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The Synergistic Effect of Raloxifene, Fluoxetine, and Bromocriptine Protects Against Pilocarpine-Induced Status Epilepticus and Temporal Lobe Epilepsy

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Abstract

The present antiepileptic drugs pose several problems in the management of seizures owing to their meager neuroprotective potential, adverse effects on bone, detrimental effects on cognitive function, chronic toxicity, drug interactions, side effects including aggression, agitation, and irritability and sometimes exacerbation of seizures. We followed up progressive preclinical investigation in mice against pilocarpine (PILO)-induced status epilepticus (SE) and temporal lobe epilepsy (TLE). To determine the response of raloxifene (RF) (4 and 8 mg/kg), fluoxetine (FT) (14 and 22 mg/kg), bromocriptine (BC) (6 and 10 mg/kg), and their low-dose combinations, oral treatment was scheduled for 28 days followed by PILO (300 mg/kg, i.p). The response was stalked for intensive behavioral monitoring of convulsions, hippocampal neuropeptide Y (NPY), and oxidative stress discernment along with histomorphological studies. The resultant data confirmed the therapeutic potential of triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) compared to monotherapy with raloxifene (4 mg/kg), and bromocriptine (6 mg/kg) as otherwise monotherapy with fluoxetine (14 mg/kg) was ineffective to suppress convulsions; an effect better than sodium valproate (300 mg/kg), a standard AED, was validated. Most profoundly, PILO-induced compensatory increases in hippocampal NPY levels (20.01%), which was escalated (100%) with the triple drug combination. The same pattern of results was superseded for oxidative stress indices and neuronal damage. The results for the first time demonstrate the propitious role of triple drug combination in the management of SE and TLE. Therapeutically, this enhancing profile of drugs fosters a safer and more effective drug-combination regimen.

Keywords Epilepsy · Serotonin · Dopamine · Neurodegeneration · Neuropeptide Y · Status epilepticus

Abbreviations

SE	Status epilepticus
TLE	Temporal lobe epilepsy
NPY	Neuropeptide Y
PILO	Pilocarpine
KA	Kainic acid
SRS	Spontaneously recurring seizures
RF	Raloxifene

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SV Sodium valproate
BC Bromocriptine
FT Fluoxetine

Introduction

Epilepsy is a serious neurological disorder manifested by recurrence of unprovoked seizures resulting in devastating consequences on 75 million of the world population [1, 2]. Antiepileptic drugs (AEDs) derive only symptomatic relief, while the underlying pathology remains unabated, and 20–30% of patients remain refractory to treatment [3], while 20–30% have associated neurological disorders. Therefore, their remains an exorbitant need for novel and more effective AEDs, which would exhibit simultaneous membrane stabilizing, as well as neuroprotective property. Temporal lobe epilepsy (TLE) is the most common type of partial complex seizure in adulthood [4]. The animal models of TLE inculpate experimental transaction in which the epileptic condition results as a downstream sequel of brain damage induced by an acute episode of status epilepticus (SE). This episode is elicited by administration of chemical convulsants (e.g., pilocarpine; and Kainic acid) or with electric shock. It has been shown that duration of SE during the initial insult is critical to the duration of latent period and subsequent development of spontaneously recurring seizures (SRSs) and brain damage [5]. Thus, if a drug abolishes the onset of SE or restricts its duration to few minutes, the subsequent development of chronic epilepsy can be prevented.

Anti-psychotics (antagonist at 5-HT and Dopamine (D2) receptors) are reported to lower seizure threshold in epileptic patients and promote seizures in patients with no previous history of the disease [6]. It means depletion of serotonin and dopamine at 5-HT and dopamine (D2) receptors, respectively, predisposes to seizures. Alterations in signaling through 5-HT receptors have also been implicated in multiple types of epilepsies, e.g., genetically engineered mouse for elevated serotonin levels exhibited increase in threshold to kainic acid (KA)-induced seizures. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has been claimed to ameliorate short-term metabolic impairment and signs of neuronal damage in surviving rats evaluated against lithium-PILO model of epilepsy [7]. Besides, fluoxetine in combination with existing AEDs, i.e., phenytoin, carbamazepine, and ameltolide, has been reported to produce a dose-dependent reduction in their ED50 values against maximal electroshock (MES)-induced tonic hind limb extension (THLE) in mice [8]. In genetically epilepsy-prone rats (GEPRs), fluoxetine in combination with LY 206130 (1-[1-H-indol-4-yloxy]-3-[cyclohexylamino]-2-propanol maleate) and 5-HT1A somatodendritic autoreceptor antagonists (-)-pindolol enhanced their anti-seizure effects [9].

Several lines of investigation have also confirmed inadequacy of dopaminergic system in progression of epileptic seizures. For example, Bozzi et al. (2000) inactivated D2R gene in two mouse strains known to be previously resistant to (KA-induced seizures and excitotoxicity and demonstrated their increased sensitivity, susceptibility, and hippocampal cell death on KA administration compared to WT mouse [10]. Bromocriptine, a dopamine (D2) receptor agonist, has been reported to lower spike frequencies in both primary and secondary epileptic foci following implantation of cobalt in conscious rats as inscribed by electrocorticogram [11]. It has also shown protection against hippocampal neurodegeneration induced by ischemia in gerbil model of global cerebral ischemia [12].

Far from being given, fluoxetine and bromocriptine would restrain generation of epileptic seizures and even act as mild neuroprotectants, but intricate character of epileptic seizures is severe neurodegeneration coupled with hypersyncronous neuronal activity. Thus, neurodegeneration would, in the progression of disease, degenerate serotonergic and dopaminergic neurons, deplete post-synaptic receptor densities of serotonergic and dopaminergic receptors, and change receptor properties by altering the properties of binding site towards binding of serotonin and/or dopamine. Thereby, subsequent effect of serotonergic and dopaminergic agonists would evanesce, leading to clinical onset of drug refractory epilepsy, as is the problem with existing AEDs.

This problem could have been circumvented with the use of estrogens which although are neuroprotective but are reported to facilitate seizures. The effect of 17β-estradiol on neurons is well established during menopause, i.e., state of diminished levels of 17β-estradiol leading to increased cascade of neurodegenerative diseases, while post-liminary administration of 17β-estradiol around menopause delays the onset of neurodegenerative disorders [13]. 17β-estradiol increases excitatory (glutamatergic) transmission, and that facilitation of transmission extends to seizures [14, 15]. Hence, its use to prevent seizure-induced neuronal death is inexpedient. These limitations have been overcome by selective estrogen receptor modulators (SERMs), which act as estrogen agonist in the bone, cardiovascular system, and brain and as an antagonist in uterus and breast [16], and thus have emerged as surrogate for providing neuroprotection [17]. Raloxifene, a SERM, has been shown to protect hippocampus from excitotoxic effects of KA [18], ameliorate seizures and hippocampal neuronal damage in postmenopausal temporal lobe epileptic mouse model [19], and attenuate seizure severity, prolong latency to seizures, and reduce morbidity against PILO-induced SE in rats [20]. Hence, raloxifene would exert neuroprotective effect as like estrogens but would simultaneously withhold proconvulsive behavior amalgamated with estrogens. Thus, a combination of test drugs, raloxifene with fluoxetine and bromocriptine, was sought to exhibit synergistic effect against SE and TLE in mice through abolishing irregular neuronal electrical activity and augmenting neuroprotective effect, while a comparison was drawn with individual drugs.

Epilepsy leads to cascade of pathophysiological events in the brain in the form of changes in hippocampal neuropeptide (NPY) levels, induction of oxidative stress, and degeneration of different regions of hippocampus. NPY is a 36 aa polypeptide widely distributed in the CNS, where it is involved in various physiological functions [21]. NPY behaves as a natural barrier against progression of seizures in hippocampus from perforant path to dentate gyrus granular cell [22]. NPY reduces glutamate release by a presynaptic mechanism involving suppression of voltage-dependent Ca^{2+} influx [23]. NPY-deficit and knockout mice that had no detectable levels of NPY were reported as more prone to convulsions, while transgenic rats overexpressing NPY were less susceptible to seizures compared to their wild type (WT) counterparts [24, 25]. Synthesis of NPY is induced by brain derived neurotropic factor-tyrosine receptor kinase-B (BDNF-TrkB) signaling upon estrogen administration. Further estrogen in inhibitory presynaptic boutons promotes loftier release of NPY during seizures (i.e., viable mechanism of estrogen induced neuronal survival in the DG and CA1–CA3 hippocampal pyramidal cell layers) [26]. This signaling pathway was sought to emulate with triple drug combination of raloxifene with fluoxetine and bromocriptine (in rescission of epileptogenesis).

Oxidative stress (OS) and nitrosative stress (NS) are defined as imbalances between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS), the disparity in which is associated with neurodegeneration and mitochondrial dysfunction spotted with epileptogenesis [27]. Seizures activate NMDA receptors by increasing levels of glutamate, reducing extracellular Ca^{2+} concentration and along with increase in cytosolic Ca^{2+} concentration [28]. The effects mediated by Ca^{2+} during excessive glutamate receptor activation (excitotoxicity) escorts to neuronal degeneration and overload of mitochondria with Ca^{2+}, so free radicals are generated. Overload of this type of Ca^{2+} leads to oxidative stress (OS), cellular damage, and eventually cell death because of Ca^{2+}-mediated opening of mitochondrial permeability transition (MPT) pores associated with apoptosis [29]. Thus, treatment strategies ameliorating seizure-induced oxidative stress could avert neuronal death.

