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Research Article

The Impact of Environmental Enrichment in Rats Subjected to the Lithium-Pilocarpine Model: Behavioral Assessment and Molecular Changes in the Hippocampus

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- Environmental enrichment
- Epilepsy
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Abstract

Epilepsy is a neurological condition characterized by unpredictable recurrent seizures that affects about 50 million people worldwide. In Temporal Lobe Epilepsy (TLE), the most common form of epilepsy in adults, seizures are often resistant to drug treatment. Environmental enrichment (EE) has positive effects on the psychological and physiological well-being of the animals and provides news insights into mechanisms of experience-dependent plasticity, including neurogenesis and synaptic plasticity.

Objectives: Evaluate the impact of EE on behavioral changes (latency and frequency of seizures, cognitive process and anxiety) in rats subjected to lesional epilepsy model induced by lithium- pilocarpine (LIP) and changes in BDNF levels in the hippocampus.

Methods: Wistar rats were exposed to an EE protocol and to a standard environment since weaning (PND 21) for 5 weeks. After this period, the animals of both groups (EE and standard) were randomized and injected with LIP or lithium saline solution and video-monitored for 60 days to evaluate the latency and the frequency of seizures. After this period we performed behavioral tests: Elevated Plus Maze (EPM), Open Field Test (OFT), Rearing and Novel Object Recognition (NOR) to evaluate memory and anxiety. The BDNF expression was assessed in the hippocampus by ELISA method.

Results: EE decreased hyperactivity, preserved short-term memory and increased the latency to the onset of spontaneous seizures of LIP rats compared to LIP rats of conventional environment. However, there were no difference in anxiety level and in the total number of seizures between conventional and EE groups. BDNF expression was increased in the hippocampus of rats LIP exposed to EE compared to LIP of conventional environment.

Conclusions: EE is a safe and effective strategy to reduce behavioral changes caused by spontaneous seizures.

ABBREVIATIONS

ANOVA: Analysis of Variance; BDNF: Brain-Derived Neurotrophic Factor; EE: Environmental Enrichment; ELISA: Enzyme-Linked Immunosorbent Assay; EPM: Elevated Plus Maze; LIP: Lithium-Pilocarpine; OFT: Open Field Test; NOR: New Object Recognition; SE: Status Epilepticus; TLE: Temporal Lobe Epilepsy.

INTRODUCTION

Epilepsy is a neurological condition characterized by unpredictable recurrent seizures that affects about 50 million people worldwide. Is a common disorder, particularly in poor areas of the world, and can have a devastating effect on people with the disorder and their families [1]. Mesial Temporal Lobe Epilepsy (MTLE) is the most common form of partial epilepsy in adult, affecting approximately 60% of patients with epilepsy. Hipocampal sclerosis (HS) is frequently associated with MTLE and is characterized by the selective loss of neurons in hippocampal subfields, gliosis, atrophy and synaptic reorganization, and has been associated with seizure generation and propagation [2,3]. Nevertheless, about 20% of patients with TLE do not show cellular loss, but present reactive gliosis [4].

Structural and functional changes resulting from the MTLE can reach not only structures in the temporal lobe, but also in

