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ORIGINAL ARTICLE

Late Anxiety-Like Behavior and Neuroinflammation in Mice Subjected to Sublethal Polymicrobial Sepsis

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Abstract Sepsis can lead to long-term cognitive changes, including memory and learning deficits, which are known as septic encephalopathy (SE). SE also includes behavioral changes. The underlying mechanism of SE is unknown, and several mechanisms have been proposed. This study investigated late anxiety-like behavior, serum cytokine levels and brain cytokine production in C57BL/6 mice subjected to polymicrobial sepsis induced by sublethal cecum ligature and puncture (CLP). Anxiety-like behavior and locomotor activity were assessed in mice 10 days after sham operation or CLP procedure using the elevated plus maze, contextual fear conditioning, and open field test. Brain and serum concentrations of the cytokines TNF-α, IFN-γ, IL-1β, IL-6, and IL-10 were determined by ELISA. We found that mice subjected to polymicrobial sepsis presented anxiety-like behavior, which was accompanied by increased serum TNF-α and brain TNF-α, IFN-γ, IL-1β, and IL-6 levels, 10 days after the surgical procedure. These findings suggest an involvement of central nervous system inflammatory mediators in the anxiety-like symptoms found in SE.

Keywords Septic encephalopathy · Anxiety · Elevated plus maze · Contextual fear conditioning · Open field · Cecum ligature and puncture · CLP · Cytokine · TNF-α · IFN-γ · IL-1β · IL-10 · IL-6

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Introduction

Sepsis is a systemic inflammatory response caused by a pathogenic or potentially pathogenic microorganism. Despite the use of antimicrobial agents and advanced life support, the mortality rate for patients with sepsis has remained between 20 and 30 % throughout the past two decades (Marik 2011).

The unregulated inflammatory response and tissue hypoperfusion induced by sepsis can lead to the dysfunction of multiple organs, as well as to central nervous system (CNS) changes, which results in a condition described as septic encephalopathy (SE).

The diagnosis of SE is challenging and based on the exclusion of other conditions, including direct infection of the CNS, head trauma, fat embolism, drug side effects, and electrolyte disturbances. Furthermore, its clinical presentation is not specific, presenting symptoms that range from mild cognitive signs such as the slowing of mentation, inattention, and agitation, to more severe ones, such as coma. Recently, several studies have demonstrated that intensive care unit survivors present long-term cognitive impairment, including alterations in memory, attention, and/or global loss of cognitive function (McCartney and Boland 1994; Hopkins et al. 2005). Symptoms of anxiety can also be present, with a frequency that varies from 10 to 60 % (Streck et al. 2008).

The pathophysiology of SE is not completely understood, but direct neuronal damage, endothelial dysfunction, unbalanced neurotransmission, blood–brain barrier damage, and cytokine dysregulation appear to be involved (Zampieri et al. 2011). High cerebral levels of pro-inflammatory cytokines have been associated with SE in experimental models (Imamura et al. 2011; Clark et al. 2010). Among the pro-inflammatory cytokines involved in SE, TNF-α seems to be one of the most significant. TNF-α induces neutrophil infiltration into brain tissue, neuronal cell apoptosis, and brain edema (Alexander et al. 2008).

In an attempt to study the long-term effects of sepsis in mice, this study aims to verify the presence of late anxiety-like behavior and changes in serum and brain cytokine levels in animals subjected to cecum ligature and puncture (CLP).

Experimental Procedure

Animals and Surgical Procedure

To perform this study, male C57BL/6 mice (20–25 g) aged 8–12 weeks were obtained from the Animal Care Facilities of the Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG). Animals were housed in cages in temperature-controlled rooms and received food and water ad libitum. All experiments were approved by the Animal Ethics Committee of the UFMG.

Polymicrobial sepsis induced by CLP is currently the most widely used animal model of sepsis (Doi et al. 2009). Animals were subjected to CLP as previously described (Wichterman et al. 1980). Briefly, mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. Under aseptic conditions, a 2-cm midline laparotomy was performed to expose the cecum with the adjoining intestine. The cecum was ligated with a 4.0 silk suture at its base, below the ileocecal valve, and was perforated three times with a 22 gage needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and returned to the peritoneal cavity. The laparotomy was then closed with 4.0 silk sutures. Animals were resuscitated with normal saline (50 mL/kg intraperitoneally) immediately after CLP. All animals were returned to their cages after complete recovery from anesthesia, with free access to food and water. In the sham-operated group, mice were subjected to all surgical procedures, but the cecum was neither ligated nor perforated.

