Increased neurogenesis after ACEA and levetiracetam treatment in mouse pilocarpine model of epilepsy

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Abstract

Introduction and objectives. The aim of the study was to assess the impact of long-term therapy with the second generation antiepileptic drug levetiracetam (LEV) with arachidonyl-2'-chloroethylamide (ACEA), a highly selective cannabinoid CB1 receptor agonist, on the process of neurogenesis in a mouse pilocarpine model of epilepsy (PILO). Additionally, a relationship was established between the treatment with ACEA in combination with LEV, and hippocampal neurogenesis in mouse PILO brain.

Materials and method. All experiments were performed on adolescent male CB57/BL mice injected i.p. with LEV (10 mg/kg), ACEA (10 mg/kg) and PMSF (30 mg/kg) (phenylmethylsulfonyl fluoride — a substance protecting ACEA against degradation by the fatty-acid amidohydrolase), pilocarpine (PILO, a single dose 290 mg/kg) and methylscopolamine (30 min before PILO to stop the peripheral cholinergic effects of the pilocarpine, 1 mg/kg). The process of neurogenesis was evaluated after 10 days treatment with LEV and ACEA.

Results. Obtained results indicated that the combinations of ACEA+PMSF+LEV and ACEA+PMSF increased the total number of newborn cells compared to the control. Moreover, ACEA+PMSF administered alone and in combination with LEV had a significant impact on neurogenesis increasing the total number of newborn neurons compared to the control group. Neither LEV nor PMSF had a significant impact on the number of proliferating cells and newborn neurons when compared to the control PILO group. In turn, LEV administered alone decreased significantly the number of astrocytes. However, ACEA+PMSF has demonstrated significant increase of astrocytes compared to control mice.

Conclusions. These data provide substantial evidence that the combination of LEV+ACEA significantly increases the level of newborn neurons in the PILO dentate subgranular zone.

Key words

Levetiracetam, ACEA, neurogenesis, neurons, astrocytes, pilocarpine

INTRODUCTION

Levetiracetam (LEV), is one of the most common second-generation antiepileptic drugs, mainly used in the treatment of partial onset seizures with and without secondary generalization in adults and adolescents with newly-diagnosed epilepsy [1]. LEV is known to be ineffective in classic seizure models which screen potential compounds for antiseizure efficacy, such as maximal electroshock and pentylenetetrazol in rodents [2–5], whereas it has shown protective activity against acute seizures induced by 6 Hz electrical stimulation — a model of psychomotor seizures [6, 7]. Additionally, its activity mechanism varies from that of other antiepileptic drugs and this may be related to a brain-specific stereo-selective binding site, synaptic vesicle protein 2A (SV2A). SV2A appears to be important for the availability of calcium dependent neurotransmitter vesicles ready to release their content [8]. Numerous studies have suggested that levetiracetam has considerable neuroprotective properties in both epileptic and nonepileptic disorders [9–13]. Its neuroprotection was demonstrated in several brain injury as well as neurodegenerative disease prototypes. These include brain damage resulting from status epilepticus (SE) or acute seizures, spontaneous epilepsy, closed head trauma, subarachnoid haemorrhage (SAH), hypoxic-ischemia, and stroke [14]. Nowadays, researchers are focused on the development of new potential anticonvulsants that also protect neurons against degeneration [15–18]. In searching for the best neurological drug protecting neuronal cells, stimulating neurogenesis, but also without developing side-effects, cannabinoids have proved to be a strong group of substances with many beneficial properties [19–25]. There is much evidence suggesting that both exogenous and endogenous cannabinoids can control cell genesis in the brain, although the effects can vary considerably depending on the cannabinoid, dose and duration of administration [26–28]. For example, chronic administration of the CB2 selective agonist HU-308 increased neural progenitor cell proliferation [28]. Similarly, chronic treatment with another synthetic cannabinoid — HU-210, a drug that has
a high affinity for both CB1 and CB2 receptors, enhances proliferation and survival of cells in the rat dentate gyrus [29]. Interestingly, chronic administration of another synthetic CB1/CB2 agonist WIN55,212-2 administered to rats during adulthood was found to have no effect on the number of immature neurons in the dentate gyrus. However, interestingly, administration during adolescence decreased the number of immature neurons, an effect that is attributed to selective suppression of dorsal but not ventral hippocampal neurogenesis [30]. The endocannabinoid anandamide (AEA) or the CB1-specific agonist ACEA displayed a higher degree of neural stem cells neuronal differentiation and maturation in vitro, and enhanced neuronal stem cell differentiation into neurons, but not astrocytes and oligodendrocytes [31].