the frontal lobe. These changes may be associated with problems in cognitive processes, including executive function, working memory, attention, decision, language, planning and judgment. Furthermore, they may be associated also with anxiety, psychosis and depression, dramatically reducing the quality of life of these people [5-7]. The Pilocarpine model, whether or not associated with lithium, is a well-studied model of MTLE, which has been widely used, as it reproduces the main pathologic characteristics observed in humans [8]. Following systemic application of high doses of Pilocarpine, rats exhibit uninterrupted seizures in an acute period lasting 15-18 hours. Then, animals show normal behavior and electroencephalographic recording patterns for a period of approximately 14 days, also denominated latent period. This period terminates with the appearance of spontaneous epileptic seizures initiating a chronic phase maintaining for the remaining animal's life time [9]. All rats displaying SE for at least 1 h develop hippocampal sclerosis that is characterized by selective cell loss in the CA1, CA3 subfields and hills of the hippocampal formation, dispersion of granular cells of the dentate gyrus, mossy fiber sprouting and neurogenesis [9-11]. Rats subjected to Pilocarpine usually present cognitive impairment, being a suitable model to study the mechanisms involved in the generation of seizures and behavioral changes, and new therapeutic strategies [7]. Studies have shown that neuronal injury induced by pilocarpine contributes to cognitive deficit, which appears in the late stage of the model [9]. Many studies have shown that environmental enrichment (EE) enhances performance of rats in various behavioral task assessing motor, learning and memory and emotional functions for reviews see [12]. EE has also demonstrated beneficial effects on the recovery of several disorders of the central nervous system such as Alzheimer's, Parkinson's disease, Huntington's and epilepsy [13,14]. It has been shown that EE had beneficial effect in temporal lobe epilepsy (TLE) models. EE reduced the injury caused by kainic acid and improved the performance of rats subjected to lithium pilocarpine model (LIP) in spatial memory tasks [15], increased cell proliferation and survival decreasing seizures and improving cognitive impairments [16].

The BDNF (brain-derived neurotrophic factor) has been cited as one of the factors responsible for neuro plasticity caused by EE [17-20].

The present study was aimed to determine the effect of EE on behavioral changes caused by Lithium-Pilocarpine (latency and frequency of spontaneous seizures, cognitive process and anxiety). Rats were video-monitored for better define the latency for the onset of the first spontaneous seizure and the frequency of spontaneous seizures. The BDNF level was determined in the hippocampus as a marker of neuroplasticity.

MATERIALS AND METHODS

Animals

Sixty adult male Wistar rats (350 ± 30 g) provided by the biotery of Federal University of São Paulo (CEDEME-UNIFESP) were housed under controlled conditions (22 ± 1°C, 12/12h light/dark cycle, lights on at 7:00 a.m.) with water and food ad libitum. All experiments were approved by the ethics research committee of the UNIFESP (CEUA N° 8740200814). Efforts were made to minimize pain or discomfort of animals. The experiments were performed following the principles outlined in the ARRIVE (Animal Research: Reporting of *InVivo* Experiments) guidelines and the Basel declaration (http://www.basel-declaration.org). The 3R concept (Replacement, Refinement and Reduction of Animals in research) has been considered when planning the experiments.

Enrichment Protocol

After weaning (21 days after birth), rats were grouped randomly, and maintained during 5 weeks in two types of environments, standard or enriched. For enriched environment rats (N = 8/box) were housed in a box with total area of 66 X 80 X 34 cm built on from the junction of two boxes joined by a PVC pipe with 15 cm of diameter (Figure 1A). In this box were placed objects that allowed the practice of voluntary exercise (wheels, ramps, pipes), objects to shelter (pots or boxes) and objects to sensory stimulation (colored balls, pet bottle, wooden batons) of different colors, textures and materials (metal, plastic and wood). The objects were changed, rearranged and washed three times per week to ensure the effect of novelty and challenge.

The age of onset, duration and other parameters of the environmental enrichment protocol were chosen based on previous data [19,21]. Animals kept in cages with no environmental enrichment (N = 5/cage) were housed in plastic cages measuring 33 x 40 x 17 cm (Figure 1B). After 5 weeks rats of both conditions were taken and subjected to systemic application of lithium-pilocarpine to induce epilepsy, or saline.