Elevated Plus Maze Test

Anxiety-like behavior was assessed in CLP and sham-operated mice 10 days after the surgical procedure using the elevated plus maze (EPM; Insight®, SP, Brazil) (seven animals per group). The chosen time point was based on previous data from Tuon et al. (2008). This widely used test for anxiety-like behavior in rodents has been previously described by Handley and Mithani (1984). Briefly, the apparatus consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) extending off a central platform (5 × 5 cm). The arms were arranged such that the two arms of each type opposed each other. The maze was 50 cm high. Mice were placed in the center of the maze facing an open arm and were allowed to freely explore the EPM for 5 min. The animal was considered in the arm if it placed all four paws onto the arm; otherwise, the animal was considered within the center of the maze. The time spent within each arm of the EPM and entries made into the open and closed arms were recorded for each mouse. The percentage (%) of time spent in the open arms and the number of open arm entries were used as measures of anxiety. The number of closed arm entries was used as a measure of locomotion. In the EPM paradigm, reduced open arm activity indicates increased anxiety levels. Between each trial, the maze was wiped clean with a damp sponge and dried with paper towels. After behavioral assessment, the animals were killed by decapitation, and the brain and serum were collected.

Contextual Fear Conditioning Test

Contextual fear conditioning is a paradigm used to assess anxiety-like behavior (Blanchard and Blanchard 1969) and was performed using an apparatus consisting of a clear rectangular Plexiglas® box with a floor of parallel metallic rods for the delivery of electric current (Insight®, SP, Brazil). Ten days after surgery, five CLP and five sham-operated mice were allowed to freely explore the new environment for 3 min. Then, three pulses of electric current (0.5 mA) were delivered through the metallic rods for 2 s with an interval of 40 and 80 s between pulses. Animals were then left in the cage for an additional 1 min before returning to their home cage. Twenty-four hours later, animals were introduced to the conditioning box again and monitored for freezing for 5 min before being returned to their home cage.

Open Field Test

The open field (Insight®, SP, Brazil) test was performed as previously described by Hall (1934) and Walsh and Cummins (1976) and conducted to evaluate the locomotor activity of CLP and sham-operated mice 10 days after the surgical procedure (seven animals per group). Briefly, animals were gently placed in the central area and left to explore the arena for 5 min. Total distance traveled and

velocity of motion during open field activity were automatically analyzed by tracking the center of the animal using "ANY-maze" video-tracking software (Stoelting Co., Wood Dale, IL, USA).

Serum and Brain Cytokine Measurements

To perform the ELISA assay, the entire right hemisphere of the brain was homogenized in extraction solution containing aprotinin. The concentration of the cytokines TNF-α, IFN-γ, IL-1β, IL-10, and IL-6 was then determined by ELISA (R&D Systems, Minneapolis, MN, USA and Pharmingen, San Diego, CA, USA). Five sham-operated animals and seven CLP mice were processed for ELISA. In addition, serum was obtained from five animals of each group to determine the concentration of these same cytokines by ELISA (R&D Systems, Minneapolis, MN, USA and Pharmingen, San Diego, CA, USA).

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Student's *t* test was used to analyze anxiety-like behavior in the EPM and fear conditioning tests and locomotor activity in the open field test, as well as to compare cytokine levels from brain and serum. A value of $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Sublethal Polymicrobial Sepsis Promoted Anxiety-Like Behavior in the EPM and Contextual Fear Conditioning Tests

CLP mice exhibited a significant reduction in the percentage (%) of time spent in and the number of entries into the open arms as compared with sham-operated mice 10 days after surgery (Fig. 1a: $p = 0.015$, $t = 2.85$, $n = 7$ animals per group; Fig. 1b: $p = 0.024$, $t = 2.58$, $n = 7$ animals per group). No difference was found in the number of entries into the closed arms between CLP and sham-operated mice, indicating that both groups exhibited similar locomotor activity in the EPM (Fig. 1c: $p = 0.14$, $t = 1.59$, $n = 7$ animals per group).

