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Formalin-induced nociceptive behavior and c-Fos expression in middle-aged female rats

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ABSTRACT

The impact of the estrous cycle on the nociceptive response in middle-aged female rats was assessed using the formalin test and c-Fos immunoreactivity as a marker of neural activation. Young (2-month-old) and middle-aged (11-month-old) rats were examined, dividing the middle-aged rats into two groups based on their estrous cycle: regular 4-day estrous cycle and irregular estrous cycle. The right hind paw was subcutaneously injected with 50 μl of 2% formalin or saline as a control. Behavioral changes were observed for 1 h. Cycling rats were used during proestrus. Middle-aged female rats had a significantly higher score for nociceptive behavior compared to young rats, irrespective of estrous cyclicity, which suggests that aging, not the ability to maintain estrous cyclicity, causes hypersensitivity to the formalin injection. Immunohistochemical analysis found that the brain response to formalin injection was also more sensitive in middle-aged rats than young rats; a significant increase in the number of c-Fos immunoreactive cells was found in the ventral portion of the lateral septum of middle-aged rats injected with formalin compared to young and middle-aged rats injected with saline, irrespective of estrous cyclicity. Based on these results, we conclude that the sensitivity to painful stimuli in middle-aged female rats, which are in a neuroendocrine state similar to pre- and peri-menopausal women, is associated with age and not affected by reproductive ability.

1. Introduction

Women often report pain-related symptoms during menopause [1,2], and pain is a significant health problem for middle-aged women [3]. However, the factors that affect the pain response in menopausal females are not clear. A lack of understanding regarding these factors and mechanisms is mostly due to limited studies on age-related changes in nociception and pain behavior, which have only been performed in males [4]; no studies have focused on females during the arrest of spontaneous ovulation. For example, an age-associated change has been demonstrated in the sensitivity to formalin-induced tonic pain, which peaks at mid-life [5], but the study only used male rats.

Reproductive aging in female rodents is characterized by an arrest of spontaneous ovulation, with a transition from regular to irregular cycling in middle age [6,7]. Because the menopausal transition in women and female rats share significant characteristics, including patterns of luteinizing hormone secretion and responsiveness to estradiol, middle-aged female rats are an appropriate model for studying reproductive aging [7]. Importantly, changes in the blood estrogen levels of pre- and peri-menopausal women during aging [8] are similar to those of middle-aged rats [9–11]; the responsiveness to estrogen in the brain plays an essential role in the initiation of reproductive senescence before any changes in circulating estrogens [7].

In females, estrogens are not only essential for reproduction [12], but they are also an important factor in the modulation of pain responses. Interestingly, although the formalin test is a model of behavioral responses to tissue injury (phase 1) and inflammation (phase 2) [13,14], an estrogen effect is evident during interphase [15–18]. Thus, estrogens, the responsiveness to estrogens, and/or aging per se may alter pain responses in aged female rats, and the formalin test is suitable for measuring these alterations.

If 17β-estradiol is an important factor for pain responses, a change in pain-related behavior would not be observed unless serum 17β-estradiol is altered. If the responsiveness to 17β-estradiol is important, pain-related behavior would be different between regular-cycling and irregular-cycling groups of female rats. If aging itself is important, female rats would exhibit altered pain responses regardless of reproductive state. Therefore, the present study examined changes in the response to formalin-induced nociceptive stimuli using a behavioral test and focusing on the effect of the estrous cycle in middle-aged rats with unchanged serum 17β-estradiol levels [9–11]. In addition to behavioral examinations, we analyzed c-Fos expression

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as a marker of neural activity in order to determine the cerebral region associated with middle-age-related changes in the pain response.

2. Methods

2.1. Animals

Female 2-month-old (young) and 11-month-old (middle-aged) Wistar rats (Charles River, Yokohama, Japan) were maintained at a constant temperature of 24–26 °C under controlled lighting conditions (lights on 05:00–19:00) with food and water available ad libitum. Daily vaginal smears were obtained, and rats exhibiting three or more consecutive 4-day estrous cycles were defined as having a regular cycle. For young rats, only those exhibiting a regular cycle were used. Middle age was defined as 11 months of age because it is when rats transition from a regular estrous cycle to an irregular cycle [7]. The middle-aged rats were divided into two groups: rats exhibiting a regular 4-day estrous cycle (R group) and those that had ceased cycling (IR group). Regular-cycling rats were used on the day of proestrus. Irregular-cycling rats exhibiting persistent estrus or persistent diestrus were examined on these days. Twenty-four hours before formalin injection, a silicone cannula for intravenous anesthetic injection was implanted into the right atrium of all rats under ether anesthesia. This procedure did not interfere with the estrous cycle. The right hindpaws of some young (173 ± 3.3 g, $n = 8$) and middle-aged rats (R group 376.3 ± 3.4 g, $n = 8$; IR group 374.3 ± 4.5 g, $n = 11$) were injected with 50 µl of 2% formalin and behavioral changes observed for 1 h. The remaining young (172.1 ± 4.1 g, $n = 7$) and regular middle-aged rats (375 ± 7.6 g, $n = 6$) were injected with saline on the day of proestrus as a control. All rats were sacrificed by an overdose of intravenous pentobarbital (100 mg/kg) and subjected to immunohistochemical analysis.

