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Research report

# Evaluation of the brain-derived neurotrophic factor, nerve growth factor and memory in adult rats survivors of the neonatal meningitis by *Streptococcus agalactiae*

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ABSTRACT

*Streptococcus agalactiae* (GBS) is a major cause of severe morbidity and mortality in neonates and young infants, causing sepsis, pneumonia and meningitis. The survivors from this meningitis can suffer serious long-term neurological consequences, such as, seizures, hearing loss, learning and memory impairments. Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) control the neuronal cell death during the brain development and play an important role in neuronal differentiation, survival and growth of neurons. Neonate Wistar rats, received either 10 μL of sterile saline as a placebo or an equivalent volume of *GBS* suspension at a concentration of $1 \times 10^6$ cfu/mL. Sixty days after induction of meningitis, the animals underwent behavioral tests, after were killed and the hippocampus and cortex were retired for analyze of the BDNF and NGF levels. In the open-field demonstrated no difference in motor, exploratory activity and habituation memory between the groups. The step-down inhibitory avoidance, when we evaluated the long-term memory at 24 h after training session, we found that the meningitis group had a decrease in aversive memory when compared with the long-term memory test of the sham group. BDNF levels decreased in hippocampus and cortex; however the NGF levels decreased only in hippocampus. These findings suggest that the meningitis model could be a good research tool for the study of the biological mechanisms involved in the behavioral alterations secondary to GBS meningitis.



## 1. Introduction

*Streptococcus agalactiae* or Group B Streptococcus (GBS) is a major cause of severe morbidity and mortality in neonates and young infants (Baker and Edward, 2003). The incidence of this infection varies in different parts of the world, of which maternal colonization with GBS in the genitourinary or gastrointestinal tracts is the primary risk factor for illness (Allardice et al., 1982). Neonatal infection occurs principally when GBS ascends from the vagina to the amniotic fluid after onset labor or membranes rupture; it also can be aspirated into the fetal lungs while traversing the birth canal (Desa and Trevenen, 1984; Verani et al., 2010). GBS is an important pathogen that causes invasive bacterial infections, such as sepsis, pneumonia and meningitis in neonates during the first week of life (Baker, 2000). The development of this illness shows a resistance to immune clearance allowing bloodstream survival and the ability to breach the endothelial blood–brain barrier (BBB) (Maisey et al., 2008). GBS crosses the BBB without any evidence of intracellular tight-junction disruption or microorganism detection between the cells (Kim, 2008), and it can grow within the cerebrospinal fluid (CSF), which stimulates the neuroinflammatory molecules release (Yadav et al., 2009), occurring the neuronal death. In animal models (Leib et al., 1996a,b; Grandgirard et al., 2007) and in patients suffering from meningitis the neuronal damage is characterized in cortical, subcortical, and hippocampal regions (Free et al., 1996).

The survivors from this meningitis can suffer serious long-term neurological consequences, such as, seizures, hearing loss, learning and memory impairments (Edwards and Baker, 2005).

*Abbreviations:* BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CFU, unity formation of colony; CSF, cerebrospinal fluid; GBS, group B Streptococcus; LTM, long-term memory; NGF, nerve growth factor; OD, optical density; PBS, phosphate buffer solution; PMSF, phenylmethylsulfonyl fluoride; STM, short-term memory.

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The rat model allows for a refined assessment of clinical and neurological symptoms (Leib et al., 2001). Previous studies, we verified impairment in aversive memory in neonatal rats (Barichello et al., 2010a), and decrease of the habituation memory, aversive memory and impairment in recognition objects by adult rats submitted to pneumococcal meningitis (Barichello et al., 2010b).

Neurotrophins control the neuronal cell death during the brain development and play an important role in neuronal differentiation, survival and growth of neurons in peripheral and central nervous system. The nerve growth factor (NGF) was the first neurotrophin discovered with these characteristics (Colafrancesco and Villoslada, 2011), furthermore other important nurotrophin is the brain-derived neurotrophic factor (BDNF), that exert continuing effects on neuronal function in adulthood (Nagahara and Tuszynski, 2011). BDNF protein is in great part distributed throughout the adult brain in almost all cortical areas, as well as various subcortical and spinal cord regions (Soppet et al., 1991). In addition the BDNF reduces multiple forms of neuronal injury in bacterial meningitis (Bifrare et al., 2005). Therefore, the aim of this study was to evaluate the BDNF, NGF levels and memory in adult Wistar rats submitted to meningitis in neonatal period by *S. agalactiae*.

## 2. Materials and methods

### 2.1. Infecting organism

*S. agalactiae*, serotype III which has been isolated from a patient with invasive disease, was cultured overnight in 10 mL of Todd Hewitt broth, diluted in fresh medium and grown to logarithmic phase. The culture was centrifuged for 10 min at ($5000 \times g$) and resuspended in sterile saline to the concentration $1 \times 10^6$ cfu/mL. The accuracy of the inoculum size was confirmed by quantitative cultures (Barichello et al., 2010a; Grandgirard et al., 2007).