Lithium Pilocarpine Model

Animals from EE or NE environments were subjected to intra peritoneal injection (i.p.) of lithium chloride (Sigma - LiCl, 127.17 mg / ml) diluted in saline 0.9% administered 16 to 20 hours prior to subcutaneous injection (s.c.) of methyl scopolamine nitrate (ME, 1mg / kg) diluted in 0.9% saline. ME was used to limit peripheral cholinergic effects of pilocarpine hydrochloride, such as diarrhea, piloerection, orofacial automatisms associated with salivation, wink, vibrissae contractions, yawning [11]. Pilocarpine was injected intraperitoneally (30 mg/kg s.c., Sigma-Aldrich) 30 min after ME to induces SE. The control group received lithium and saline instead of pilocarpine. After two hours of SE duration, rats were treated with thiopental (30 mg / kg, i.p.) and diazepam (1 mg / kg s.c.) to minimize behavioral seizures. This procedure was used to increase animals survival rate presenting SE. Following 48 hours, the surviving animals were rehydrated with

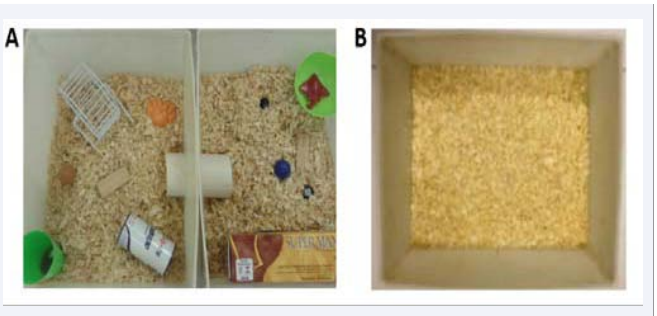

Figure 1 (A) Environmental enrichment cage; (B) Standard cage.

2 ml of 0.9% saline (s.c.) for 2 days. The following groups were used in the study (N=15 rats/group)

1. Animals exposed to EE and subjected to the LIP model
2. Animals exposed to EE and treated with saline
3. Animals exposed to standard condition and subjected to the LIP model
4. Animals exposed to standard condition and treated with salines

Video-Monitoring

Starting 4 days after SE-induction, video-monitoring (24 hours / 7 days) was performed over a 2-month period in order to assess the latency for the first seizure and the frequency of seizures. At the end of the video-monitoring, rats were subjected to behavioral tests for cognitive evaluation.

Open Field

The open field test (OFT) was performed in a single session of 10-minutes. The test consists in placing the animal in the center of the apparatus (circular field of 1 m diameter) and observes the distance walked and the immobility time in the apparatus. Two natural behaviors of rodents, i.e. the tendency to explore new environments and aversion to light and open spaces were evaluated [22-24].We used video-monitoring coupled to the software Any Maze® to analyze these behaviors.

Elevated plus Maze

The Elevated Plus Maze (EPM) is one of the classic tests used to measure anxiety [25-27]. The apparatus consists two closed and dark arms, and two open arms and clear, with ground elevation by 50 cm.

The test consists in placing the animal in the center of the apparatus, and measure the movement toward the closed or open arms during 5 min. Less anxious animals usually remain more time in the open arm. The numbers of entrances in the arms are quantified using Any Maze® software.

Novel Object Recognition

The novel object recognition (NOR) is a test of learn and memory. Rats are exposed to specific objects in a circular field of 1 meter diameter, bordered by a transparent acrylic cylinder of 50 cm diameter. The animals undergo a period of adaptation of 20 minutes for 3 consecutive days to reduce the effect of the environment novelty. After the adaptation period, the animals are exposed to two identical objects, with same color and shape, for 5 minutes, and the time that the animal explores each object is measured by stopwatches. Following this, one familiar object is removed and replaced with a novel object. After two hours of the first exposure, rats are put back in the same place and for 5 minutes the time spent exploring the novel object is recorded. The exploratory movement is determined when the animal touches with his nose each object. The NOR is assessed by the preference in explore the novel object, and the time spent by the animal in exploring each object on test trial. Studies have shown that animals usually spend more time exploring the novel object in the second exposure [28].