Heparinized phosphate buffer (PB; pH 7.5) at approximately 4 °C was perfused through the cardiac ventricle, followed by paraformaldehyde (4%) in PB. After perfusion, the brain was removed from the cranium, fixed at 4 °C overnight in PB containing 4% paraformaldehyde, and incubated at 4 °C overnight in 25% sucrose in PB. The brains were then frozen with powdered dry ice and stored at −70 °C until processed for immunohistochemistry.

Blood samples were taken before the injection and the serum 17β-estradiol concentrations determined using an EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA). Serum samples were extracted once with diethyl ether and reconstituted with assay buffer. All animal housing and surgical procedures were in accordance with the guidelines specified by the institutional animal care and use committee of Yokohama City University School of Medicine. The experiments followed the ethical guidelines of the International Association for the Study of Pain [10].

2.2. Behavioral test

Immediately after the formalin injection, the rat was placed in a transparent Plexiglas box ($30 \times 30 \times 30$ cm) with a transparent floor positioned over a mirror angled at 45° to allow for observations of nociceptive behavior. The nociceptive behavior assessment [13,14] was performed on a single parameter by scoring the time of elevation for the injected paw. This simple method successfully detects sex differences in the formalin test [18] and was chosen because changes in single behavioral responses may be overlooked when using the weight-scores method [19]. We determined the length of time that the paw was elevated from the floor every 5 min for 1 h, and a mean response was calculated for each phase. For statistical purposes, the data were separated into three phases: phase I (0–5 min after formalin injection), interphase (5–15 min after injection), and phase II (15–60 min after injection).

2.3. Immunohistochemistry

Frozen coronal sections (30 µm) were cut using a Bright cryostat and washed with 0.1 M phosphate buffered saline (PBS). Samples were incubated overnight with rabbit polyclonal c-Fos antibody diluted 1:40,000 (PC38, Ab-5, Calbiochem) in PBS containing 1.5% normal goat serum and 0.1% Triton X-100. The next day, sections were incubated with biotinylated anti-rabbit IgG (1:200), followed by incubation with streptavidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Vectastain Lab).

Bound peroxidase was visualized by incubating the sections for 8 min in 0.05% 3,3′-diaminobenzidine with H_2O_2. Samples were then mounted on glass slides, dehydrated in graded alcohol, cleared in xylene, and coverslipped with Permount.

The lateral septal nucleus (LS) was subdivided into the dorsal (LSD), intermediate (LSI), and ventral (LSV) regions [20] (Fig. 1), and the number of c-Fos immunoreactive (c-Fos-ir) cells in each region was determined by an investigator blinded to the experimental conditions. Two sections per rat were selected and matched across all animals in all experimental groups. Microscopic images (5×10 magnification) were imported into a computer with a Penguin 600CL digital camera (Pixera Corporation, Los Gatos) and analyzed using Image-Pro plus version 5.1 (MedikaCybernetics, Inc.). The cut-off value was defined so that the number of nuclei obtained by this method was consistent with the number obtained by visual inspection.

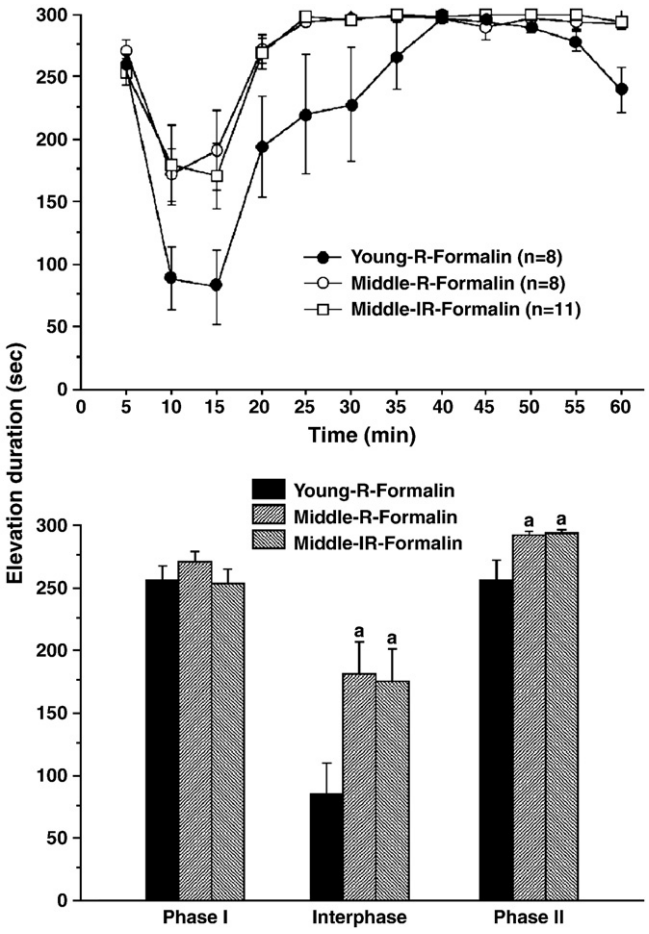

Fig. 1. Behavioral responses of young and middle-aged female rats during the formalin test. R, regular 4-day estrous cycle; IR, irregular estrous cycle. Each data point in the upper panel represents the amount of time the animals raised the injected paw (elevation duration) every 5 min during 1 h of observation. For statistical purposes, the data were separated into three phases in the lower panel: phase I (0–5 min after formalin injection), interphase (5–15 min after injection), and phase II (15–60 min after injection). The data are presented as mean ± SEM. a $P<0.05$ vs. young rats.