Previous results from neurogenesis research by the authors of the current study using ACEA and other LPP, valproate (VPA) in PILO mice, indicated that ACEA alone and in combination with VPA increased the total numbers of BrdU-positive cells, newly-born neurons and astrocytes, compared to the VPA group [32]. Taking into consideration all the above-mentioned information, the authors hypothesize that the combination of ACEA with LEV may have an impact on neurogenesis, as well as on LEV administered alone in PILO mice. To confirm this hypothesis, it was decided to evaluate the relationship between treatment with LEV+ACEA combination and hippocampal neurogenesis in a mouse PILO model of epilepsy.

**MATERIALS AND METHOD**

**Animals and experimental conditions.** All experiments were performed on adolescent male CB57/BL mice (6 weeks old). The mice were kept in colony cages with access to food and tap water *ad libitum*, under standardized housing conditions (natural light-dark cycle, temperature 20 °C). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of 8 mice in each group. Each mouse was used only once. All tests were performed between 09:00–14:00. Procedures involving animals and their care were conducted in conformity with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures listed below conformed also to the Guidance for the Care and Use of Laboratory Animals and approved by the Local Ethics Committee at the Life Science University of Lublin, Poland (License No.: 23/2013).

**Drugs.** The following drugs were used in this project:levitracetam (LEV; UCB Pharma, Braine-l’Alleud, Belgium), ACEA (arachidonyl-2-chloroethylamide; N-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; pre-dissolved in anhydrous ethanol (5 mg/ml); Tocris Cookson Ltd., Bristol, UK), dissolved in distilled water and phenylmethylsulfonylfluoride (PMSF; ICN Biomedicals Inc., Irvine, CA, USA), used to limit the degradation of ACEA by inhibition of fatty acid amid hydrolase [33]. Pilocarpine (290 mg/kg) All drugs were injected intraperitoneally (i.p.) as a single injection, in a volume of 0.005 ml/g.

**Pilocarpine-induced convulsions.** Mice were housed individually on a 12-hour day/night cycle at least 7 days prior to treatment with free access to food and tap water *ad libitum*. Pilocarpine study was performed in accordance to the procedure described by Bahaskaran and Smith [34] with minor changes. Mice were administered an i.p. injection of methylscopolamine (1 mg/kg) 30 min prior to injection of pilocarpine to reduce the peripheral cholinergetic effects of the pilocarpine. Experimental animals were then injected i.p. with a single dose of pilocarpine 290 mg/kg [35]. Mice were carefully observed after pilocarpine injection to catch the first symptoms of convulsions. Seizure behaviour occurred about 2 hours after the pilocarpine injection and determined according to Racine’s 1 – 5 scale [36, 37]. The most important were convulsive seizures (categories 3 – 5) which correlate with the eventual development of spontaneous seizures and mossy fibre sprouting. A mouse that experienced a minimum of 3 generalized convulsive seizure events within 2 hours following pilocarpine injection was considered to have undergone status epilepticus (SE). The category 3–5 spontaneous seizures was assessed by passive observation 2 hr/day, for one week after SE. Animals with spontaneous seizures (PILO mice) were used for the next step of the experiment. For animals where no seizures were observed, euthanasia was performed using carbon dioxide inhalation.