Brain damage or neuronal injury caused by epileptic seizures or SE critically alters morphologic and functional characteristics of neurons leading to clinical onset of chronic epilepsy or TLE accompanied by learning and memory deficits [30]. AEDs like valproic acid have demonstrated neuroprotection against ischemia in streptozotocin (STZ)-induced hyperglycemic rats [31], while topiramate has demonstrated neuroprotective effects against glutamate excitotoxicity in rat hippocampal neurons via modulating BDNF/TrkB-dependent ERK pathway [32]. However, neuroprotective effect of said drugs is frequently masked by severe side effects posed by them, during long-term therapy. Hence, there is an unmet need of drug(s) or drugs combinations which would simultaneously raise seizure threshold, restrict the anatomical and functional damage to neurons exposed to initial insult, and promote endogenous fight against seizures with meager side effects. In view of above facts, we assessed the effects of raloxifene, fluoxetine, bromocriptine, and their low-dose combinations against PILO-induced SE in mice. Besides, an attempt was made to explore the role of hippocampal NPY, oxidative stress, and neuroprotection in mediation of anti-epileptic effects of the above drug(s).

Materials and Methods

Animals

Swiss albino mice (both sexes), 10–12 weeks old in the weight range of (25–35 g), were obtained from central animal house facility IIM Jammu (India). Animals were housed in polypropylene cages with dust-free rice husk as a bedding material. The animals were provided with a commercial diet (Ashirwad feed, India) and water ad libitum under controlled temperature (25 ± 2 °C), humidity (60 ± 10%), and lighting (12:12 light/dark cycle) conditions. All the experimental procedures involving animals were conducted as per CPCSEA guidelines after getting proper approval by Institutional Animal Ethical Committee (IAEC), PG Department of Pharmaceutical Sciences, University of Kashmir, Srinagar [No: F-IAEC(Pharm. Sc.) APPROVAL/2013/15]. Utmost care was taken to ensure that animals were treated in the most humane and ethically acceptable manner.

Drugs and Dosing Schedule

Sodium valproate (SV) and fluoxetine (FT) were gifts from Sun Pharmaceuticals Ltd. (India). Raloxifene (RF) was purchased from Cipla Ltd. (India), bromocriptine mesylate (here to referred as bromocriptine (BC)) and diazepam were sourced from Monarch Pharmaceuticals (India), and PILO was obtained from Himedia Laboratories Pvt. Ltd. (India). Sodium valproate and PILO were prepared from pyrogen-free sterile water for injection. Raloxifene, fluoxetine, bromocriptine and diazepam were suspended in 2% Tween 80. All treatments were administered orally except PILO and diazepam, which were injected intraperitoneally (i.p) in a volume not exceeding 10 ml/kg. Raloxifene was administered in doses of 4 and 8 mg/kg (the dose of 8 mg/kg was derived from the corresponding dose (60 mg) in humans for the treatment of osteoporosis [33]). Fluoxetine was administered in doses of 14 and 22 mg/kg (the dose of 22 mg/kg was reputed to induce granule cell dematuration and enhance serotonergic modulation [34]) and bromocriptine was administered in doses of 6 and 10 mg/kg (the dose of 10 mg/kg was reported to protect against MPTP-induced neurotoxicity in mice [35]). All drug(s) were administered for a period of 28 days prior to PILO (300 mg/kg) injection in mice. Diazepam (10 mg/kg) was used to reduce PILO-induced mortality in susceptible groups.

Experimental Design

One hundred thirty mice were randomly selected and divided into 13 experimental groups consisting of ten mice in each group. The division followed was the following: Group I: normal control; Group II: toxic control; Group III: standard anti-epileptic drug (AED); Groups IV–XIII were administered raloxifene (RF), fluoxetine (FT), and bromocriptine (BC) alone and in different combinations; Groups IV–IX as monotherapy; Groups X–XII as duo drug combination therapy; and Group XIII as triple drug combination therapy. The monotherapy with each of raloxifene, fluoxetine, and bromocriptine had been evaluated at two dose levels, while in combination, the lower doses of each were used in order to achieve desired therapeutic response at the cost of minimum adverse effects.

Pilocarpine-Induced Behavioral Seizures

The dose of PILO (300 mg/kg; i.p) was standardized in our laboratory for induction of SE in 100% of normal mice, which was found in conformity with that of Mazzuferi et al. (2012) [36]. The mice were placed in plexiglas chambers for behavioral monitoring of seizure progression for 60 min. The continuous seizure activity was recorded as per slight modification of Kim et al. (2010) [37] as follows: Stage 1: immobilization and staring; Stage 2: head nodding; Stage 3: rearing accompanied by forelimb clonus and wet dog shakes; Stage 4: falling and wobbling; Stage 5: jumping, circling, or rolling; and Stage 6: severe tonic-clonic seizures. A highest score was recorded at intervals of every 3 min, i.e., 20 scores for 60 min. The mean for 20 scores was calculated, which gave a score for each animal. The mean of scores from all animals in a group was tailed, i.e., seizure severity score for each group was established. A substance was considered to possess anticonvulsant activity if it reduced seizure severity score compared to score in toxic control. Six (06) mice from each of the groups were promptly sacrificed post-monitoring and processed for hippocampal NPY and biochemical estimations, while remaining four (04) mice from each of the said groups were kept under controlled conditions for 24 h, thereafter sacrificed for histomorphological studies. A compound was considered to possess anticonvulsant activity if it decreased seizure severity score when compared to score in mice treated with PILO alone.

Pilocarpine-Induced Neuronal Damage

PILO-induced neuronal damage was assessed by histopathological evaluation of hippocampus 24 h after PILO injection. Animals from each group were deeply anesthetized with ether, and the brains were removed from the skull and immediately preserved in formalin. Brains were cut in 8-μm thick coronal sections, every 100 μm from 2.3 to 4.3 mm posterior to the bregma with the help of microtome and mounted the sections on clean marked glass slides. Hematoxylin and eosin (H/E) staining was used for staining to access neuronal terminal and cellular degeneration. The prepared slides were examined under light microscope, and digital photographs were taken by a pathologist unaware of treatment regimen from hippocampal Cornu ammonis sub regions, i.e., CA1, CA2, CA3, and DG of both hemispheres (entire hippocampus was studied under 4×, while hippocampal subregions, cornu ammonis—CA1, CA2, CA3—and dentate gyrus were considered under 40×). Normal neurons were skillfully differentiated from deformed neurons by their typical large size, medium-intensity staining, and dark nucleoli. After seizures pyramidal cells often were shrunken, pyknotic or few had entirely disappeared. The analysis of neurons was executed with a 1-cm^2 10 × 10-box microscopic grid. The grid of counting was positioned on a well-defined zone of hippocampus, i.e., CA1, CA2, CA3, and DG, and counting was conducted out with a microscopic enlargement of 200- or 400-fold defined for each single hippocampal subfield. Neurons touching the inferior and right edges of the grid were not counted.

Estimation of Hippocampal NPY Levels

Succeeding behavioral experiments, animals were sacrificed, band the rains were removed and hippocampus was separated. NPY was estimated using mouse NPY ELISA kit (Ray Biotech, Norcross, USA) according to the manufacturer's protocol. The kit allows in vitro quantitative determination of NPY levels using immunoassay techniques.

Estimation of Hippocampal Oxidative Stress Indices

Post-behavioral monitoring of seizures in the mice, homogenate of hippocampus was processed in ice-cold phosphate buffer (pH 7.4) and centrifuged at 3000 rpm for 15 min at 4 °C to obtain the supernatant, which was used for the following:

Protein Estimation

Briefly, 0.2 ml of PMS (10% *w/v*) was pipetted off in a fresh autoclaved calibrated glass tube and processed with 0.8 ml of MilliQ water, followed by addition of 5 ml Copper Reagent. The samples were incubated at room temperature for 10 min. Finally, 1 ml of Folin's reagent was added to each sample, vortexed, and again incubated for 30 min at room temperature. The blue color developed was read at 700 nm against appropriate blank using bovine serum albumin (BSA 0.1 mg/ml) as standard [38].

Thiobarbituric Acid Reactive Substances (TBARS) Estimation

Briefly, 1 ml of PMS (10% *w*/*v*) was pipetted off in a fresh autoclaved calibrated glass tube, followed by addition of 1.0 ml of TCA (10%) and 1.0 ml of TBA (0.67%). The tubes were covered with thin aluminum sheets and incubated in boiling water for 45 min. After cooling, the tubes were centrifuged at 2500 rpm for 10 min. The absorbance of supernatant was read spectrophotometrically at 525 nm against appropriate blank. Rate of lipid peroxidation (LPO) was estimated by measuring MDA content as nanomole MDA formed per gram wet tissue at 37 °C by using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [39].

Catalase (CAT) Estimation

Briefly, 0.05 ml of PMS (10% *w*/*v*), 1.95 ml of phosphate buffer (0.1 M, PH 7.4), and 1.0 ml of H_2O_2 (0.019 M) were pipetted off and mixed in a fresh autoclaved calibrated glass tube to make total assay mixture of 3.0 ml. Changes in absorbance were recorded spectrophotometrically at 240 nm. Catalase activity was calculated as nanomole H_2O_2 consumed per minute per milligram of protein [40].

Superoxide Dismutase (SOD) Estimation

Briefly, 0.1 ml of PMS (10% *w*/*v*), 3 ml of Tris HCl buffer (PH 8.5), and 25 μl of pyrogallol (24 mM) were pipetted off and mixed in a fresh autoclaved calibrated glass tube. Changes in absorbance were recorded at 1-min intervals for every 3 min spectrophotometrically at 420 nm. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation [41].

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. The data was represented as mean ± SEM. $P < 0.05$ was considered as significant in all the cases.

