Effects of experimental cerebral malaria in memory, brain-derived neurotrophic factor and acetylcholinesterase activity in the hippocampus of survivor mice


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HIGHLIGHTS

- Cerebral malaria causes cognitive impairment in the survivors mice.
- Cerebral malaria decrease BDNF protein levels in the hippocampus.
- Cerebral malaria does not alters AChE activity in the hippocampus.

ABSTRACT

Malaria is the most important human parasitic disease and cerebral malaria (CM), its main neurological complication, is characterized by neurological and cognitive damage in both human and animal survivors. The brain-derived neurotrophic factor (BDNF) appears to be involved with activity-dependent synaptic plasticity. There is great interest regarding its role in learning and memory as well as acetylcholinesterase activity (AChE) that is implicated in many cognitive functions and probably plays important roles in neurodegenerative disorders. In the present work, we evaluated BDNF protein levels and AChE activity in the hippocampus and habituation in an animal model of CM using C57BL/6 mice after fifteen days of the induction. The results demonstrated that there was a decrease in BDNF levels in the hippocampus of C57BL/6 mice infected with PbA when compared with C57BL/6 non-infected mice and C57BL/6 non-infected mice that received treatment with chloroquine. However, no difference was observed in AChE activity in the hippocampus. When habituation was evaluated there was memory impairment in the C57BL/6 mice infected with Plasmodium berghei ANKA (PbA). In conclusion, we believe that the decreased BDNF levels in the hippocampus may be related with memory impairment without alterations on AChE activity.

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1. Introduction

Malaria is the most economically debilitating parasitic disease in humans and its neurological complication, cerebral malaria (CM), is the most severe neurological complication of the infection with Plasmodium falciparum. Also, it is arguably one of the most common non-traumatic encephalopathies in the world, particularly in terms of morbidity, mortality and deleterious economic consequences [1]. The cognitive impairment is a common sequel and persists far longer than physical and neurologic deficits in both human and animal models [2,3]. Various aspects of the cellular and molecular pathogenesis of malaria that can be associated with cognitive impairment remain incompletely defined. However, CM is known to be a complex disease and several factors are involved in its development such as the immune system, platelets, nitric oxide (NO), oxidative stress, among others [1,3–5]. These factors integrate a systemic inflammatory response during the
clinical course of a malarial infection that acts in the brain and is responsible, at least in part, for the neurological symptoms and signs presented by both patients and animals [6].

In addition, during the process of learning and memory formation there is an important peptide involved: the brain-derived neurotrophic factor (BDNF) – a member of the neurotrophin family and the most widespread growth factor in the brain. It has diverse functions in the adult brain as a regulator of neuronal survival, fast synaptic transmission, activity dependent synaptic plasticity, and learning and memory formation. Moreover, the hippocampus, a brain area which is required for many forms of long-term memory in both humans and animals, appears to be a particularly important site of BDNF action [7]. Other system that also is very important for cognitive function is the cholinergic system. The major marker of cholinergic metabolism is the activity of the hydrolytic enzyme acetylcholinesterase (AChE) that makes possible a precise temporal control of synaptic activation by rapidly hydrolyzing neurotransmitter acetylcholine (ACh) into acetate and choline. It’s known that ACh is a chemical substance found in the brain that has an important role in memory, attention, reason and language, all behavior parameters. Even though there are only 5–10% of cholinergic synapses in the hippocampus, ACh is released widely near dendrites and it is an essential player in the formation, processes, and its deficits may play important roles in the etiology of neurodegenerative disease [8].

Thus, knowing the fact that the neurobiology of cognitive impairment in CM is not yet entirely clear and that the neurotransphins and the cholinergic system have great importance in the acquisition of memory, the objective of this study was to determine whether the BDNF protein levels and AChE activity in the hippocampus fifteen days after CM induction in survivor mice that received treatment with chloroquine, a classic antimalarial drug.