We also performed the fear conditioning test in CLP and sham-operated mice 10 days after surgery and found a clear increase in the freezing response, as measured by time spent freezing (Fig. 2: $p = 0.04$, $t = 2.34$, $n = 5$ animals per group).

Sublethal Polymicrobial Sepsis Did Not Affect Motor Activity

No differences were observed in CLP and sham-operated mice with respect to total distance traveled or average velocity (Fig. 3a: $p = 0.93$, $t = 0.09$, $n = 7$ animals per group, Fig. 3b: $p = 0.90$, $t = 0.13$, $n = 7$ animals per group).

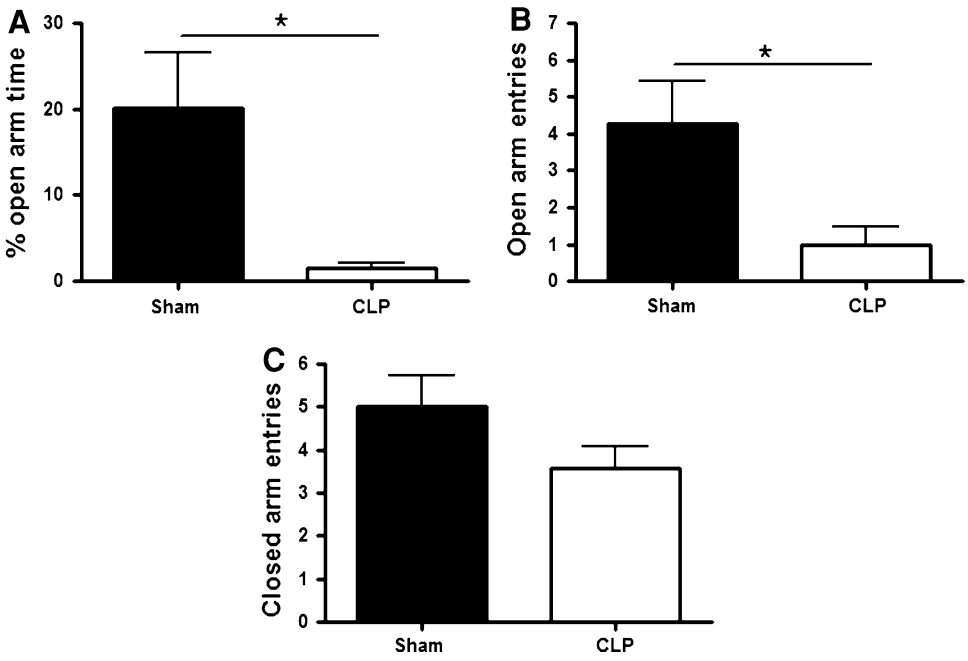

Fig. 1 Elevated plus maze test 10 days after sham operation or sublethal CLP. **a** % open arm time, **b** open arm entries, and **c** closed arm entries. The results are expressed as the mean ± SEM from seven animals per group. An *asterisk* indicates statistical significance, $^{*}p < 0.05$

Effects of Sublethal Polymicrobial Sepsis on Serum and Brain Cytokines

Serum levels of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β, and IL-6, and the anti-inflammatory cytokine IL-10 were measured in mice subjected to CLP or the sham procedure 10 days after surgery (Fig. 4). CLP mice presented higher serum TNF-α levels when compared to sham-operated animals (Fig. 4a: $p = 0.02$, $t = 2.86$, $n = 5$ animals per group). Serum levels of IFN-γ (Fig. 4b: $p = 0.31$, $t = 1.08$), IL-1β (Fig. 4c: $p = 0.30$, $t = 1.14$), IL-10 (Fig. 4d: $p = 0.47$, $t = 0.78$), and IL-6 (Fig. 4e: $p = 0.14$, $t = 1.72$) did not differ significantly between CLP and sham-operated mice (five animals per group).