**Drugs administration.** Drug administration paradigm is similar as that described by Andres-Mach [32]. Animals were injected with ACEA and LEV for 10 days. Drug solutions were prepared *ex tempore* each day and administered once a day at the following doses: LEV-10 mg/kg, ACEA-10 mg/kg and PMSF-30 mg/kg. The doses of ACEA and PMSF were chosen based on previous publications [32, 38, 39] by the authors of the presented study. Animals were given BrdU (a marker of cell proliferation) as an additional injection for the last 5 days of treatment.

**Tissue preparation.** Three weeks after the last BrdU injection, the mice were anesthetized and perfused with ice-cold saline followed by freshly prepared, ice-cold 4% paraformaldehyde (PFA) in PBS. The brains were removed, post-fixed in fresh 4% PFA for 24 h, and, subsequently, fifty-micrometer coronal sections were cut using a vibratome (VT1000S, Leica Biosystems, Wetzlar, Germany).

**Immunohistochemical staining.** Fifty-micrometer sections were stored at 4°C in cryoprotectant until needed. Free-floating sections were immunostained using primary antibodies and working concentrations: rat anti-BrdU (1:200; Chemicon, Temecula, CA, USA); goat anti-NeuN (1:200; Chemicon, Temecula, CA, USA); anti-GFAP (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, sections were rinsed with TRIS buffered saline (TBS), pH 7.4, and then blocked for 30 min at room temperature with TBS containing 5% preimmune donkey serum and 0.3% Triton-X100 (TBS++). In the next step, sections were incubated for 24 h at 4°C with primary anti-NeuN and anti-GFAP antibodies diluted in TBS++ and then washed extensively with TBS buffer at room temperature. Subsequently, sections were incubated overnight at 4°C with secondary fluorochrome-conjugated antibodies in TBS++, and again washed extensively at room temperature. For the
Impact of ACEA and LEV on total newborn cells in the dentate subgranular zone of PILO mice. Results from the neurogenesis study indicated that the combination of ACEA+PMSF+LEV PILO as well as ACEA+PMSF PILO increased the total number of BrdU positive cells in comparison to the control PILO group (Fig. 1). As mentioned above, in the control PILO group, the total number of BrdU positive cells in the dentate gyrus of mice averaged 1,766±150, while in ACEA+PMSF+LEV PILO mice, the average value was 4,237±479 ($F_{2,3}=13.41; n=5; p<0.001$) (Fig. 1), and ACEA+PMSF PILO mice the average value was 4068±457 ($p<0.01$ for comparisons). No statistical significance was observed when comparing LEV PILO and PMSF PILO groups to the control PILO group ($p>0.05$ for comparisons).

Impact of ACEA and LEV on newborn astrocytes in the dentate subgranular zone of PILO mice. In the control PILO group, the number of BrdU positive cells colocalised with NeuN in the dentate gyrus of mice averaged 876±74 while, for ACEA+PMSF PILO-treated mice averaged 2542±287; the difference was statistically significant ($F_{2,3}=16.42; n=5; p<0.001$) (Fig. 1). In ACEA+PMSF+LEV PILO-treated mice averaged 2542±287; the difference was statistically significant ($F_{2,3}=16.42; n=5; p<0.001$ for comparison) (Fig. 2). The total number of NeuN/BrdU positive cells in PMSF PILO and LEV PILO mice showed no significant difference compared to control PILO group ($p>0.05$ for comparisons).