2. Methods

2.1. Animals

We used 6–8 weeks old C57BL/6 mice (n = 10/group per behavior experiment and n = 5/group per biochemical experiment) from our breeding colony (UNESC), weighing 20–25 g. The animals were kept at controlled temperature (25 °C) with free access to chow and water in a room with a 12-h light/dark cycle (lights on at 7 a.m.). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. All protocols performed were approved by the Ethics Committee at UNESC.

2.2. Design

PbA parasitized red blood cells (PRBC) of C57BL/6 mice donor strains were kept in liquid nitrogen and were thawed and passed into normal mice that served afterwards as parasite donors. Six to eight weeks old C57BL/6 mice were inoculated intraperitoneally with 0.2 mL suspension of 106 parasitized red blood cells (n = 15/group). As a control group (n = 30/group) for infection, mice were inoculated with 106 non-parasitized red blood cells (RBC). Parasitaemia on days 3, 5, 7, and 10 were recorded (data not shown) [3].

The alterations of autonomic function and muscle tone and strength are specific and early signs of CM. Using these criteria, the SHIRPA protocol was prospectively applied on day 6 to identify CM. Positive results diagnosing CM were then taken as an indication to start chloroquine treatment, and to conduct further assessment of cognitive function in CM-positive animals. Interestingly, when we started treatment with chloroquine at 25 mg/kg on day 6, signs of neurological involvement were rapidly responsive and were abolished by day 7 post infection (data not shown) [3]. At the 15th day post infection, 30 animals (10 animals = RBC + saline; 10 animals = RBC + chloroquine and; 10 animals = PbA + chloroquine) were submitted to habituation to the open-field test, and 15 animals (5 animals = RBC + saline; 5 animals = RBC + chloroquine and; 5 animals = PbA + chloroquine) were killed by decapitation, followed by the harvesting of the hippocampus and immediately stored at −70 °C for further evaluation of BDNF protein levels and AChE activity. At the 15th day post infection, 30 animals (10 animals = PbA + saline; 10 animals = PbA + chloroquine and; 10 animals = PbA + chloroquine) were submitted to habituation to the open-field test, and 15 animals (5 animals = RBC + saline; 5 animals = RBC + chloroquine and; 5 animals = PbA + chloroquine) were killed by decapitation, followed by the harvesting of the hippocampus and immediately stored at −70 °C for further evaluation of BDNF protein levels and AChE activity.

2.3. Drug

Chloroquine (Farmanguinhos, Oswaldo Cruz Foundation, Brazil) was directly dissolved in water (w/v). The solutions were prepared immediately before use and protected from light before administration.

2.4. Biochemical analyses

2.4.1. BDNF protein levels

The BDNF levels in hippocampus were measured by anti-BDNF sandwich-ELISA, according to the manufacturer instructions (Chemicon, USA). Briefly, brain slices were homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM (EGTA). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent and standard curve ranged from 7.8 to 500 pg/mL of BDNF. The plates were then washed four times with sample diluent and a monoclonal anti-BNDF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After the addition of streptavidin–enzyme, substrate and stop solution the amount of BDNF was determined by absorbance in 450 nm. The standard curve demonstrates a direct relationship between Optical Density (OD) and BDNF concentration. Total protein was measured by Lowry’s method using bovine serum albumin as a standard.

2.5. AChE activity

Briefly, hydrolysis rate was measured at ACh(S) concentration of 0.8 mM in 1 mL assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of structures homogenate was added to the reaction mixture and pre-incubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25 °C.

All samples were run in duplicate. Protein was measured by Lowry’s method using bovine serum albumin as standard.

2.6. Behavioral test

2.6.1. Habituation to the open field test

This task evaluates motor performance in the training section and non-associative memory in the retention test session. Habituation to an open field was carried out in a 40 cm × 80 cm open field surrounded by 50 cm high walls made of brown plywood with a
frontal glass wall. The floor of the open field was divided into 9 equal rectangles by black lines. The animals were gently placed on the left rear quadrant and left to explore the area for 5 min (training session). Immediately following this, the animals were taken back to their home cage and submitted again to a similar open-field session 24 h later (test session). Crossing of the black lines and rearing performed in both sessions were counted. The decrease in the number of crossings and rears between the two sessions was taken as a measure of the retention of habituation.