Cytokine levels of TNF-α, IFN-γ, IL-1β, IL-6, and IL-10 were measured in brain extracts of mice 10 days after sepsis induction or sham operation (Fig. 5). There was a significant increase in brain TNF-α (Fig. 5a: $p = 0.04$, $t = 2.31$), IFN-γ (Fig. 5b: $p = 0.0003$, $t = 5.37$), IL-1β (Fig. 5c: $p < 0.0001$, $t = 7.18$), and IL-6 (Fig. 5e: $p = 0.04$, $t = 2.35$) levels in CLP mice compared with the sham-operated group. Similar brain levels of IL-10 (Fig. 5d: $p = 0.57$, $t = 0.57$) were detected in both the CLP and sham groups. Five sham-operated animals and seven CLP-treated mice were used.

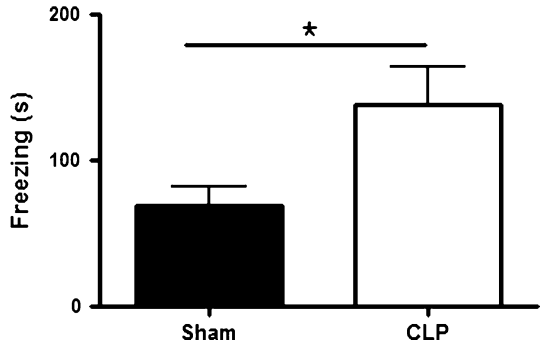

Fig. 2 Total freezing time in contextual fear conditioning test 10 days after sham operation or sublethal CLP. The results are expressed as the mean ± SEM from five animals per group. An *asterisk* indicates statistical significance, $*p < 0.05$

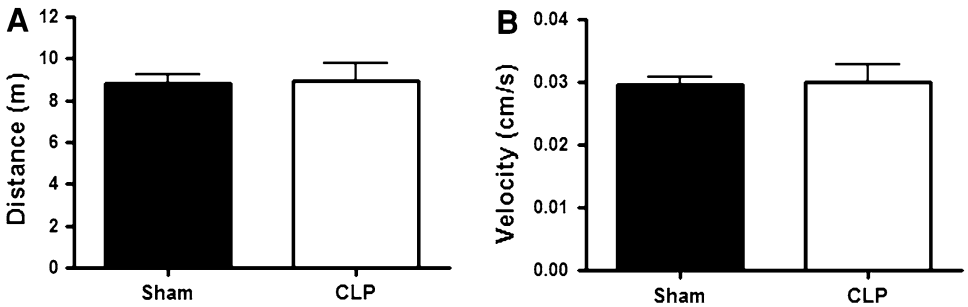

Fig. 3 Open field test 10 days after sham operation or sublethal CLP. **a** Total distance (m) and **b** average velocity (cm/s). The results are expressed as the mean ± SEM from seven animals per group. No statistical differences were found