Results

Effect of Raloxifene, Fluoxetine, Bromocriptine, and their Combinations on Pilocarpine-Induced Seizure Severity in Mice

PILO (300 mg/kg, i.p) induced severe seizures in mice. Monotherapy with low doses of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) significantly ($p < 0.05$) reduced seizure severity score, while no such effect was observed with their corresponding higher doses, i.e., raloxifene (8 mg/kg) and bromocriptine (10 mg/kg). Also, fluoxetine (14 and 22 mg/kg) and its duo drug combinations each with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) were devoid of anti-seizure effects. The combination of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) rendered no additional therapeutic benefit over individual drugs. However, interestingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) exhibited synergism in abrogation of PILO-induced seizures ($p < 0.001$), and an anti-seizure effect greater than observed with sodium valproate ($p < 0.01$), a standard AED, was exhibited (Fig. 1).

Effect of Raloxifene, Fluoxetine, Bromocriptine, and their Combinations on Hippocampal NPY Levels in Mice

PILO (300 mg/kg, i.p) upsurged NPY levels by 20.01% ($p < 0.05$) in the hippocampus. NPY levels were further raised by 29.71 and 31.25% ($p < 0.05$, $p < 0.01$) upon raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) administration, while their corresponding higher doses, i.e., raloxifene (8 mg/kg) and bromocriptine (10 mg/kg) insignificantly elevated hippocampal NPY levels. The minuscule elevation of NPY levels was also observed with fluoxetine (14 and 22 mg/kg) and its combination with each of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg). Low-dose combination of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) inflated NPY levels by 31.52%, ($p < 0.01$), and an effect similar to monotherapy with bromocriptine (6 mg/kg) was observed. However, interestingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) exhibited 100% escalation in NPY levels ($p < 0.001$), which was greater than observed with sodium valproate (300 mg/kg) (31.80%; $p < 0.01$) (Fig. 2).

Effect of Raloxifene, Fluoxetine, Bromocriptine, and their Combinations on Hippocampal Oxidative Stress in Mice

Protein Levels

PILO (300 mg/kg, i.p) lured decrease in hippocampal protein levels. Administration of raloxifene (4 mg/kg), bromocriptine (6 mg/kg), and their combination significantly ($p < 0.05$) resisted abatement in protein levels. Captivatingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) also significantly ($p < 0.001$) resisted alleviation in protein levels, and an effect greater than standard AED, i.e., sodium valproate ($p < 0.01$), was surmountable, while remaining drug(s) and their combinations failed to resist such change (Fig. 3).

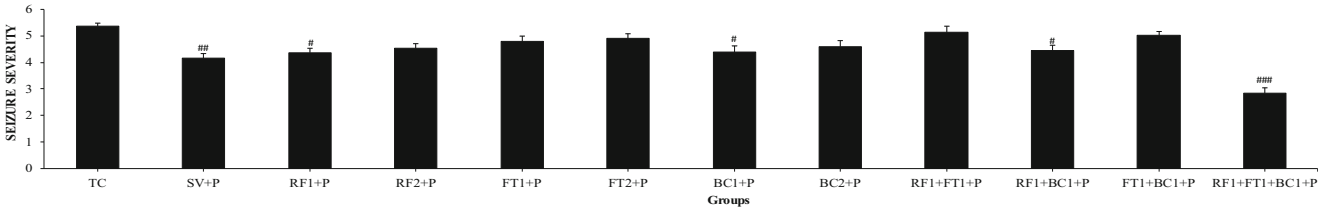


Fig. 1 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations against PILO-induced seizure severity in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of 10 mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

TBARS

Pilocarpine (300 mg/kg, i.p) impelled increase in hippocampal MDA levels. Administration of raloxifene (4 mg/kg), bromocriptine (6 mg/kg), and their combination significantly ($p < 0.05$) dwindled MDA levels. Engrossingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) also significantly ($p < 0.001$) attenuated MDA levels, and an effect greater than sodium valproate ($P < 0.01$) was surmountable, while remaining drug(s) and their combinations failed to resist such change (Fig. 4).

Catalase Levels

Pilocarpine (300 mg/kg, i.p) magnified hippocampal catalase levels. Administration of raloxifene (4 mg/kg), bromocriptine (6 mg/kg), and their combination further significantly ($p < 0.05$) raised catalase levels. Engagingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) furthermore significantly ($p < 0.001$) escalated catalase levels. The said combination increased catalase levels more than standard AED, i.e., sodium valproate, while remaining drug(s) and their combinations failed to significantly contribute to the variation (Fig. 5).

Superoxide Dismutase (SOD) Levels

Pilocarpine (300 mg/kg) induced insignificant curtailment in hippocampal SOD levels. Hence, all drugs and their combinations also exhibited insignificant augmentation in SOD levels (Fig. 6).

Effect of Raloxifene, Fluoxetine, Bromocriptine, and their Combinations on Pilocarpine-Induced Hippocampal Neuronal Damage in Mice

Hippocampal scrutiny of normal mice administered with vehicle (2% Tween 80) displayed normal size, shape, and regularly ordered compact array of neuronal cells, with prominent dark-colored nucleus in all hippocampal subregions. In contrast, PILO (300 mg/kg) administration revealed marked effects of SE in the form of comprehensive loss of pyramidal neurons in CA1, CA2, and CA3 regions, while extensive neuronal loss and gross neuronal shrinkage were palpable in the DG region. Treatment with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) protected against neurodegeneration as discernible from mild apoptosis in CA1, CA2, and CA3 regions. However, their combination bestowed no additional benefit than individual drugs, while their corresponding higher doses, i.e., raloxifene (8 mg/kg) and bromocriptine (10 mg/kg), replenished meager neuroprotection. Fluoxetine (14 mg/kg and 22 mg/kg) and its low-dose combination each with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) failed to produce neuroprotection, apparent from boundless loss of neurons in CA1, CA2, and CA3 regions, while the DG region revealed large number of shrunken neurons. Amusively, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) was foreseen with overwhelming neuroprotective effect substantiated with mild

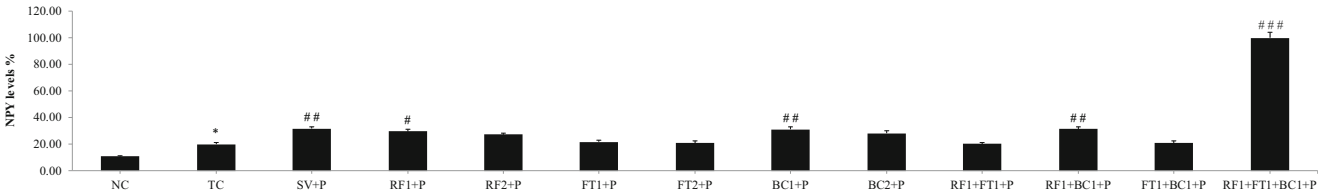


Fig. 2 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations on hippocampal NPY levels after PILO-induced SE in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of six mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

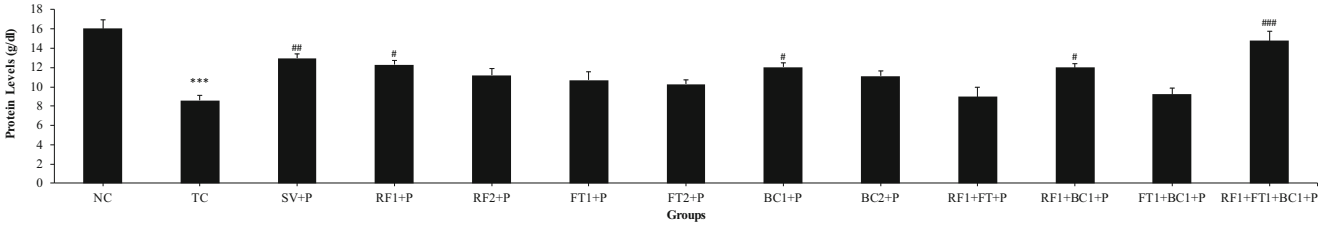


Fig. 3 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations on hippocampal protein levels post-PILO-induced SE in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of six mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

pyknosis in the CA1 region, while CA2, CA3, and DG revealed compactly arranged organized layer of pyramidal cells with prominent nucleus and very few pyknotic neurons. The neuroprotective effect of triple drug combination was much superior than sodium valproate (Figs. 7 and 8).

Discussion

The present AEDs pose several problems in the management of seizures owing to their meager neuroprotective potential, adverse effects on the bone, detrimental effects on cognitive function, chronic toxicity, drug interactions, side effects including aggression, agitation, and irritability, and sometimes exacerbation of seizures [42, 43]. Long-term outcome studies [3] and randomized trials [44–47] suggest that fewer than 50% of patients become seizure-free on the first AED. Even prognosis and efficacy of new AEDs are discouraging, and no AED has been found to possess exemplary properties [48, 49]. The probable choices of AEDs alone or in combination are so innumerable that often it is not possible for a prescriber to try every permutation within a single lifetime. The introduction of newer AEDS to the established drugs brings their total number up to around 20 for use in the common epilepsies [50]. This qualifies the likelihood possibility of about 200 duo-therapies or more than 1000 combinations of three AEDs. Most patients with refractory epilepsy take two, three, or even four AEDs [50]. However, the experimental and clinical evidence in support of "rational polytherapy" is sparse, with only the combinations of topiramate each with lamotrigine, gabapentin and felbamate, and sodium valproate with lamotrigine described as reliable synergistic combinations, while the latter combination is also fortified with human data [50, 51]. However, increase in the practicability of intensified adverse effects even with such combinations cannot be abandoned [51]. Much to the contrary, the combination of carbamazepine with lamotrigine was found to be antagonistic involving pharmacodynamic mechanism, resulting in extravagant drug loading [52], while brimming teratogenic risks are reported with combination of phenobarbital, phenytoin and primidone, carbamazepine, sodium valproate, and phenobarbital with or without phenytoin (i.e., cramping use in pregnant women) [53]. Thus, epileptic disorders have remained a serious challenge for researchers; the reason we could ascribe is that we are missing the right therapeutic target.