Euthanasia

One day after the behavioral tests, the animals were anaesthetized with ketamine and xylazine (150 mg/kg and 30 mg/kg, respectively), and then euthanized by decapitation using a guillotine. The brains were removed from the skull over ice plate and the hippocampi (right and left) were dissected and placed in tubes, frozen in liquid nitrogen and stored in -80°C freezer until the experimental day.

Enzyme-linked Immunosorbent Assay

BDNF was quantified in the hippocampus samples using the enzyme-linked immunosorbent assay test - ELISA (Phoenix Pharmaceuticals, Inc.). Tissues were homogenized in a proportion of 100 mg of tissue per 1ml, of lysis buffer (TBS, 1% NP40, Triton X-100 1% and 10% glycerol) containing protease inhibitor (Sigma-Aldrich) (1 µl per 100 µl of lysis buffer). The samples were centrifuged (12,000 rpm at 4 ° C for 20 minutes), the supernatant transferred to a new micro tube and the pellet discarded. Bradford method was used to estimate protein and 400µg of protein was used in the assay [29].

BDNF of samples was estimated using a standard curve obtained with serial dilutions of BDNF provided by the kit. Analyses were in duplicate. The plates were incubated for two hours at room temperature under agitation (300-400 rpm). The wells were washed 4 times with 350 µl of buffer solution, the supernatant discarded and the plates were pressed on blotting paper to dry. After this, 100 µl of biotinylated anti-BDNF were added into each well, and incubated for two hours at room temperature. After new washing sequence, 100 µl of SA-HRP were added into each well, the plates were incubated for 30 minutes at room temperature. After washing, 100 µl of the solution (TMB) were added in each well and the plates were again incubated during 30 min. Finally, a stop solution (hydrochloric acid, 100ul/well) was used to block the reaction and the plates were read at 450nm using Epoch® Gene5® reader software.

Statistical analysis

The analysis was performed using SPSS software. (IBM – version 17.0 or superior).For evaluation of the Open field test, Elevated Plus Maze and BDNF we used a two-way analysis of variance (ANOVA), to analyze the factors Environment and Pilocarpine. For analysis of New Object Recognition we used ANOVA with repeated measures. Bonferroni was used as post-hoc test. The latency and the frequency of seizure were evaluated by Student´s t Test. Data were expressed as mean ± standard deviation and a $p < 0,05$was considered significant.

Words

Rats housed in an EE took longer to manifest the first recurrent seizure after LIP administration compared to those housed in a conventional environment ($t(13) = 2.939$, $p < 0.05$) (Figure 2). No significant differences was observed in the total number of seizures in LIP rats housed in EE compared to rats housed in a standard environment (Figure 3).

The analysis of the total locomotion (central and peripheral) by two-way ANOVA revealed a significant effect on environmental factor ($F(1,38) = 10.302$; $p < 0.05$) and in the pilocarpine factor

(F (1,38) = 15.266; p <0.05), but no interactions was observed between these factors (Figure 4). Rats from LIP group showed higher activity (total locomotion inside the apparatus) than the saline group, and EE reduced the locomotion behavior in both LIP and saline groups.

Rats raised in an EE had lower frequency of rearing than rats raised in a standard environment (F (1, 38) = 4.484; p <0.05) (Figure 5).

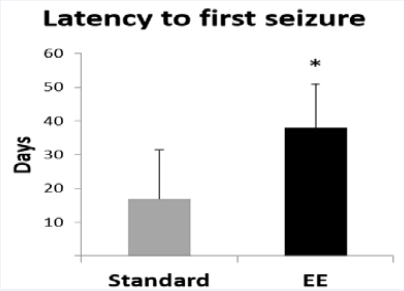

Figure 2 Latency for the appearance of the first spontaneous seizure after SE in rats housed in an EE (38 ± 13,09) compared to rats housed in a standard environment (17 ± 14,61). Data are expressed as mean ± standard deviation. * P <0.05.

