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Alterations in the threshold of the potassium concentration to evoke cortical spreading depression during the natural estrous cycle in mice

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ABSTRACT

Cortical spreading depression (CSD) has been implicated in a variety of neurological disorders. However, the relationship between serum sex hormones and susceptibility to the development of CSD in naturally estrous cycling female animals is largely unknown. The natural estrous cycle of mice consists of four stages, namely, proestrus, estrus, metestrus and diestrus. We measured the serum concentration of estradiol and progesterone in estrus and diestrus and compared the minimum potassium concentrations necessary to evoke CSD in each stage and in males. In diestrus, the minimum potassium concentration required to evoke CSD was significantly lower compared to the other three phases and male animals. The serum level of estradiol is significantly higher and serum level of progesterone is significantly lower in diestrus compared to estrus. Furthermore, when we administered an estrogen receptor antagonist, the susceptibility to the development of CSD was decreased. Conversely, the administration of a progesterone receptor antagonist increased the susceptibility to CSD. Our results demonstrated that neuronal excitability related to CSD induction differs among the natural estrous phases in mice.

1. Introduction

The phenomenon of cortical spreading depression (CSD) was initially reported by Leão as a reversible response of the rabbit cerebral cortex that manifested itself as the near-complete depolarization of neurons and glial cells, followed by sustained suppression of spontaneous neuronal activity (Leão, 1944). CSD is provoked by electrical, mechanical or chemical stimulation with potassium to a point of the cortical tissue in various animals. CSD is known to spread through the cortical tissue from the initiation site at a rate of 2–5 mm/min, with the deflection of DC potential and subsequent suppression of electroencephalogram activity (Ayata and Lauritzen, 2015). CSD research in animal models has provided important information about its pathophysiological role in many neurological disorders, including migraine and stroke (Bolay et al., 2002; Dreier, 2011; Iwashita et al., 2013; Moskowitz et al., 2004; Sukhotinsky et al., 2010; Toriumi et al., 2016).

Migraine headaches are known to predominantly affect women; their prevalence in women is approximately three times higher than that in men (Lipton et al., 2002; Sakai and Igarashi, 1997). The precise mechanism underlying migraines has not been established, but CSD is thought to have a fundamental role in migraines, especially in the development of the aura that accompanies a migraine (Hadjikhani et al., 2001; Hauge et al., 2009; Moskowitz et al., 2004).

In addition to neuronal activity related to the estrus cycles observed in seizure or hypoxic tolerance (Kasischke et al., 1999; Scharfman and MacLusky, 2006), much attention has been focused on the relationship between CSD and sex steroid hormones, such as estrogen and progesterone, because of the epidemiological predominance of women among migraine sufferers (Bolay et al., 2011; Brennan et al., 2007; Eikermann-Haerter et al., 2009; Guedes et al., 2009; Sachs et al., 2007). However, most studies exploring this relationship have been conducted in experimental settings involving ovariectomy or the exogenous administration of female sex steroids, and there is little data on alterations in the susceptibility to the development of CSD with regard to the natural estrous cycle. In the present study, we examined the threshold for evoking CSD after determining the stage of the female estrous cycle in mice. We obtained *in vivo* evidence that the susceptibility to the development of CSD differs during the stages of the natural estrous cycle in mice.

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2. Material and methods

2.1. Animals

Animals were used with the approval (No. 09058) of the Animal Ethics Committee of Keio University (Tokyo, Japan), and all experimental procedures were in accordance with the university's guidelines and the ARRIVE (animal research: reporting *in vivo* experiments) reporting guidelines for the care and use of laboratory animals. All of the procedures were undertaken with utmost caution to minimize the suffering of the animals. To maintain the regularity of the natural estrous cycle, the animals were housed in temperature-controlled rooms on a 12-h light–dark cycle. A total of 112 C57BL/6J mice (13 male, 99 female; CLEA Japan, Inc., Tokyo, Japan) aged 7–18 weeks were used. For CSD measurement, mice were assigned to the different experimental groups: (i) proestrus stage ($n = 13$), (ii) estrus stage ($n = 16$), (iii) metestrus stage ($n = 15$), (iv) diestrus stage ($n = 13$), (v) male ($n = 13$), (vi) mifepristone administration ($n = 13$), (vii) tamoxifen administration ($n = 9$). For the assessment of the serum hormone levels, we used 20 mice; estrus stage ($n = 10$) and diestrus stage ($n = 10$).

2.2. Identification of the mouse estrous cycle

In mice, an estrous cycle consists of four distinct stages: proestrus, estrus, metestrus and diestrus. To classify the approximate stage of the estrous cycle, we carried out visual inspection of the vagina as shown in the supplemental file (Supplemental file 1). Then, vaginal cytology was performed to verify the precise stage of the mouse estrous cycle (McLean et al., 2012). For cytology, vaginal mucous membrane cells were collected on a slide glass. After being air-dried, the collected cells were stained with Giemsa stain solution. The staging of the estrous cycles was carried out by observing the presence of leukocytes, cornified epithelial cells, and nucleated epithelial cells in the fluid. All of the animals used for the experiments were found to repeat the natural estrous cycle every 4–5 days. The regularity and reproducibility of the natural estrous cycles of the mice was confirmed by the above methods for at least 10 consecutive days (once a day) before the mice were used for the CSD experiments.