It has been hypothesized that combining two or more AEDs with different MOA results in heightening of their anticonvulsant effects. To ratify this hypothesis, a study was carried out to ascertain synergistic interaction between duo and triple drug combinations of raloxifene (anti-osteoporosis drug), fluoxetine (antidepressant drug), and bromocriptine (dopamine (D2) receptor agonist). The comparison was figured with individual drugs and standard AED, i.e., sodium valproate. Results of this study clearly stipulated the superlative activation of hippocampal NPY signaling pathway by triple drug combination of raloxifene with fluoxetine and bromocriptine and subsequent mitigation of PILO-induced SE and TLE. The anticonvulsant effect of the said combination was better than sodium valproate, which was further confirmed from histomorphological findings and measurement

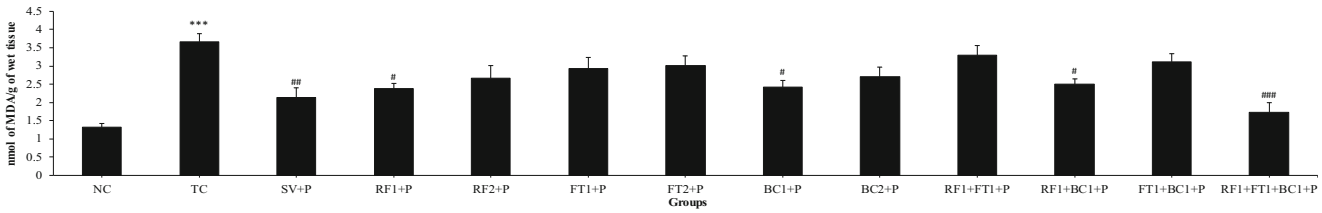


Fig. 4 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations on hippocampal lipid peroxidation levels following PILO-induced SE in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of six mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

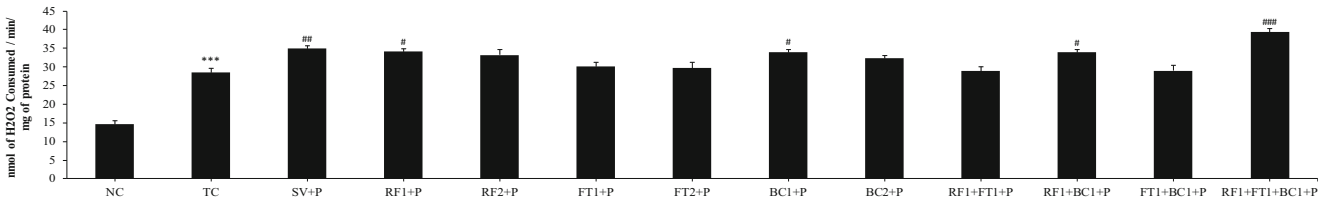


Fig. 5 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations on hippocampal catalase (CAT) levels post-PILO-induced SE in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of six mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

of oxidative stress indices. Hence, there are compelling reasons to state that the present study has significant clinical potential in patients with SE, TLE, and refractory epilepsies.

In agreement with previous literature, raloxifene (4 mg/kg) was found to be effective in restricting chemically (PILO) induced seizures as evidenced from scaling down of seizure severity score [19]. Raloxifene is delineated to act via regulating opiate and GABAergic neurotransmission by modulating the levels of β-endorphin and neuroactive steroids. Chronic raloxifene administration to postmenopausal women increases plasma levels of β-endorphin and tetrahydroprogesterone (allopregnanolone), an anxiolytic metabolite of progesterone that modulates GABAA receptors, and exerts anti-epileptic effects [54, 55]. Bromocriptine (6 mg/kg) was also found to mitigate PILO-induced seizures, which is in alliance with previous literature demonstrating anti-epileptic effects of bromocriptine, in case of self-induced, drug-resistant epilepsy [56] and neuroprotective efficacy against KA-induced brain damage [57]. In clinical studies, bromocriptine has been shown to reduce seizure severity in TLE patients affected by pituitary prolactinomas [58]. Interestingly, prolonged use of bromocriptine treatment was without any severe side effects [58]. Exceptionally, higher doses of raloxifene (8 mg/kg) and bromocriptine (10 mg/kg) unveiled no significant effect on attenuating seizure severity, while their low-dose combination rendered no additional therapeutic benefit than individual drugs at lower doses. Fluoxetine (14 and 22 mg/kg) was also devoid of anti-seizure effect although recounted in literature to suppress maximal electroshock-induced seizures in rats [59], pentylenetetrazole-induced convulsions in mice, focally evoked limbic motor seizures in rats [59], and audiogenic seizures in genetically epilepsy-prone DBA/2J or genetically epilepsy-prone rats (GEPRs) [60]. Fluoxetine has also been reported to significantly enhance the doses of picrotoxin needed to produce tonic hindlimb extension and death in unstressed and swim-stressed mice, and in stressed mice also the dose of picrotoxin producing running/bouncing clonus [61]. It also raised the density of GABAA receptors in DG, CA1, and CA2 regions of lithium–PILO-treated rats, and indicative of anti-epileptic effect [7]. However, failure of fluoxetine (14 and 22 mg/kg) to restrict PILO-induced seizures suggests that pathophysiological involvement of brain is different in the case of seizures involving chemical or electric shock or in genetically epilepsy prone rats. The combination of fluoxetine (14 mg/kg) either with raloxifene (4 mg/kg) or bromocriptine (6 mg/kg) tended to inverse their anti-seizure effects, whilst in literature, fluoxetine has been reported to enhance the anticonvulsive action of sodium valproate, but not that of ethosuximide against PTZ-induced clonic convulsions in mice [62]. The reason behind this anomaly needs to be investigated. Nevertheless, triple drug low-dose combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) exhibited synergism in abrogation of PILO-induced seizures ($p < 0.001$). Here, fluoxetine (14 mg/kg) augmented the anti-seizure effect of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg), and an antiepileptic effect better

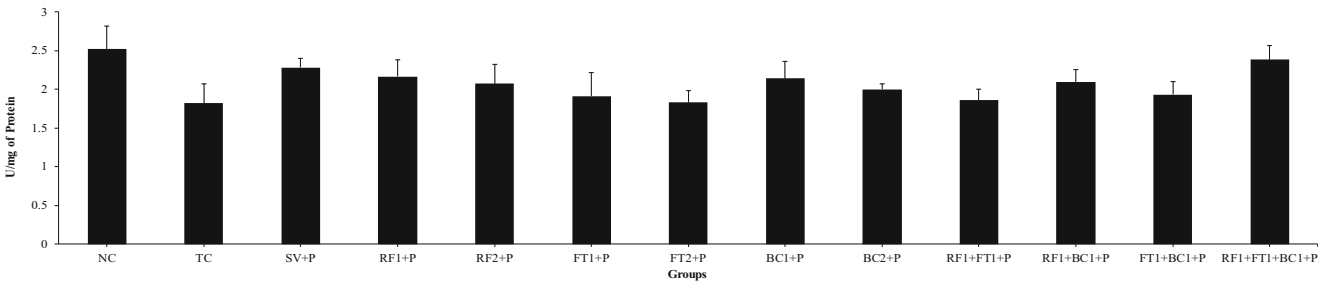


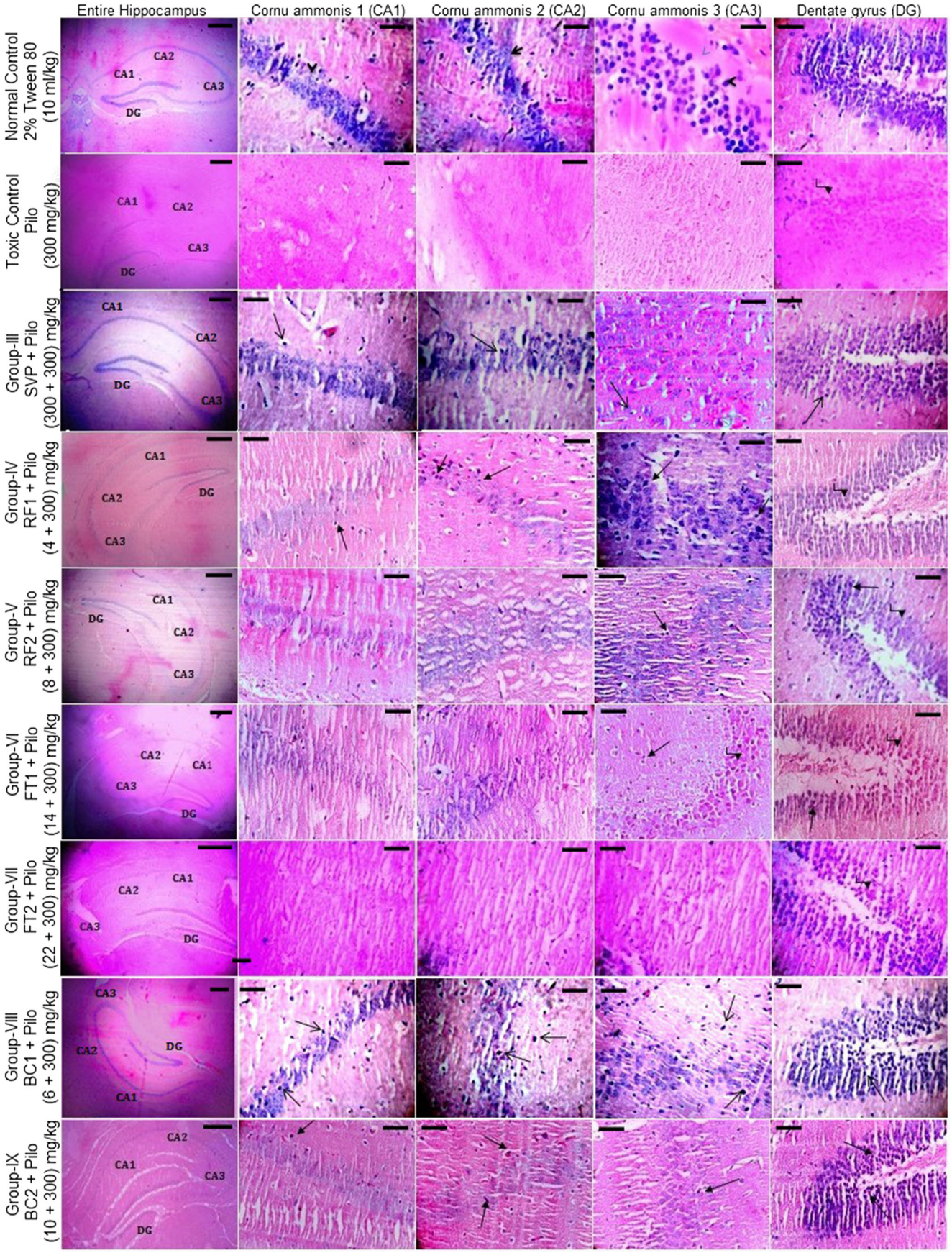
Fig. 6 Effect of RF (4 and 8 mg/kg), FT (14 and 22 mg/kg), BC (6 and 10 mg/kg), and their combinations on hippocampal superoxide dismutase (SOD) levels after PILO-induced SE in mice using SV (300 mg/kg) as standard AED. Data is represented as mean ± SEM of six mice per group. Analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (compared with normal control group). $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (compared with toxic control group)