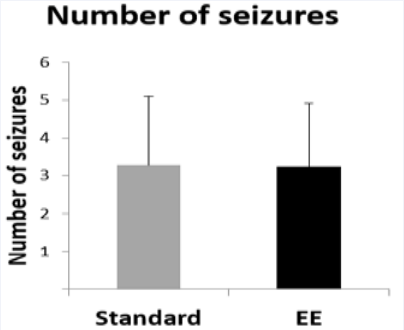

Figure 3 Total number of spontaneous and recurrent seizures of rats LIP, previously housed in an EE (3 ± 1,7) or in a standard environment(3 ±1,8).

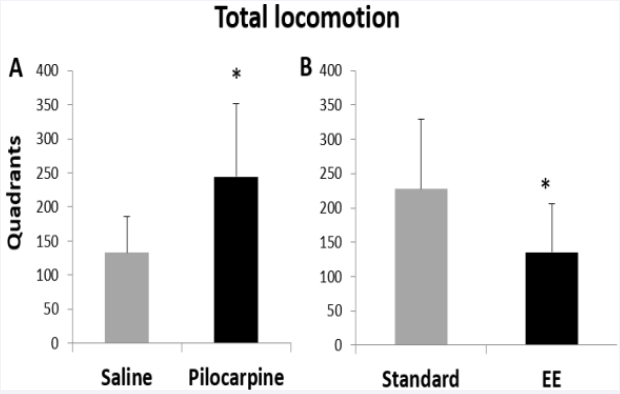

Figure 4 Total locomotor activity of rats LIP or saline (A), housed in an EE or in a standard environment (B). Data are expressed as mean ± standard deviation. * P <0.05.

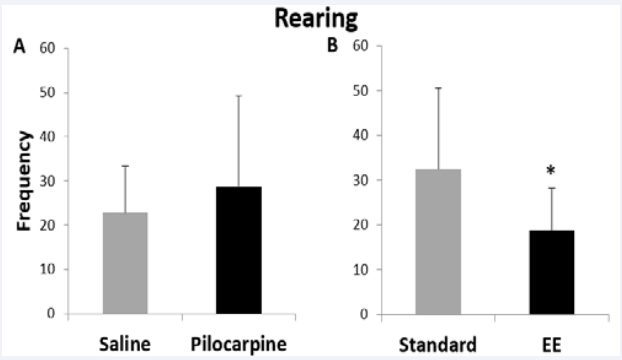

Figure 5 Frequency of rearing of animals subjected to LIP and saline (A), housed in an EE or in a standard environment (B). Data are expressed as mean ± standard deviation. * P <0.05.

The analyze of the time spent in the open and in the closed arms by rats from LIP and saline group by two-way ANOVA revealed no significant effect of the factors pilocarpine and environment, nor the interaction between these factors (Figure 6A, 6B).

The time spent by rats in exploring novel object was analyzed by ANOVA with repeated measures. Rats from LIP group raised in a standard environment spent similar time exploring usual object (A1) as the novel object (B). However, rats LIP raised in an EE spent much more time exploring novel object indicating an improving in the memory deficits caused by seizures (F (1,31) = 21,829; p<0,05) (Figure 7).

The BDNF expression in the hippocampus of rats LIP raised in both conditions, EE and standard environment, increased significantly compared to their respective control group (F (3,38) = 8.960; p <0.05) (Figure 8). However, the increase observed in rats raised in EE was higher than rats LIP raised in a standard environment, showing that EE intensified the BDNF expression.

DISCUSSION

This study aimed to investigate the impact of EE on behavioral aspects (anxiety, hyperactivity, latency and frequency of seizures) and in the hippocampal BDNF level of LIP rats compared to LIP rats housed in a conventional environment. Several studies using EE have shown changes in hippocampal neurogenesis and plasticity, with positive effects on animal welfare exposed to an experimental model [30,31].

In the present study, EE caused a significant increase in the latency for the appearance of the first spontaneous seizure in LIP rats compared with LIP rats kept in a standard condition. No difference in the total number of seizures was observed in LIP rats exposed to EE compared to conventional environment.