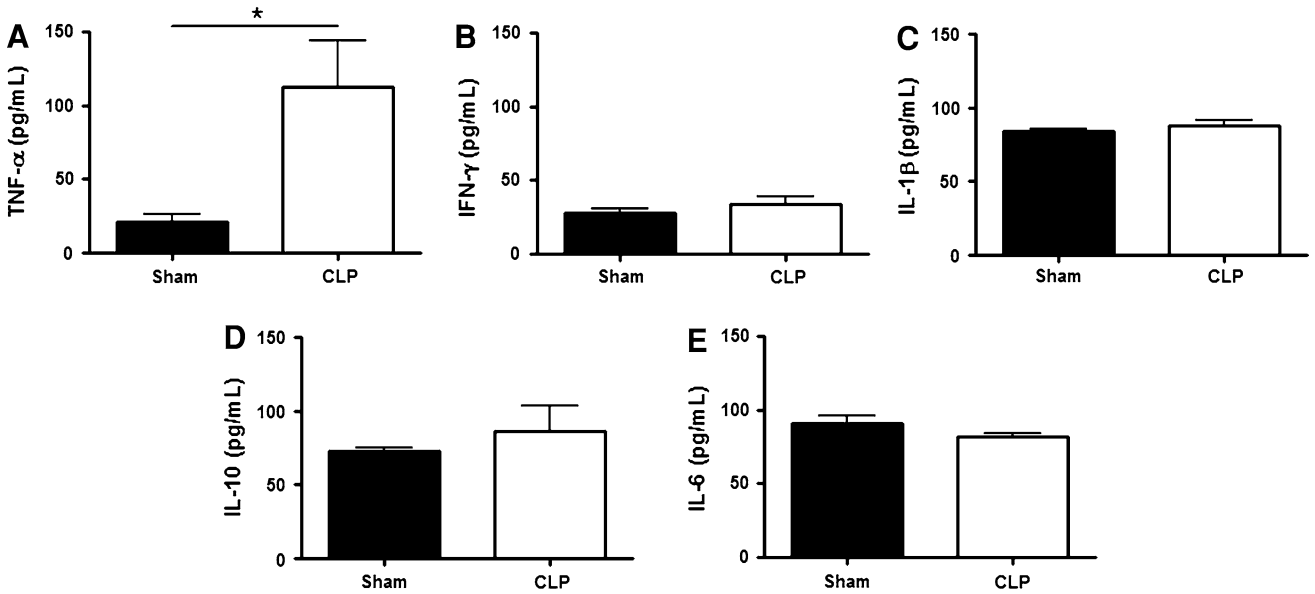

Fig. 4 Comparative analysis of serum levels of pro-inflammatory cytokines TNF-α (**a**), IFN-γ (**b**), IL-1β (**c**), and IL-6 (**e**), and anti-inflammatory cytokine IL-10 (**d**) in CLP and sham mice 10 days after surgery. The results are expressed as the mean ± SEM from five animals per group. An *asterisk* indicates statistical significance, $*p < 0.05$