than sodium valproate ($p < 0.01$) was contemplated. The decrease in seizure severity is related to delay in initiation of seizures, as well as reduction in the number of occurrences of tonic–clonic seizures induced by PILO. This might suggest mitigation of M1 mAChR as well as *N*-methyl-D-aspartate (NMDA) receptor activation by the said drug(s), in particular triple drug combination. Further, it has been shown that duration of SE during the initial insult is critical to the duration of latent period and subsequent development of SRSs and brain damage. Lemos and Cavalheiro (1995) conducted first study of its kind where adult male Wistar rats were injected with a single dose of PILO (300–320 mg/kg, i.p.) followed by a combined diazepam (10 mg/kg) and pentobarbital (30 mg/kg) treatment, and a progressive increase in the mean latency to the first spontaneous seizure and a decrease in seizure frequency in animals with shorter SE (1 and 2 h) were observed along with less severe neuropathological alterations in the animals. Finally, study ended up showing that animals experiencing only 30 min of SE did not develop SRSs [5]. On the contrary, Klitgaard et al. (2002) observed SRSs in rats that had experienced 30 min SE and had terminated SE by injecting diazepam (10 mg/kg, i.v.) [63]. Nevertheless, the probability, latency, and severity of SRSs and of neuropathological change enfeeblements with depreciating SE duration [5, 64]. Hence, if a drug abolishes the onset of SE or restricts its duration to few minutes, the subsequent development of chronic epilepsy (TLE) can be prevented. Yet, it can be comprehended that triple drug combination prevents SE as well as TLE.

Neuropeptide-Y (NPY) is an endogenous neuropeptide widely distributed across rat brain with eminent expression in the hilar region of the hippocampus, arcuate nucleus of hypothalamus, and cerebral cortex [65]. Anti-seizure effect of NPY has been reported against absence seizures in Genetic Absence Epilepsy Rats of Strasbourg (GAERS) via modulating pathological oscillatory thalamocortical activity [66]. In our study, PILO-induced seizures with a concomitant increase in hippocampal NPY levels, which is in agreement with previous report of Dube 2007, who reported upregulation of hippocampal NPY expression after experimentally induced febrile seizures (FS) and concluded that inhibitory actions of NPY, released after seizures, exert a protective effect that reduces the risk of seizure recurrence in the brain. The hippocampal NPY was further escorted with significant (29.71%, 31.25%; $p < 0.05$, $p < 0.01$) rise from chronic treatment with low doses of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) alone, while their combination rendered an effect similar to bromocriptine (6 mg/kg). This finding braces behavioral result from this study that dual drug combination of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) is no better than individual drugs. An insignificant elevation of hippocampal NPY levels was revealed with fluoxetine (14 mg/kg and 22 mg/kg) alone and its combination each with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg). It also emphasizes results from behavioral study that fluoxetine (14 mg/kg) inverses therapeutic potential each of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg). Yet, though, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) displayed an enormous upsurge (100%; $p < 0.001$) in hippocampal NPY levels. Further, our study corroborated with the earlier experimental reports indicating that sodium valproate boosts NPY levels [67]. However, the rise in NPY levels observed with triple drug combination was substantially higher than sodium valproate (31.80%, $p < 0.01$).

The miraculous effect of antagonist fluoxetine (14 mg/kg) on seizure severity, neurodegeneration, oxidative stress, and hippocampal NPY levels, in combination with agonists raloxifene (4 mg/kg) and bromocriptine (6 mg/kg), is charismatic, as synergism was not observed with low-dose combination of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg). The mechanistic approach hypothesized for therapeutic potential of triple drug combination involves elevated synthesis and release of NPY (estrogen induces NPY formation through BDNF-TrkB signaling, which was predicted to mimic with raloxifene, a component of triple drug combination) and activation of hippocampal NPY (Y2) receptors (presynaptic) and NPY (Y5) receptors (postsynaptic). Invigoration of former receptor inhibits glutamate release at CA1-CA3 synapse via inhibiting N-type, P/Q type, and VGCC at presynaptic neuronal terminal, while incitement of latter receptor advances coupling with Gi/o—inhibiting AC, activating MAPK—opening of K^+ channels and obstruction of excitatory hilar-CA3 synaptic transmission, leading to synergistic anti-epileptic and neuroprotective effects [23, 68, 69]. Taken together, NPY is perhaps co-released with serotonin and dopamine and synergizes with them in mediation of antiepileptic and neuroprotective effects. The co-transmission and synergism of NPY with NA had formerly been reported [70].

Oxidative stress is defined as deficit between generation and neutralization of ROS species such as hydroxyl radical ($HO^{\cdot}$), superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($HOO^{\cdot}$), and high amounts of nitric oxide ($NO^{\cdot}$) and its derivative reactive nitrogen species (RNS) [71], which play an important role in the generation and progression of epileptic seizures [72, 73]. Anomalous levels of oxidant and antioxidant enzymes have been reputed in children with refractory epilepsies [74]. Inflation of hippocampal oxidative stress in mice on PILO administration had been disclosed [75]. In the present study too, there was significant ($p < 0.001$) elevations in hippocampal MDA (a marker of lipid peroxidation) levels in PILO-SE mice, which is in conformity with previous reports [76, 77]. Raloxifene (4 mg/kg), bromocriptine (6 mg/kg), and their combination facilitated significant ($p < 0.05$) reduction in MDA levels, while fluoxetine (14 and 22 mg/kg) alone and its combination each with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) did not significantly


Entire Hippocampus
Cornu ammonis 1 (CA1)
Cornu ammonis 2 (CA2)
Cornu ammonis 3 (CA3)
Dentate gyrus (DG)
Normal Control 2% Tween 80 (10 ml/kg)
Toxic Control Pilo (300 mg/kg)
Group-III SVP + Pilo (300 + 300) mg/kg
Group-IV RF1 + Pilo (4 + 300) mg/kg
Group-V RF2 + Pilo (8 + 300) mg/kg
Group-VI FT1 + Pilo (14 + 300) mg/kg
Group-VII FT2 + Pilo (22 + 300) mg/kg
Group-VIII BC1 + Pilo (6 + 300) mg/kg
Group-IX BC2 + Pilo (10 + 300) mg/kg
CA1
CA2
CA3
DG

◀ **Fig. 7** Representative photomicrographs depicting H/E stained hippocampal sections of Swiss albino mice comparing PILO-induced neuronal damage in different monotherapy groups. Scale bar of 4X images (EH) = 0.5mm (500 μm) and 40X images (CA1, CA2, CA3 & DG) = 0.05 mm (50 μm). Hippocampal transverse section of mice observed 24 h following PILO (300 mg/kg, i.p) delineates complete loss of pyramidal neurons in CA1, CA2, and CA3 regions, while extensive neuronal loss and gross neuronal shrinkage (↳) is evident in DG region (Group II). Nominal loss of healthy neurons, mild pyknosis (↓) is discerned in CA1 and CA2 regions, while scattered arrangement of healthy neurons in CA3 region along with grossly normal morphology of neurons is ascertained in DG region upon sodium valproate administration (group III). Treatment with raloxifene (4 mg/kg) prevented morphological changes induced by PILO, as evidenced from mild apoptosis in CA1, CA2, and CA3 regions along with failure to resist gross neuronal shrinkage in the DG region (group IV). However, raloxifene (8 mg/kg) displayed minimal influence on deranged morphology (group V). Treatment with fluoxetine (14 and 22 mg/kg) rather aggravated PILO-induced neuronal damage (groups VI and VII). Bromocriptine (6 mg/kg) also exhibited neuroprotective effect evidenced with mild pyknosis in CA1 CA2, CA3, and DG regions (group VIII), while with bromocriptine (10 mg/kg), neuroprotective effect was not substantial (group IX)

alter raised MDA levels. However, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) showed exorbitant potential in diminishing ($p < 0.001$) MDA levels.

