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Evaluation of potential gender-related differences in behavioral and cognitive alterations following pilocarpine-induced *status epilepticus* in C57BL/6 mice

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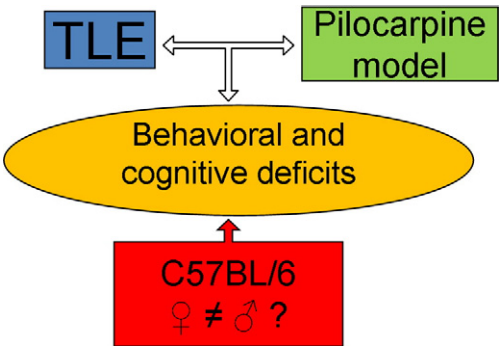
HIGHLIGHTS

- Pilocarpine-induced *SE* elicits long-term behavioral disturbances in C57BL/6 mice.
- Epileptic mice, regardless of gender, display increased anxiety-like behavior.
- Long-term object recognition memory is impaired in epileptic mice of both genders.
- Only male mice show altered performance in the forced swim test.
- Hilar cell loss is of similar magnitude in epileptic mice of both genders.

GRAPHICAL ABSTRACT



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ABSTRACT

Together with pharmacoresistant seizures, the quality of life of temporal lobe epilepsy (TLE) patients is negatively impacted by behavioral comorbidities including but not limited to depression, anxiety and cognitive deficits. The pilocarpine model of TLE has been widely used to study characteristics of human TLE, including behavioral comorbidities. Since the outcomes of pilocarpine-induced TLE might vary depending on several experimental factors, we sought to investigate potential gender-related differences regarding selected behavioral alterations in C57BL6 mice. We found that epileptic mice, independent of gender, displayed increased anxiety-like behavior in the open-field test. In the object recognition test, epileptic mice, regardless of gender, showed a decreased recognition index at 24 (but not at 4) hours after training. On the other hand, no significant differences were found regarding mice learning and memory performance in the Barnes maze paradigm. Motor coordination and balance as assessed by the beam walk and rotarod tests were not impaired in epileptic mice of both genders. However, female mice, independent of epilepsy, performed the beam walk and rotarod tasks better than their male counterparts. We also found that only male epileptic mice displayed disturbed behavior in the forced swim test, but the mice of both genders displayed anhedonia-like behavior in the taste preference test. Lastly, we found that the extent of hilar cell loss is similar in both genders. In summary, both genders can be successfully employed to study behavioral comorbidities of TLE; however, taking the potential gender differences into account may help choose the more

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appropriated gender for a given task, which may be of value for the minimization of the number of animals used during the experiments.

1. Introduction

Epilepsy is a common neurological disease that affects approximately 0.6% of the entire population [1]. Temporal lobe epilepsy (TLE) is the most prominent of the acquired epilepsies, it is considered the most common type of partial complex seizure in adulthood [2]. TLE is commonly preceded by an initial brain injury, such as an episode of prolonged seizures or *status epilepticus* (*SE*), complicated childhood febrile seizures, hypoxia or traumatic brain injury, which leads to chronic epilepsy with spontaneous recurrent seizures (SRS) [1]. In addition to SRS, most epilepsy patients display behavioral comorbidities, including but not limited to depression, anxiety and psychosis, and impaired cognitive performance [3].

Most of the characteristics of TLE can be reproduced in chronic animal models, such as the pilocarpine model [4]. Pilocarpine-induced *SE* elicits the appearing of SRS, and also abnormal behaviors that are similar to behavioral abnormalities in patients with TLE. Therefore, the pilocarpine model has been very useful to the study of the relationship between epilepsy and its behavioral comorbidities [4].

Outcomes of pilocarpine-induced *SE* might vary depending on species and strain, mainly with respect to seizure sensitivity and seizure-induced effects [5]. In fact, several studies have shown differences in seizure susceptibility and seizure-induced effects between different mouse strains [6], and even between mice of the same strain that have been bred separately [7,8].