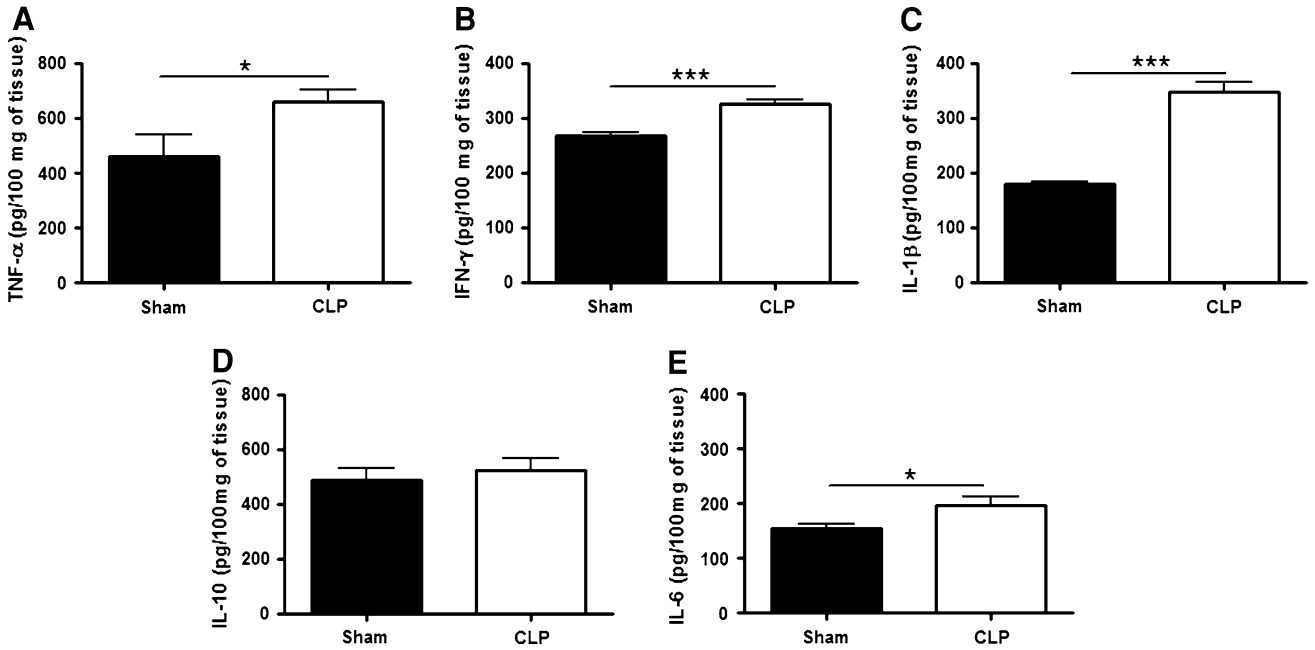

Fig. 5 Comparative analysis of brain levels of pro-inflammatory cytokines TNF-α (**a**), IFN-γ (**b**), IL-1β (**c**), and IL-6 (**e**), and anti-inflammatory cytokine IL-10 (**d**) in CLP and sham mice 10 days after surgery. The results are expressed as the mean ± SEM from five sham-operated and seven CLP-treated mice. *Asterisks* indicate statistical significance, $*p < 0.05$ and $***p < 0.001$

Discussion

To the best of our knowledge, this is the first study to demonstrate late anxiety-like behavior in mice submitted to sublethal CLP. This behavioral change was accompanied by elevated levels of inflammatory cytokines in the brain and increased serum levels of TNF-α.

Previous clinical and experimental studies have demonstrated the association between anxiety and inflammatory cytokine levels in the periphery and CNS (Reichenberg et al. 2001; Bauhofer et al. 2004; Swiergiel and Dunn 2007; Comim et al. 2011). For instance, it has been shown that the activation of the immune system via low doses of endotoxin induces anxiety in humans. The severity of anxiety was positively correlated with serum levels of TNF-α and IL-6 (Reichenberg et al. 2001). Our group has demonstrated that in the early course of CLP-induced sepsis there was enhanced production of the cytokines IL-1β, IL-10, and TNF-α in the brain, which was associated with changes in performance in behavioral tasks assessing fear and anxiety (Comim et al. 2011).

TNF-α is a pivotal cytokine in sepsis, and high serum levels of TNF-α predict poor clinical outcomes in septic patients (Pinsky et al. 1993). Although TNF-α was initially thought to cause cell death in the CNS, it is now widely appreciated to be a physiological gliotransmitter that functions in normal communication among astrocytes, microglia, and neurons, and therefore plays a role in synapse regulation (Clark et al. 2010). TNF-α also seems to alter synaptic function through its ability to regulate neuronal morphology (Kubota et al. 2009).

Some studies have demonstrated that although IL-1β is required for normal learning and memory processes, excessive levels of IL-1β produce detrimental cognitive and behavioral effects (Rachal Pugh et al. 2001). Interleukin-1 receptor null mice show decreased anxiety-related behavior, but no change in locomotor activity, indicating an anxiogenic effect of IL-1β (Koo and Duman 2009). Intracerebroventricular administration of IL-1β and TNF-α also elicit similar behavioral responses in the elevated plus maze, with both cytokines causing an anxiogenic response (Connor et al. 1998). Moreover, it is known that a synergistic interaction between IL-1β and other cytokines, such as TNF-α and IFN-γ, leads to a greater level of neurotoxicity, as observed in traumatic, ischemic, or excitotoxic animal models (Allan et al. 2005). Additionally, elevated circulating levels of IL-6 predict depressed mood and anxiety in healthy and clinical populations (Morozink et al. 2010).

Regarding IFN-γ, animal and clinical findings support a main role for this cytokine in mood disorders and anxiety (Kwant and Sakic 2004). Severity of anxiety in humans has been correlated with serum levels of IFN-γ (Maes et al. 1998). Therapeutic administration of interferons may induce an array of psychiatric side effects including fatigue, depression, anxiety, and irritability, as well as cognitive changes (Kwant and Sakic 2004).

There are limited data about the influence of anti-inflammatory cytokines on cognition. It has been hypothesized

that IL-10 may affect emotionality or anxiety levels in animals through its inhibitory effect on IL-1β, IFN-γ, and TNF-α production, and not through a direct effect on neuronal circuits (Mesquita et al. 2008).

As a limitation of our study, we note that this is a descriptive study and cannot show causality. Other inflammatory mediators may also be involved in anxiety-like behavior during SE. More studies are needed to better describe late behavioral changes and their association with inflammatory parameters and brain damage.

In conclusion, we observed late anxiety-like behavior accompanied by elevated levels of brain pro-inflammatory cytokines after induction of experimental SE, suggesting a possible involvement of CNS inflammation in this neuropsychiatric syndrome.

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