Catalase (CAT) is an enzyme present mainly in mitochondria and peroxisomes that catalyzes the decomposition of hydrogen peroxide to water and oxygen [78]. A significant rise in hippocampal CAT levels was observed in PILO-SE mice, which is in agreement with previous literature [79, 80]. It has been documented that increase in ROS generated because of PILO-SE that leads to increase in CAT as a compensatory mechanism [81]. Further significant ($p < 0.05$) escalation in CAT levels was observed upon administration of raloxifene (4 mg/kg), bromocriptine (6 mg/kg), and their combination. However, fluoxetine (14 and 22 mg/kg) and its combination each with raloxifene (4 mg/kg) and bromocriptine (6 mg/kg) failed to signify any effect. Convincingly, triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) showed exorbitant potential in sprouting ($p < 0.001$) CAT levels.

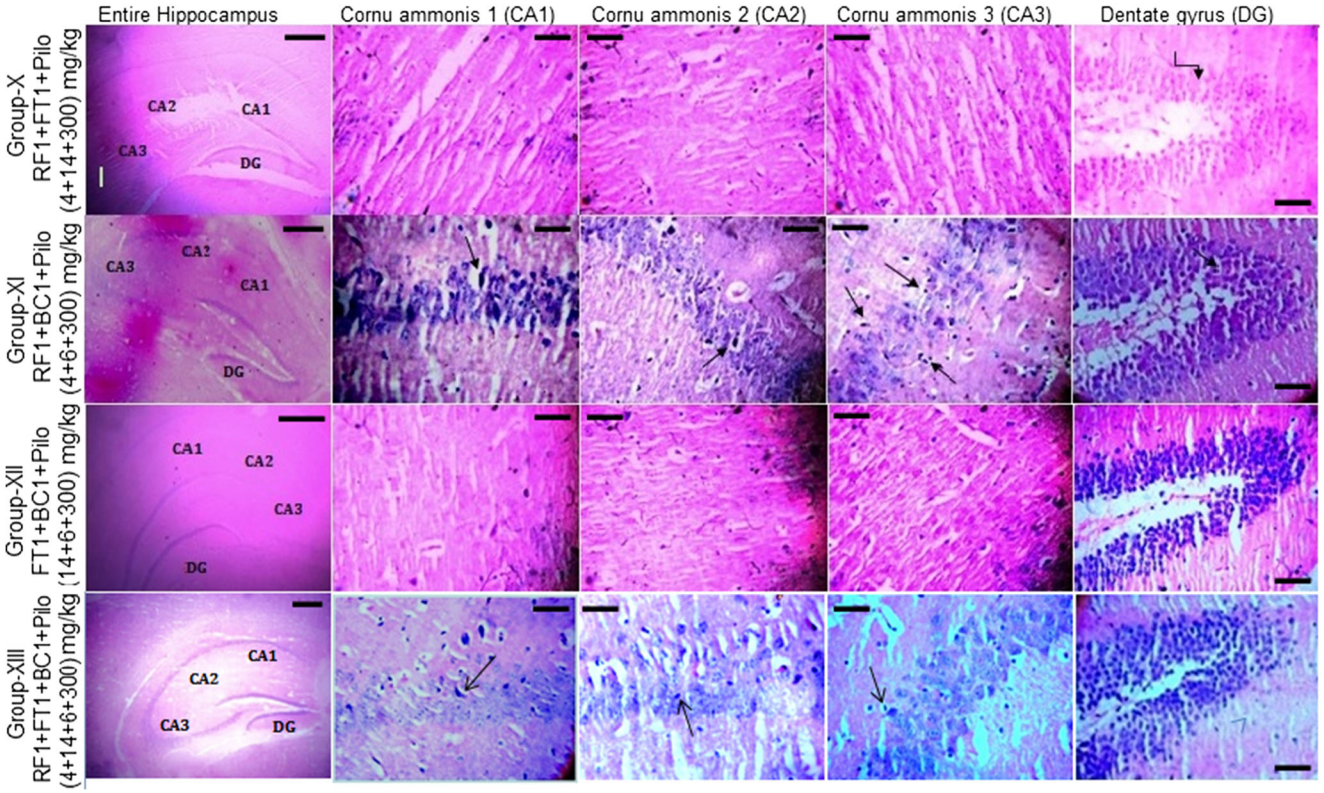


Fig. 8 Representative photomicrographs depicting H/E stained hippocampal sections of Swiss albino mice comparing PILO-induced neuronal damage in different combination treatment groups. Scale bar of 4X images (EH) = 0.5mm (500 μm) and 40X images (CA1, CA2, CA3 & DG) = 0.05mm (50 μm). Hippocampal transverse section of mice reveals inversion of neuroprotective effect of raloxifene (4 mg/kg) when combined with fluoxetine (14 mg/kg) (group X). The combination of raloxifene (4 mg/kg) with bromocriptine (6 mg/kg) retrieved PILO-induced damage as observed by mild pyknosis (↓) in CA1, CA2, CA3, and DG (group XI). Again, the combination of fluoxetine (14 mg/kg) with bromocriptine (6 mg/kg) rather peeved PILO-induced neuronal damage (group XII). Yet, though triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) effectively prevented the morphological changes induced by PILO as evident by intact neuronal structure with very few pyknotic nuclei in the CA1, CA2, and CA3 regions (group XIII)

The SOD enzyme plays indispensible role in detoxifying superoxide anions in hydrogen peroxide and oxygen. It can scavenge the superoxide anion by catalyzing it to H_2O_2 and O_2, which prevents the oxidative stress-induced cellular damage [82]. In the present study, there was no significant change in SOD activities in PILO-SE mice. Hence, all the treatment groups failed to show any significant alteration in SOD levels. Thusly, SOD is not likely the key enzyme involved in intracellular antioxidant defense system of the brain against seizures (i.e., is not engaged in a crucial role against oxidative stress induced with PILO-SE). The present results further suggest that triple drug combination has superlative antioxidative effect via modulating levels of MDA and catalase than via superoxide dismutase activity, compared to other treatment groups and could well restrict oxidative stress-induced hyperpolarizations and neuronal damage.

After an initial insult preceding epileptic seizures, the change in neuronal network functions is set in motion, leading to onset of chronic seizures in the presence of both neuronal damage and over excitation [83]. Neurodegeneration involves change in receptor properties of neurons thereby critically altering neuronal response towards anti-epileptic drugs leading to clinical onset of pharmacoresistant seizures sometimes accompanied by cognitive decline [84]. Unfortunately, not all AEDs exhibit neuroprotective potential. Drugs like barbiturates and benzodiazepines have been reported to be neutral or even promote neurodegeneration especially on immature brain tissue, although novel AEDs especially lamotrigine, tiagabine, and topiramate seem out to be neuroprotectants [85]. However, extrapolation of data from animals to humans presents several challenges, as most of the studies concerning neuroprotective effects of AEDs have been conducted in ischemic models of neuronal injury. Hence, therapeutic interventions, which would counteract neurodegeneration along with hyper-synchronous neuronal depolarizations, can halt the progression of epileptic seizures. Hippocampal neuronal loss in patients with epilepsy especially those with generalized epileptic seizures and long duration of the epileptic disorder has been reported [86]. Histopathological examination of normal and hippocampal sections from PILO (300 mg/kg) administered mice revealed marked effects of SE in the form of extensive neuronal death in CA1, CA2, and CA3 areas, hallmark of extensive neuronal pyknosis, with extensive shrinkage of neurons in the DG region, which is in agreement with previous studies [87, 88]. These changes were markedly improved by triple drug combination of raloxifene (4 mg/kg) with fluoxetine (14 mg/kg) and bromocriptine (6 mg/kg) and were bolstered up to a lesser extent by low doses of raloxifene (4 mg/kg) and bromocriptine (6 mg/kg), while their corresponding higher doses exhibited no such neuroprotective effect. Further, the low-dose combination of raloxifene (4 mg/kg) with bromocriptine (6 mg/kg) rendered no additional neuroprotective effect than the individual drugs at low doses. The sodium valproate seemed out to be neuroprotectant also, but less so than triple drug combination.