C57BL/6 mice are one of the oldest and most widely used inbred strains in biomedical research, and are commonly used as a genetic background to create transgenic and knockout mice [9]. Using a behavioral test battery for studying behavioral and cognitive alterations in the pilocarpine model of TLE in C57BL/6 mice, Müller and colleagues in 2009 found that epileptic mice exhibit behavioral and cognitive alterations reflecting the several disturbances that are associated with epilepsy in humans [8]. Given the importance of pilocarpine model of TLE in C57BL/6 mice and in light of the need for prevent, limit, and reverse the comorbidities associated with epilepsy and its treatment [4], further characterization of the behavioral alterations elicited by pilocarpine-induced *SE* is important. In this context, to the best of our knowledge, the influence of the gender in the behavioral alterations following pilocarpine-induced *SE* in C57BL/6 mice has not been studied yet. Therefore, the present study aimed to investigate the occurrence of gender-related differences regarding selected behavioral alterations in C57BL6 mice after pilocarpine-induced *SE*, in order to shed some light on the impact of gender in this model of TLE.

2. Materials and methods

2.1. Animals and reagents

Male and female C57BL/6 mice (20–30 g; 30–60 day-old at the time of *SE* induction) were used. Animals were maintained under controlled light and environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to water and food (SupraTM, Santa Maria, RS, Brazil). All experimental protocols were designed in the aim to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislations (guidelines of Brazilian Council of Animal Experimentation — CONCEA — and of U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals — PHS Policy), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process 098/2012).

Methylscopolamine, pilocarpine and diazepam were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri) and were dissolved in 0.9% NaCl to 0.1 mg/mL, 32 mg/mL and 2 mg/mL, respectively.

2.2. Induction of SE by pilocarpine

SE was defined by continuous stage 3 to 5 seizures [10] during at least 30 min without regaining consciousness (unresponsiveness to any environmental stimuli) together with loss of postural control. This definition is consistent with that being commonly used in the rat pilocarpine model [11].

To induce the *SE* in mouse, pilocarpine was injected intraperitoneally (i.p.). In order to avoid peripheral cholinergic effects, methylscopolamine (1 mg/kg; i.p.) was administered 30 min before the application of pilocarpine. For induction of seizures, an initial injection of 320 mg/kg of pilocarpine was administrated (i.p.). If during the first hour the animal was not in *SE*, a second dose of 225 mg/kg of pilocarpine was injected, and if 40 min after the second dose the mice was not in *SE* another injection of pilocarpine (225 mg/kg) was applied, thus, the maximum number of repeated pilocarpine injections was restricted to three.

All the mice that developed *SE* received diazepam (10 mg/mL) after 60 min in *SE*, to stop seizure activity. To facilitate recovery after *SE*, all the mice were injected with 0.5 mL of Ringer solution (NaCl 130 mmol/L, KCl 4 mmol/L, $CaCl_2$ 1,5 mmol/L, glucose 20 mmol/L) in the afternoon following *SE* as well as at least twice daily during the next three days. No systematic observations were done to detect spontaneous seizures in all the mice, but we noted clear convulsive behavior in most mice during handling, weighing or other manipulations. In addition, several studies have shown that all the C57BL/6 mice experiencing *SE* become epileptic [7–9].

2.3. Behavioral testing

The general procedures for behavioral tests in pilocarpine-epileptic mice were carried out according to Gröticke et al. [12]. Accordingly, behavioral tests started 2 months after *SE* (i.e. in the chronic phase of pilocarpine-induced epilepsy). In the first set of experiments, the animals were evaluated in the following paradigms: open-field, object recognition, beam walk, taste preference and forced swim tests. The sequence of behavioral tests was organized from the least to the most aversive, with an inter-test interval of at least 1 day [12]. In the second set of experiments a separated group of animals was subjected to the Barnes maze test, and a third independent group of animals was used for the rotarod test and for histological analysis. All behavioral tests were carried out under artificial light and controlled conditions of temperature (22 ± 1 °C). Any animal presenting a clearly visible seizure (stage ≥3 in the Racine's scale) at least 1 h before the test was not tested in that same day. If an animal presented a seizure during the test, it was not included in the data analysis pertinent to that parameter/test. By using this approach, one *SE* animal of each gender was excluded from all analyses.

The experiments were arranged in a way that eliminated every disturbance or affection by the presence of other mice or the experimenter of the respective mouse that was subjected to a behavioral experiment, except when the mice needed to be handled and observed by an experimenter.

2.4. Open field

The animals were placed in the central area of a round open field (56 cm in diameter), which had its floor divided into 10 equal areas.