The representative pictures of vaginal fluid cytology are shown in Fig. 1. There was a predominance of nucleated epithelial cells in the proestrus stage (Fig. 1A). In the estrus stage, we observed many cornified epithelial cells (Fig. 1B). In the metestrus stage, a large number of leukocytes and nucleated cells were observed (Fig. 1C). In the diestrus stage, there were a few leukocytes, nucleated and cornified epithelial cells (Fig. 1D).

2.3. Drug administration

Mifepristone (Cayman Chemical Company, MI, USA; 10 mg/kg, in sesame oil), which is a progesterone receptor antagonist, or tamoxifen (Cayman Chemical Company, MI, USA; 10 mg/kg, in sesame oil), which is an estrogen receptor antagonist, were intraperitoneally administered to female mice for four consecutive days. We checked the vaginal cytology to examine possible effects of these drugs on estrous cycling, and the threshold for CSD induction was measured on the next day of the last injection.

2.4. Measurement of DC potential

Female C57BL/6J mice (CLEA Japan, Tokyo) were anesthetized with isoflurane (1.0% in room air with the flow rate 400 mL/min) *via* a concentration-controllable anesthesia unit (400; Univentor, Zejtun, Malta). Body temperature was maintained with a heating pad and thermocontroller (BWT-100; Bioresearch Center Co., Nagoya, Japan). Each mouse was fixed to a head-holder (SG-4N, modified to be flexible around the horizontal axis; Narishige Scientific Instrument Laboratory, Tokyo).

For the measurement of DC potentials, an Ag/AgCl electrode (tip diameter = 200 μm, EEG-5002Ag; Bioresearch Center Co.) was inserted 200 μm under the pia mater (2 mm posterior and 2 mm lateral to the bregma) and fixed with dental cement. Ag/AgCl reference electrodes (EER-5004Ag; Bioresearch Center Co.) were placed in the subcutaneous tissue. DC potentials were amplified at 1–100 Hz and digitized at 1 kHz with a differential headstage and differential extracellular amplifier (Models 4002 and EX1; Dagan Co., Minneapolis, MN) (Unekawa et al., 2012). Mean blood pressure and heart rate were continuously recorded through the tail using a non-invasive blood pressure monitor as physiological parameters (Model MK-2000ST; Muromachi Kikai Co., Tokyo, Japan).

CSD was induced by applying a 5-μL drop of KCl solution of varying concentrations into an additional posterior 0.5 mm-diameter hole with its center 4 mm posterior and 2 mm lateral to the bregma. Initially, 0.025 M potassium was applied to the cranial window, and the potassium concentration was gradually increased by 0.025 M until CSD was induced. We compared the minimal potassium concentrations required to evoke CSD in each stage of the natural estrous cycle.

2.5. Assessment of the serum hormone levels

We also measured the serum concentrations of estradiol and progesterone in the estrus and diestrus stages of the estrous cycle by liquid chromatography (*i.e.*, the tandem mass spectrometry method [LC–MS/MS]).

2.6. Statistical analysis

Unless otherwise stated, all of the numerical data are expressed as the mean ± SD. SPSS for Windows (SPSS Inc., Chicago, IL) version 22 was used for all statistical analyses. The minimal potassium concentrations necessary to evoke CSD in each stage of the natural estrous cycle were compared using Kruskal–Wallis tests, followed by Mann–Whitney *U* tests. The serum concentrations of estradiol

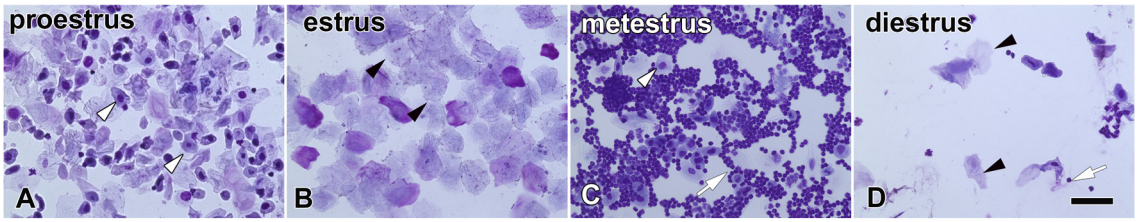

Fig. 1. The four stages of the mouse estrous cycle indicating the cytological findings of the vaginal fluid from each stage. (A) Proestrus stage, (B) estrus stage, (C) metestrus stage and (D) diestrus stage. Arrows indicate leukocytes, white arrowheads show nucleated epithelial cells, and black arrowheads indicate cornified epithelial cells. Scale bar = 50 μm.

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