Conclusions

The present study recites that synergistic low-dose triple drug combination of raloxifene with fluoxetine and bromocriptine as optimum strategy against chemically induced seizures and neurodegeneration enriched with meager adverse effects and malformation rate. The study further emphasizes that optimum level of serotonin and dopamine acts synergistically with NPY against deteriorating process of epileptogenesis.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

References

1. Puttachary S, Sharma S, Stark S, Thippeswamy T (2015) Seizure-induced oxidative stress in temporal lobe epilepsy. Biomed Res Int 2015:745613–745620. https://doi.org/10.1155/2015/745613
2. Sankaraneni R, Lachhwani D (2015) Antiepileptic drugs—a review. Pediatr Ann 44:e36–e42. https://doi.org/10.3928/00904481-20150203-10
3. Kwan P, Brodie MJ (2000) Early identification of refractory epilepsy. N Engl J Med 342:314–319. https://doi.org/10.1056/NEJM200002033420503
4. Wieser H-G, ILAE Commission on Neurosurgery of Epilepsy (2004) ILAE commission report. Mesial temporal lobe epilepsy with hippocampal sclerosis. Epilepsia 45:695–714. https://doi.org/10.1111/j.0013-9580.2004.09004.x
5. Lemos T, Cavalheiro EA (1995) Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats. Exp Brain Res 102:423–428
6. Starr MS (1996) The role of dopamine in epilepsy. Synapse 22: 159–194 . doi: https://doi.org/10.1002/(SICI)1098-2396(199602)22:2<159::AID-SYN8>3.0.CO;2-C
7. Shiha AA, de Cristóbal J, Delgado M, Fernández de la Rosa R, Bascuñana P, Pozo MA, García-García L (2015) Subacute administration of fluoxetine prevents short-term brain hypometabolism and reduces brain damage markers induced by the lithium-pilocarpine model of epilepsy in rats. Brain Res Bull 111:36–47. https://doi.org/10.1016/j.brainresbull.2014.12.009
8. Leander JD (1992) Fluoxetine, a selective serotonin-uptake inhibitor, enhances the anticonvulsant effects of phenytoin, carbamazepine, and ameltolide (LY201116). Epilepsia 33:573–576
9. Browning RA, Wood AV, Merrill MA, Dailey JW, Jobe PC (1997) Enhancement of the anticonvulsant effect of fluoxetine following blockade of 5-HT1A receptors. Eur J Pharmacol 336:1–6. https://doi.org/10.1016/S0014-2999(97)01215-6

10. Bozzi Y, Vallone D, Borrelli E (2000) Neuroprotective role of dopamine against hippocampal cell death. J Neurosci 20:8643–8649
11. Farjo IB, McQueen JK (1979) Dopamine agonists and cobalt-induced epilepsy in the rat. Br J Pharmacol 67:353–360
12. O'Neill MJ, Hicks CA, Ward MA et al (1998) Dopamine D2 receptor agonists protect against ischaemia-induced hippocampal neurodegeneration in global cerebral ischaemia. Eur J Pharmacol 352:37–46
13. Rocca WA, Grossardt BR, Shuster LT (2011) Oophorectomy, menopause, estrogen treatment, and cognitive aging: clinical evidence for a window of opportunity. Brain Res 1379:188–198. https://doi.org/10.1016/j.brainres.2010.10.031
14. Lange SC, Julien RM (1978) Re-evaluation of estrogen-induced cortical and thalamic paroxysmal EEG activity in the cat. Electroencephalogr Clin Neurophysiol 44:94–103
15. Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW (1999) 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol 81:925–929. https://doi.org/10.1152/jn.1999.81.2.925
16. Ibrahim NK, Hortobagyi GN (1999) The evolving role of specific estrogen receptor modulators (SERMs). Surg Oncol 8:103–123
17. Arevalo MA, Santos-Galindo M, Lagunas N, Azcoitia I, Garcia-Segura LM (2011) Selective estrogen receptor modulators as brain therapeutic agents. J Mol Endocrinol 46:R1–R9. https://doi.org/10.1677/JME-10-0122
18. Ciriza I, Carrero P, Azcoitia I et al (2004) Selective estrogen receptor modulators protect hippocampal neurons from kainic acid excitotoxicity: differences with the effect of estradiol. J Neurobiol 61:209–221. https://doi.org/10.1002/neu.20043
19. Pottoo FH, Bhowmik M, Vohora D (2014) Raloxifene protects against seizures and neurodegeneration in a mouse model mimicking epilepsy in postmenopausal woman. Eur J Pharm Sci 65:167–173. https://doi.org/10.1016/j.ejps.2014.09.002
20. Scharfman HE, Malthankar-Phatak GH, Friedman D, Pearce P, McCloskey DP, Harden CL, MacLusky NJ (2009) A rat model of epilepsy in women: a tool to study physiological interactions between endocrine systems and seizures. Endocrinology 150:4437–4442. https://doi.org/10.1210/en.2009-0135
21. Brothers SP, Wahlestedt C (2010) Therapeutic potential of neuropeptide Y (NPY) receptor ligands. EMBO Mol Med 2:429–439. https://doi.org/10.1002/emmm.201000100
22. Velísková J, Velísek L (2007) Beta-estradiol increases dentate gyrus inhibition in female rats via augmentation of hilar neuropeptide Y. J Neurosci 27:6054–6063. https://doi.org/10.1523/JNEUROSCI.0366-07.2007
23. Qian J, Colmers WF, Saggau P (1997) Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca2+entry. J Neurosci 17:8169–8177. https://doi.org/10.1523/JNEUROSCI.17-21-08169.1997
24. Vezzani A, Michalkiewicz M, Michalkiewicz T, Moneta D, Ravizza T, Richichi C, Aliprandi M, Mulé F et al (2002) Seizure susceptibility and epileptogenesis are decreased in transgenic rats overexpressing neuropeptide Y. Neuroscience 110:237–243
25. Weinshenker D, Szot P, Miller NS, Rust NC, Hohmann JG, Pyati U, White SS, Palmiter RD (2001) Genetic comparison of seizure control by norepinephrine and neuropeptide Y. J Neurosci 21:7764–7769
26. May RM, Tabatadze N, Czech MM, Woolley CS (2014) Estradiol regulates large dense core vesicles in the hippocampus of adult female rats. Brain Struct Funct 219:1947–1954. https://doi.org/10.1007/s00429-013-0614-7
27. Nigar S, Pottoo FH, Tabassum N, Verma S, Javed M (2016) Molecular insights into the role of inflammation and oxidative stress in epilepsy. J Adv Med Pharm Sci 10:1–9
28. White HS, Smith MD, Wilcox KS (2007) Mechanisms of action of antiepileptic drugs. Int Rev Neurobiol 81:85–110. https://doi.org/10.1016/S0074-7742(06)81006-8
29. Zoratti M, Szabò I (1995) The mitochondrial permeability transition. Biochim Biophys Acta 1241:139–176
30. Chepurnov SA, Suleĭmanova EM, Guliaev MV, Abbasova KR, Pirogov IuA, Chepurnova NE (2012) Neuroprotection in epilepsy. Usp Fiziol Nauk 43:55–71
31. Suda S, Ueda M, Nito C, Nishiyama Y, Okubo S, Abe A, Aoki J, Suzuki K et al (2015) Valproic acid ameliorates ischemic brain injury in hyperglycemic rats with permanent middle cerebral occlusion. Brain Res 1606:1–8. https://doi.org/10.1016/j.brainres.2015.02.013
32. Mao X-Y, Cao Y-G, Ji Z, Zhou HH, Liu ZQ, Sun HL (2015) Topiramate protects against glutamate excitotoxicity via activating BDNF/TrkB-dependent ERK pathway in rodent hippocampal neurons. Prog Neuro-Psychopharmacol Biol Psychiatry 60:11–17. https://doi.org/10.1016/j.pnpbp.2015.01.015
33. Delmas PD, Ensrud KE, Adachi JD, Harper KD, Sarkar S, Gennari C, Reginster JY, Pols HA et al (2002) Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial. J Clin Endocrinol Metab 87:3609–3617. https://doi.org/10.1210/jcem.87.8.8750
34. Kobayashi K, Ikeda Y, Sakai A, Yamasaki N, Haneda E, Miyakawa T, Suzuki H (2010) Reversal of hippocampal neuronal maturation by serotonergic antidepressants. Proc Natl Acad Sci U S A 107:8434–8439. https://doi.org/10.1073/pnas.0912690107
35. Mohanasundari M, Srinivasan MS, Sethupathy S, Sabesan M (2006) Enhanced neuroprotective effect by combination of bromocriptine and Hypericum perforatum extract against MPTP-induced neurotoxicity in mice. J Neurol Sci 249:140–144. https://doi.org/10.1016/j.jns.2006.06.018
36. Mazzuferi M, Kumar G, Rospo C, Kaminski RM (2012) Rapid epileptogenesis in the mouse pilocarpine model: video-EEG, pharmacokinetic and histopathological characterization. Exp Neurol 238:156–167. https://doi.org/10.1016/j.expneurol.2012.08.022
37. Kim YB, Ryu JK, Lee HJ, Lim IJ, Park D, Lee MC, Kim SU (2010) Midkine, heparin-binding growth factor, blocks kainic acid-induced seizure and neuronal cell death in mouse hippocampus. BMC Neurosci 11:42. https://doi.org/10.1186/1471-2202-11-42
38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
39. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. https://doi.org/10.1016/0003-2697(79)90738-3
40. Maehly AC, Chance B (1954) The assay of catalases and peroxidases. Methods Biochem Anal 1:357–424
41. Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474
42. Reynolds EH, Shorvon SD (1981) Monotherapy or polytherapy for epilepsy? Epilepsia 22:1–10
43. Brodie MJ, Besag F, Ettinger AB, Mula M, Gobbi G, Comai S, Aldenkamp AP, Steinhoff BJ (2016) Epilepsy, antiepileptic drugs, and aggression: an evidence-based review. Pharmacol Rev 68:563–602. https://doi.org/10.1124/pr.115.012021
44. Mattson RH, Cramer JA, Collins JF, Group* the D of VAECSN 264 (1992) A comparison of valproate with carbamazepine for the treatment of complex partial seizures and secondarily generalized tonic–clonic seizures in adults. N Engl J Med 327:765–771. https://doi.org/10.1056/NEJM199209103271104
45. Richens A, Davidson DL, Cartlidge NE, Easter DJ (1994) A multicentre comparative trial of sodium valproate and