There are many reports showing a beneficial effect of EE in the TLE, such as reduction in the frequency and the severity of seizures, and in neuroprotection [14,32]. Many mechanisms have been associated with the antiepileptic and a neuroprotective effect caused by EE, among them increased release of neurotrophic factors, strengthening of inhibitory circuitry mediated by GABA, neurogenesis, and neurotransmitter release [33-35].

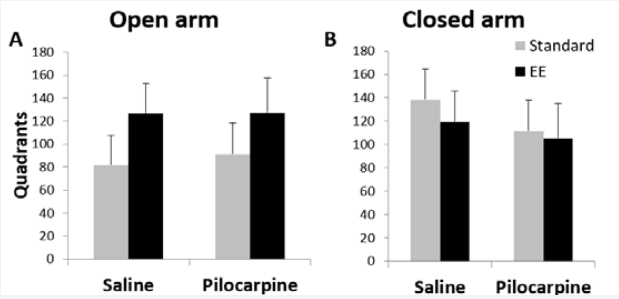

Figure 6 Time spent in the open (A) and closed (B) arms, expressed as mean ± standard deviation by rats subjected to saline and LIP groups, after house in an EE or in a standard environment. No significant differences was observed between the groups.

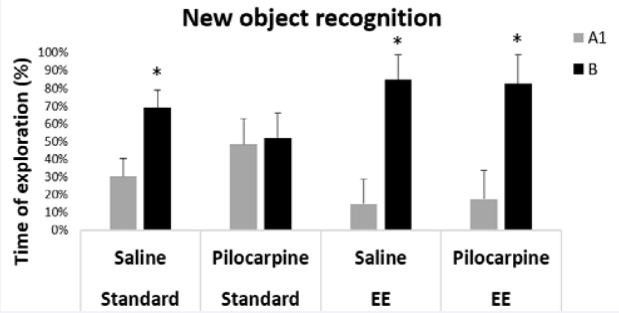

Figure 7 Time spent in exploring the novel object (B) compared to the usual object (A1)by animals subjected to LIP or saline, previously housed to an EE or to a standard environment. Data expressed as mean ± standard deviation. * P <0.05.

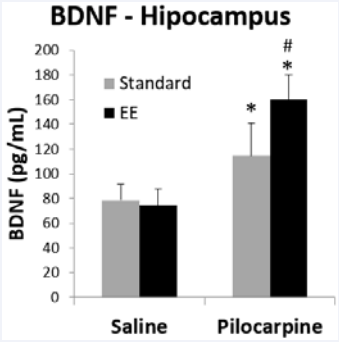

Figure 8 BDNF levels in the hippocampus of rats subjected to LIP or saline, previously housed in an EE or in a standard environment. Data are expressed as mean ± standard deviation. *P< 0,05, when compared to saline group; #P< 0,05, when compared to rats housedin a standard environment.

In a recent study, Zhang et al. [36] have reported an association between hippocampal neurogenesis and dendritic growth of newborn neurons after kainic acid-induced TLE, with reduced long-term seizure activity and cognitive improvement in spatial learning in rats exposed to EE during 1 month compared to those of conventional environment. Fares et al. [32] also demonstrated beneficial effect of EE at weaning on behavioral changes and cellular loss caused by Pilocarpine in rats. According to the authors, the improvement in cognitive decline observed in rats subjected to Pilocarpine following EE, may be a result of partial preservation of LTP and protection of hippocampal "place cell".

In the present study, we have shown that rats raised in a standard environment and subjected to LIP present hyper locomotion behavior and the EE decrease this altered behavior. Rats housed in a EE decreased the frequency of rearing and this result corroborate with the other authors [19].

