korach, & Merchenthaler, 1997; Simerly, Swanson, Chang, & Muramatsu, 1990). It was postulated that oestradiol decreases seizure severity by facilitating the release of the anticonvulsant neuropeptide Y (NPY) via ERα activity in the hippocampus (Ledoux, Smejkalova, May, Cooke, & Woolley, 2009), where the major inhibitory neurotransmitter GABA is co-localized with NPY (Colmers & El Bahh, 2003). Moreover, hippocampal GABAergic neurons, which are demonstrated to express both ERα and ERβ (Adams et al., 2002; Milner et al., 2005), were reported to be reduced in epileptic patients or animals (Sperk et al., 2004). Although the current data demonstrate that the ER agonists, apart from their inhibitory effect on c-Fos expression, did not reduce the severity of PTZ-induced seizures, treatment with either ERα or ERβ agonist supported the memory performance and abolished the reduction in the immunoreactivity of hippocampal GABA(A)α1. Thus, it appears that the maintenance of hippocampal GABA(A)α1 receptor activity could be associated with the improvement in seizure-induced

memory loss. In contrast to studies in which the memory-enhancing effect of oestradiol was mostly attributed to the involvement of ERβ (Jacome et al., 2010; Liu et al., 2008) or ERα (Frye, Duffy, & Walf, 2007), the present data suggest that both receptors mediate the restoration of memory deficits. Since increased anxiety enhances PAT performance (Luvisetto, Basso, Petronilli, Bernardi, & Forte, 2008) and oestrogens exert significant anxiolytic effects (Oyola et al., 2012), it is possible that the increased PAT performance after the seizure could be partially due to the anxiolytic effects of oestrogens. However, the non-selective ER agonist, expected to have an anxiolytic action, did not provide any memory-enhancing effect. Although the involvement of both ERs in memory-enhancing effects of oestradiol was previously demonstrated in other causes of memory impairment (Boulware, Heisler, & Frick, 2013; Hammond, Mauk, Ninaci, & Nelson, 2009), the current data prove that both receptors are equally efficient in improving seizure-induced memory loss.

Oestrogens were shown to restore hippocampal neurogenesis and enhance memory function by increasing the number of dendritic spines and synapses in the entorhinal cortex via the up-regulation of BDNF (Pietranera, Lima, Roig, & De Nicola, 2010; Scharfman & MacLusky, 2006), which is well known to play an important role in the plasticity of the brain regions related with cognitive function (Driscoll et al., 2012). Since BDNF-synthesizing neurons express ERs and the BDNF gene contains an oestrogen response element, it appears that a functional interaction exists between oestrogen and BDNF (Miranda, Sohrabji, & Dominique Toran-Allerand, 1993). In cultured hippocampal slices, E2 treatment has increased BDNF levels and provided neuroprotection against *N*-methyl-D-aspartate toxicity, suggesting a contribution of BDNF to E2-mediated neuroprotection (Aguirre & Baudry, 2009). Further supporting the crosstalk between BDNF and E2, the findings of our study suggest that PTZ-induced depletion in the cerebral BDNF was prevented only in the ERβ agonist-pretreated group, which has exhibited an improved memory performance along with a remarkable neuroprotection against oxidative injury. In parallel with the fact that E2 may modulate BDNF expression via the activation of the CREB signalling pathway (Zhou, Zhang, Cohen, & Pandey, 2005), our data also demonstrated that seizure-induced reduction in cerebral CREB expression was up-regulated by both ER agonists.

Although clinical or experimental reports have indicated that oestrogens worsen cerebral ischaemic damage (Harukuni, Hurn, & Crain, 2001; Viscoli et al., 2001), subtyping of ERs has brought clear evidence that ERβ activation is neuroprotective against ischaemia (Carswell et al., 2004). This is the first study to evaluate the effects of selective ER agonists on seizure-induced damage in comparison with oestradiol and progesterone, as well as SERMs. In conclusion, chronic administration of oestrogen, progesterone, ER modulators/agonists or blockade of testosterone exhibited anti-inflammatory and antioxidant actions on single seizure-induced neural injury. However, as compared with oestrogen, both ER agonists had additional stimulatory impact on CREB signalling and hippocampal GABA(A)α1 receptor density, which were accompanied by improved memory function, while ERβ agonist had an additional up-regulatory effect on BDNF expression. Additional experimental studies are necessary to investigate any synergistic effects of these agonists when given in combination. Nevertheless, the results of our study suggest that either ERα or ERβ receptor activation may be beneficial to protect against seizure-related oxidative brain injury and cognitive dysfunction, and further experimental studies are required to evaluate their possible therapeutic role in recurrent seizures of chronic epilepsy models.

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COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

All the experiments were performed at the Marmara University School of Medicine, Department of Physiology. Study conception and design of the work: T.K., S.A.T. and B.Ç.Y. Data acquisition, analysis and data interpretation, and drafting of the manuscript: all authors. Critical revision: T.K., S.A.T. and B.Ç.Y. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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