carbamazepine in adult onset epilepsy. Adult EPITEG Collaborative Group. J Neurol Neurosurg Psychiatry 57:682–687
46. Brodie MJ, Richens A, Yuen AW (1995) Double-blind comparison of lamotrigine and carbamazepine in newly diagnosed epilepsy. UK Lamotrigine/Carbamazepine Monotherapy Trial Group Lancet 345: 476–479
47. Beydoun A (1997) Monotherapy trials of new antiepileptic drugs. Epilepsia 38(Suppl 9):S21–S31
48. Franco V, French JA, Perucca E (2016) Challenges in the clinical development of new antiepileptic drugs. Pharmacol Res 103:95–104. https://doi.org/10.1016/j.phrs.2015.11.007
49. Gatti G, Bonomi I, Jannuzzi G, Perucca E (2000) The new antiepileptic drugs: pharmacological and clinical aspects. Curr Pharm Des 6:839–860
50. Brodie MJ, Sills GJ (2011) Combining antiepileptic drugs—rational polytherapy? Seizure 20:369–375. https://doi.org/10.1016/j.seizure.2011.01.004
51. Sarhan EM, Walker MC, Selai C (2016) Evidence for efficacy of combination of antiepileptic drugs in treatment of epilepsy. J Neurol Res 5:267–276. https://doi.org/10.14740/jnr.v5i6.356
52. Mani J (2013) Combination therapy in epilepsy: what, when, how and what not! J Assoc Physicians India 61:40–44
53. Etemad L, Moshiri M, Moallem SA (2012) Epilepsy drugs and effects on fetal development: potential mechanisms. J Res Med Sci 17:876–881
54. Bernardi F, Pluchino N, Stomati M et al (2003) CNS: sex steroids and SERMs. Ann N Y Acad Sci 997:378–388
55. Genazzani AR, Lombardi I, Borgioli G, di Bono I, Casarosa E, Gambacciani M, Palumbo M, Genazzani AD et al (2003) Adrenal function under long-term raloxifene administration. Gynecol Endocrinol 17:159–168
56. Clemens B (1988) Dopamine agonist treatment of self-induced pattern-sensitive epilepsy. A case report. Epilepsy Res 2:340–343. https://doi.org/10.1016/0920-1211(88)90044-7
57. Micale V, Incognito T, Ignoto A, Rampello L, Spartà M, Drago F (2006) Dopaminergic drugs may counteract behavioral and biochemical changes induced by models of brain injury. Eur Neuropsychopharmacol 16:195–203. https://doi.org/10.1016/j.euroneuro.2005.08.003
58. Deepak D, Daousi C, Javadpour M, MacFarlane IA (2007) Macroprolactinomas and epilepsy. Clin Endocrinol 66:503–507. https://doi.org/10.1111/j.1365-2265.2007.02759.x
59. Prendiville S, Gale K (1993) Anticonvulsant effect of fluoxetine on focally evoked limbic motor seizures in rats. Epilepsia 34:381–384. https://doi.org/10.1111/j.1528-1157.1993.tb02425.x
60. Dailey JW, Yan QS, Mishra PK, Burger RL, Jobe PC (1992) Effects of fluoxetine on convulsions and on brain serotonin as detected by microdialysis in genetically epilepsy-prone rats. J Pharmacol Exp Ther 260:533–540
61. Perićić D, Lazić J, Svob Strac D (2005) Anticonvulsant effects of acute and repeated fluoxetine treatment in unstressed and stressed mice. Brain Res 1033:90–95. https://doi.org/10.1016/j.brainres.2004.11.025
62. Borowicz KK, Piskorska B, Stępniak B, Czuczwar SJ (2012) Effects of fluoxetine on the anticonvulsant action of valproate and ethosuximide in mouse model of myoclonic convulsions. Ann Agric Environ Med 19:487–490
63. Klitgaard H, Matagne A, Vanneste-Goemaere J, Margineanu DG (2002) Pilocarpine-induced epileptogenesis in the rat: impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations. Epilepsy Res 51:93–107. https://doi.org/10.1016/S0920-1211(02)00099-2
64. Liu Z, Nagao T, Desjardins GC, Gloor P, Avoli M (1994) Quantitative evaluation of neuronal loss in the dorsal hippocampus in rats with long-term pilocarpine seizures. Epilepsy Res 17:237–247
65. Morris BJ (1989) Neuronal localisation of neuropeptide Y gene expression in rat brain. J Comp Neurol 290:358–368. https://doi.org/10.1002/cne.902900305
66. Stroud LM, O'Brien TJ, Jupp B et al (2005) Neuropeptide Y suppresses absence seizures in a genetic rat model. Brain Res 1033: 151–156. https://doi.org/10.1016/j.brainres.2004.11.022
67. Elms J, Powell KL, van Raay L, Dedeurwaerdere S, O'Brien TJ, Morris MJ (2013) Long-term valproate treatment increases brain neuropeptide Y expression and decreases seizure expression in a genetic rat model of absence epilepsy. PLoS One 8:e73505. https://doi.org/10.1371/journal.pone.0073505
68. Guo H, Castro PA, Palmiter RD, Baraban SC (2002) Y5 receptors mediate neuropeptide Y actions at excitatory synapses in area CA3 of the mouse hippocampus. J Neurophysiol 87:558–566. https://doi.org/10.1152/jn.00532.2001
69. van den Pol AN (2012) Neuropeptide transmission in brain circuits. Neuron 76:98–115. https://doi.org/10.1016/j.neuron.2012.09.014
70. Lundberg JM, Franco-Cereceda A, Hemsén A, Lacroix JS, Pernow J (1990) Pharmacology of noradrenaline and neuropeptide tyrosine (NPY)-mediated sympathetic cotransmission. Fundam Clin Pharmacol 4:373–391
71. Winyard PG, Moody CJ, Jacob C (2005) Oxidative activation of antioxidant defence. Trends Biochem Sci 30:453–461. https://doi.org/10.1016/j.tibs.2005.06.001
72. Hussein AM, Ghalwash M, Magdy K, Abulseoud OA (2016) Beta lactams antibiotic ceftriaxone modulates seizures, oxidative stress and connexin 43 expression in hippocampus of pentylenetetrazole kindled rats. J Epilepsy Res 6:8–15. https://doi.org/10.14581/jer.16002
73. Waldbaum S, Patel M (2010) Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy? J Bioenerg Biomembr 42:449–455. https://doi.org/10.1007/s10863-010-9320-9
74. Saad K, Hammad E, Hassan AF, Badry R (2014) Trace element, oxidant, and antioxidant enzyme values in blood of children with refractory epilepsy. Int J Neurosci 124:181–186. https://doi.org/10.3109/00207454.2013.831851
75. Pazdernik TL, Emerson MR, Cross R, Nelson SR, Samson FE (2001) Soman-induced seizures: limbic activity, oxidative stress and neuroprotective proteins. J Appl Toxicol 21(Suppl 1):S87–S94
76. Carrasco-Pozo C, Tan KN, Borges K (2015) Sulforaphane is anticonvulsant and improves mitochondrial function. J Neurochem 135:932–942. https://doi.org/10.1111/jnc.13361
77. Costa DA, de Oliveira GAL, Lima TC, dos Santos PS, de Sousa DP, de Freitas RM (2012) Anticonvulsant and antioxidant effects of cyano-carvone and its action on acetylcholinesterase activity in mice hippocampus. Cell Mol Neurobiol 32:633–640. https://doi.org/10.1007/s10571-012-9812-8
78. Rodrigues AD, Scheffel TB, Scola G, dos Santos MT, Fank B, Dani C, Vanderlinde R, Henriques JAP et al (2013) Purple grape juices prevent pentylenetetrazol-induced oxidative damage in the liver and serum of Wistar rats. Nutr Res 33:120–125. https://doi.org/10.1016/j.nutres.2012.12.002
79. Gao F, Gao Y, Liu Y, Wang L, Li YJ (2014) Berberine exerts an anticonvulsant effect and ameliorates memory impairment and oxidative stress in a pilocarpine-induced epilepsy model in the rat. Neuropsychiatr Dis Treat 10:2139–2145. https://doi.org/10.2147/NDT.S73210
80. Oliveira AA, Almeida JPC, Freitas RM, Nascimento VS, Aguiar LMV, Júnior HVN, Fonseca FN, Viana GSB et al (2007) Effects of levetiracetam in lipid peroxidation level, nitrite-nitrate formation and antioxidant enzymatic activity in mice brain after pilocarpine-induced seizures. Cell Mol Neurobiol 27:395–406. https://doi.org/10.1007/s10571-006-9132-y

81. Frantseva MV, Velazquez JL, Hwang PA, Carlen PL (2000) Free radical production correlates with cell death in an in vitro model of epilepsy. Eur J Neurosci 12:1431–1439
82. Kiasalari Z, Khalili M, Roghani M, Heidari H, Azizi Y (2013) Antiepileptic and antioxidant effect of hydroalcoholic extract of Ferula Assa Foetida gum on pentylentetrazole-induced kindling in male mice. Basic Clin Neurosci 4:299–306
83. Halász P, Rásonyi G (2004) Neuroprotection and epilepsy. In: Frontiers in Clinical Neuroscience. Springer, Boston, MA, pp. 91–109
84. Kimiskidis V (2008) Neuroprotection in epilepsy: the role of anti-epileptic drugs. Ann General Psychiatry 7:S74. https://doi.org/10.1186/1744-859X-7-S1-S74
85. Trojnar MK, Małek R, Chrościńska M, Nowak S, Błaszczyk B, Czuczwar SJ (2002) Neuroprotective effects of antiepileptic drugs. Pol J Pharmacol 54:557–566
86. Dam AM (1980) Epilepsy and neuron loss in the hippocampus. Epilepsia 21:617–629
87. Cho I, Cho Y-J, Kim H-W, Heo K, Lee BI, Kim WJ (2014) Effect of androsterone after pilocarpine-induced status epilepticus in mice. J Epilepsy Res 4:7–13
88. do Nascimento AL, Dos Santos NF, Campos Pelágio F et al (2012) Neuronal degeneration and gliosis time-course in the mouse hippocampal formation after pilocarpine-induced status epilepticus. Brain Res 1470:98–110. https://doi.org/10.1016/j.brainres.2012.06.008