Recent evidence indicates that EE can alters dopaminergic signaling by decreasing dopamine uptake in medial prefrontal cortex (mPFC) and increasing dopamine uptake in orbital frontal cortex (OFC) of rats. The increase in the extracellular dopamine in the mPFC caused by EE may be a mechanism involved with the decrease in the locomotor activity in rats [34]. On the other hand, the decreased extracellular dopamine in the OFC can be responsible for reducing the impulsive choice in behavioral tasks [34]. The analysis of dopamine concentration in further study may help to understand the behavioral changes observed in the present study. The analysis of data from EPM test showed that there was no significant difference on anxiety between the groups. There are many reports showing that rats subjected to temporal lobe epilepsy models (e.g. pilocarpine, lithium-pilocarpine, kainic acid) present increased locomotor activity and reduced anxiety level during spontaneous and recurrent seizures phase [33,35,37].The divergence between our study and those from the authors may be due to the use of different protocols and test conditions applied in the studies. Seizures induced by pilocarpine cause cell loss in limbic structures, mainly in the hippocampus, piriform cortex and amygdala and is frequently associated with cognitive deficit [38,39].

The analysis of recognition memory using NOR showed that EE intensified the time spent by control rats in exploring novel objects compared to rats housed to conventional environment. In LIP, the time spent exploring novel object was similar in to those spent in the first exposition. When LIP rats were exposed to EE, the time spent exploring the novel object increased compared to those housed in a conventional environment, showing that rats recovered the ability to explore novel objects. Our data are in contrast with those of Detour et al. [33] that didn´t verify differences in the tasks of object-recognition between LIP and control groups, suggesting that object discrimination is preserved despite expressive damage in regions involved with memory and anxiety as hippocampus, amygdala and entorhinal cortex.

Studies have shown that exposure to EE results in various structural and functional changes that may contribute to the preservation of memory, including increase in dendritic branching and synapse number in the cortex and hippocampus, in addition to increases in neurogenesis and in synaptogenesis in CA1 and CA3, brain areas usually affected by recurrent seizures.

Additionally, EE can attenuate expression of cytokines as IL-1B and TNF-alpha and improve hippocampal-dependent tasks [43,44]. There are many authors reporting increased level of cytokines in the hippocampus of rats subjected to pilocarpine model, including IL-1B and TNF-alpha, and these changes have been associated with hippocampal damage [45-48]. Considering this property of EE in modulate the expression of IL-1B and TNF-

alpha and attenuate hippocampal damage improving cognitive function, this may help explain why our LIP rats showed an improvement in learn and memory tasks.

Besides improving the performance in object-recognition tasks, EE induced a significant increase in the BDNF level in the hippocampus of LIP compared to saline. BDNF was also significantly increased in LIP rats housed in conventional environment, but was greater in EE rats. BDNF is a neurotrophin able to regulate Long-Term Potentiation (LTP), in particular in its primary phase, Early Long Term Potentiation (LTP-E), and is involved with learn and memory [17,49-51]. BDNF is reported to be released during neuronal activity. LIP model present cell loss in several brain areas and BDNF expression increases in the same regions [52-54]. This increase in BDNF expression caused by EE can contribute to improve the cognitive function in LIP rats.

The EE was used in this study showing beneficial effects on behavioral deficits related to epileptogenesis. Several studies used simultaneous EEG and video-monitoring for confirming behavioral changes and seizures classification after treatments aiming to modify or block seizures. However, in this study we used video-monitoring to obtain preliminary data regarding the EE effect on behavioral deficits induced by LIP. Our data indicate that activities that stimulate active social life and cognition may have a beneficial effect in the neurological clinic protecting the brain against deleterious effect of the aging.

CONCLUSION

Rats raised in an EE show increased latency for the onset of the first spontaneous seizures, normal locomotion and improvement of cognitive deficit after being injected with LIP. The beneficial effect of EE is accompanied by significant increase in BDNF expression in the hippocampus, indicating that BDNF may be a mechanism involved with memory improvement observed in LIP treated rats.

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