### 2.2. Animal model of meningitis

Neonate male Wistar rats (15–20 g body weight), postnatal day 3–4, from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of the UNESC, Brazil, and followed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

All surgical procedures and bacterial inoculations were performed under anesthesia, consisting of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Barichello et al., 2009). Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μL of sterile saline as a placebo or an equivalent volume of *S. agalactiae* suspension at a concentration of $1 \times 10^6$ cfu/mL and were subsequently returned to their cages (Barichello et al., 2011; Trampuz et al., 2007). Meningitis was documented by a quantitative culture of 5 μL of CSF obtained by puncture of the cisterna magna (Barichello et al., 2010b). Meningitis was documented 16 h after bacterial induction by a quantitative culture of 5 μL of CSF obtained by puncture of the cisterna magna (Barichello et al., 2010b). CSF was cultivated on blood agar plates at 35.2 °C and 5.5% $CO_2$ for 12–18 h (Grandgirard et al., 2007) followed by the initiation of the antibiotic treatment (ceftriaxone 100 mg/kg twice a day, i.p., for 7 days).

After 60 days, the animals seemed to be free from infection. We performed blood cultures that were all negative in this period. The animals underwent behavioral tests, after they were killed and the hippocampus and cortex were retired and stored in −80 °C for posterior analyze of the BDNF and NGF.

### 2.3. The habituation to the open-field task

Was carried out in a 40 cm × 60 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 arena for 5 min (training session), and 24 h later submitted again to a similar open-field session (test session). The crossings of the black lines and the rearings performed in both sessions were counted (Comim et al., 2009; Tuon et al., 2008).

### 2.4. The step-down inhibitory avoidance task

Was carried out in the apparatus with a 50 cm × 25 cm × 25 cm acrylic box with the floor consisting of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7 cm-wide by 2.5 cm-high platform was placed on the floor of the box against the left wall. In the training trial, the animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.4 mA, 2.0 s foot shock. A retention test trial was performed at 1.5 h (STM) short-term memory and 24 h long-term memory (LTM) after training. The retention test trial was procedurally identical to training, except that no foot shock was performed (Tuon et al., 2008). Immediately after the behavioral test, the rats were killed and the hippocampus and pre-frontal cortex were removed and stored at −80 °C.

### 2.5. NGF and BDNF levels

Were measured as previously described (Frey et al., 2006), using sandwich enzyme-linked immunosorbent assay, using commercial kits according to the manufacturer's instructions (Chemicon, USA). Briefly, brain slices were homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′N′-tetraacetic acid (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 of BDNF or NGF. Then, plates were washed four times with sample diluents. Monoclonal anti-BDNF rabbit antibody or monoclonal anti-NGF rabbit antibody diluted 1:1000 in sample diluent was incubated for 3 h at room temperature. After washing, a second incubation with anti-rabbit antibody peroxidase conjugated diluted 1:1000 for 1 h at room temperature was carried out. After addition of streptavidin-enzyme, substrate and stop solution the amount of BDNF or NGF was determined for absorbance in 450 nm. The standard curve demonstrates a direct relationship between optical density (OD) and BDNF and NGF concentration. Total protein was measured by Lowry's (1951) method using bovine serum albumin as a standard

### 2.6. Statistics

Data from the BDNF and NGF were analyzed with Student's *t*-test and variables were shown by mean ± SEM of 10 animals in each group. Differences among groups were evaluated by using analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test. In open-field, groups were compared by independent samples Student's *t*-test. Differences between training and testing sessions within each group were analyzed by the paired Student's *t*-test. Open-field data was reported as mean ± SEM. In inhibitory avoidance, groups were compared by the Mann–Whitney test. Differences between training and testing sessions were analyzed by the Wilcoxon test. Data obtained in inhibitory was reported as median ± interquartile ranges (25 and 75). Inhibitory avoidance data analysis data was nonparametric because this procedure involved a cutoff score. P-values lower than 0.05 were considered to be statistically significant.

## 3. Results

We evaluated the memory through habituation to an open-field task and the step-down inhibitory avoidance in adult Wistar rats submitted to meningitis in neonatal period. In the open-field task, Fig. 1A, there were no differences in the number of crossings and rearings between the groups in the habituation to the open-field training and test session ($p > 0.05$), demonstrated no difference in motor, exploratory activity and habituation memory between the groups. In Fig. 1B, the step-down inhibitory avoidance, we verified that there were significant differences when the animal were re-exposed to the apparatus after a gap of 1.5 h (STM) and 24 h (LTM) compared to the latency displayed in the training session, which indicates that memory was acquired for this task, however when we evaluated the long-term memory at 24 h after training session, we found that the meningitis group had a decrease in aversive memory when compared with the long-term memory test of the sham group ($p < 0.05$). The measurement of the BDNF and NGF levels are shown in Fig. 2A and B, respectively. BDNF levels decreased in hippocampus and cortex when compared to the sham group ($p < 0.05$), however the NGF levels decreased only in hippocampus when compared with the sham group ($p < 0.05$). In Fig. 3, we demonstrated that the Spearman correlation coefficient between latency LTM to step-down in the inhibitory avoidance task/BDNF was 0.8611 with $p = 0.0238$.

## 4. Discussion

In this study we showed the influence of the neonatal meningitis by *S. agalactiae* in adult animals, verifying that the animals presented habituation memory; however, in the aversive