Impact of ACEA and LEV on newborn astrocytes in the dentate subgranular zone of PILO mice. ACEA+PMSF PILO groups revealed a significant impact on newborn astrocytes (Fig. 3), compared to the control PILO mice. The average number of astrocytes for control PILO mice was 118±10, whereas for ACEA+PMSF PILO-treated mice it was 224±253 and ACEA+PMSF+LEV PILO-treated mice averaged 224±253 and ACEA+PMSF+LEV PILO-treated mice averaged 2542±287; the difference was statistically significant ($F_{2,3}=16.42; n=5; p<0.001$ for comparison) (Fig. 2). The total number of NeuN/BrdU positive cells in PMSF PILO and LEV PILO mice showed no significant difference compared to control PILO group ($p>0.05$ for comparisons).
DISCUSSION

Obtained results indicated that ACEA POLO (10 mg/kg, i.p.) co-administered with PMSF (30 mg/kg, i.p.) significantly increased the total amount of BrdU positive cells. Similarly, the combination of ACEA+PMSF+LEV POLO also enhanced BrdU+cells. Moreover, a significant increase in NeuN+cells for ACEA+PMSF+PILO and ACEA+PMSF+LEV POLO compared to the control POLO-mice, which confirms enhanced neurogenesis. Neither LEV+POLO nor PMSF+POLO administered alone for 10 days had a significant impact on the number of proliferating cells when compared to the control POLO group.

LEV as a second-generation anti-epileptic drug (AED), is mainly approved for clinical use as monotherapy and may also be used for adjunctive treatment of patients with seizures [42]. LEV was found to act through the inhibition of the synaptic vesicle protein 2A [43], but also the inhibition of HVA-Ca\(^{2+}\) channels (N-type). Moreover, it can negate the inhibition of negative allosteric modulators, such as zinc and β-carbolines of γ-aminobutyric acid (GABA)- and glycine-gated currents, and diminish the calcium release from intraneuronal stores [43, 44].

Additionally, results from different experimental research suggests that LEV provides anti-hyperalgesic effects in inflammatory pain [45–47], chronic pain [48] and neuropathic pain [49–51] models. Several studies have proposed that LEV possesses considerable neuroprotective properties in both epileptic and non-epileptic disorders [42]. Lee et al., [52] indicated that LEV treatment may effectively reduce status epilepticus-induced neuronal death in CA1 pyramidal cells, compared with diazepam treatment in rats. LEV as an add-on drug with diazepam could not alleviate status epilepticus-induced neuronal damage, compared with LEV alone.

Research using hypoxic ischemic brain injury induced by carotid artery ligation in rat pups showed that the numbers of positive apoptotic cells decreased significantly in the LEV-treated group compared with the saline group, which may be related with not only anti-epileptogenic action but also with anti-inflammatory effect of LEV [53]. Research carried out by Yan et al., [54] concerning LEV and neurogenesis, indicated a significant impact of LEV on the process of proliferation, migration and differentiation of new-born cells. They found that LEV enhanced cell proliferation and neuroblast differentiation by increasing the expression of antioxidants and P13K and the level of phosphorylated Akt in the mouse hippocampus. On the other hand, results from studies by the authors of the presented study with using LEV treatment alone and in combination with arachidonyl-2’-chloroethylamide (ACEA), indicated, that LEV injected alone decreased significantly the process of neurogenesis, whereas the combination LEV+ACEA+PMSF significantly increased the total number of new-born neurons compare to the control group [55]. Moreover, the authors reported in their previous results that ACEA alone and in combination with another antiepileptic drug valproate significantly increased neurogenesis in healthy and PILO mice [32,38]. Similar results were obtained by Compagnucci and et al. [31], where the endocannabinoid anandamide (AEA) as well as ACEA enhanced NSC differentiation into neurons, whereas the CB\(_1\)-specific agonist JWH133 was ineffective. Investigations with mouse PILO model of epilepsy allowed the observation and evaluation of the neurogenesis changes in the epileptic mouse brain.

CONCLUSIONS

From the results obtained in the presented study, it can be conclude that long-term treatment with an antiepileptic drug like LEV leads to a reduction of hippocampal proliferation, migration and differentiation of new-born cells, whereas the use of the combination of ACEA and LEV significantly increased neurogenesis. Unaffected neurogenesis certainly has great importance for patients suffering from epilepsy and
undergoing long-term treatment with antiepileptic drugs; therefore an assisted antiepileptic therapy with ACEA is worth consideration in further preclinical trials.

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