2.7. Statistical analysis

The data are presented as mean ± S.E.M. Differences among experimental groups in the experiments evaluating BDNF levels and AChE activity were determined by ANOVA. Multiple comparisons were performed by Tukey test. The data for the habituation to the Open-field were analyzed by the Student’s t test. p values less than 0.05 were considered to indicate statistical significance.

3. Results

Fig. 1 demonstrates the BDNF protein levels in the hippocampus 15 days after induction. There was a significant decrease of BDNF levels in the hippocampus of mice infected with *Plasmodium berghei* ANKA (PbA) when compared with non-infected mice that received treatment with chloroquine (*F* = 0.21; *p* = 0.0039). Fig. 2 shows the AChE activity in the hippocampus 15 days after induction where there was no difference observed (*p* > 0.05). Fig. 3 shows the statistical difference between training and test in mice infected with PbA according to the number of crossings (*t* = 1.829; *df* = 9; *p* = 0.101) and rearsings (*t* = 0.794; *df* = 9; *p* = 0.448), demonstrating cognitive impairment when compared to other groups. Finally, Fig. 4 shows the Pearson correlation. There was a negative correlation (*r* = −0.954; *p* = 0.012) between BDNF levels and number of crossing in the test session in the in mice infected with PbA.

4. Discussion

Approximately 500,000 children develop CM in sub-Saharan Africa each year and around 110,000 die [9]. However, survivors may not fully recover from CM since long-term cognitive impairment is observed in 12–14% of these individuals [2]. In animal model of CM, cognitive dysfunction during the acute phase of experimental infection with PbA is observed in mice. A test of working memory performed on the 7th day of infection demonstrated significant impairment in visual memory in C57BL/6 mice associated to significant histological alterations as well as hemorrhage and inflammation [10]. In a recent report, we demonstrated that CM survivor C57BL/6 mice presented cognitive impairment up to fifteen days after induction. Such cognitive impairment may persist up to thirty days after induction [3]. Since the hippocampus is the core for learning and memory acquisition, it has been hypothesized that adult hippocampal neurogenesis might participate in hippocampal function related to learning and memory. Despite the conflicting evidence about the adult hippocampal neurogenesis and learning and memory, it is certain that several growth factors are involved in the mechanisms regulating adult hippocampal neurogenesis [11]. We evaluated the habituation 15 days after CM induction and observed significant memory impairment.
Although the pathogenesis of CM has been extensively investigated, many aspects of the cellular and molecular mechanisms remain incompletely defined. Two neurochemical parameters, BDNF and AchE activity, that could possibly be related to memory impairment were also investigated. Additionally, the BDNF function is associated with synaptic function and plasticity [12]. Also, normal BDNF levels in the brain are essential to the maintenance of normal learning and memory functions by a process referred to as synaptic consolidation. Moreover, reductions of BDNF levels have been reported in a number of neurodegenerative diseases or associated models [12,13]. Recently, it was demonstrated in adult rats that the hippocampal BDNF levels were positively correlated with the ability to learn [14]. In other models of infectious disease, such as sepsis and meningitis, it was verified that survivor rats presented decreased BDNF levels in the hippocampus and this was correlated with memory impairment [15]. In addition, Ach is also a chemical substance found in the brain that has an important role in memory, attention, reason and language, all behavioral parameters. Ach is an essential player in the formation, maintenance, and evocation of memory processes. In this study, we observed that CM did not alter the AchE activity in hippocampus of the infected animals. The reduction of brain levels of Ach has been associated to cognitive deficits and the treatment with AchE inhibitors increases Ach levels, improving learning and memory in patients with Alzheimer’s disease [16].

In this study, we believe that (1) the cerebral malaria affected only the levels of BDNF in the hippocampus because only 5–10% of synapses in the hippocampus are cholinergics and (2) the decrease of BDNF levels in the hippocampus may be related with memory impairment. In conclusion, our initial experiment indicates that CM survivor mouse presented a decrease in BDNF levels but not in AchE activity in the hippocampus of mice and the memory impairment can be associated with low levels of BDNF in the hippocampus.

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