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Chronic stress influences nociception sensitivity of female rats in an estrous cycle dependent manner

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Abstract

Exposure to chronic stress can influence nociception, and further induce hyperalgesia. Whether stress modulation on pain in female animals occur in an estrous cycle-specific manner is still unclear. We profiled the changes in nociception (thermal, mechanical, formalin induces acute and inflammatory pain) of female Sprague-Dawley rats after treatment with chronic unpredictable mild stress (CUMS) and investigated whether these changes occur in an estrous cycle dependent manner. The results showed that CUMS female rats exhibited a lower mechanical withdrawal threshold in proestrus and estrus, a longer formalin induced licking time in metestrus and diestrus, but no changes in the latency time on the tail flick test. The present study findings suggest that chronic stress induces mechanical and formalin-evoked acute hyperalgesia of female rats in an estrous cycle dependent manner.

Keywords: chronic unpredictable mild stress; stress-induced hyperalgesia; nociception; estrous cycle; hyperalgesia; pain

Introduction

Stress is a non-specific adaptive response to a variety of stimuli which can lead to physical, immunological and psychological diseases (Schneiderman et al., 2005). Several studies have shown that exposure to chronic stressful events in life can increase the risk for psychiatric disorders and elicit reactions of hyperalgesia or allodynia (Jennings et al., 2014, Duman et al., 2016). Research findings on both human and animals have indicated that males and females show sex differences in behavioral,

psychological, endocrine and molecular responses to stress (Lu et al., 2015, Reschke-Hernandez et al., 2017). However, females are more vulnerable or susceptible to stress-related disorders compared to males (Bangasser and Valentino, 2012). Accumulated evidence also supported that, there is increased nociception and low tolerance of females to pain when compared to males (Rosen et al., 2017). This indicates that being a male or female play an important role in understanding susceptibility of an individual to stress-related responses and pain sensitivity (Rosen et al., 2017, Seo et al., 2017). The gonadal hormones including testosterone and estradiol have been reported to determine the hypothalamus-pituitary-adrenal (HPA) axis response in different sexes after acute stress (Heck and Handa, 2019).

Effects of the estrous cycle on the nociception have been reported in both clinical and animal studies. In a functional magnetic resonance imaging (fMRI) study conducted among humans, the stress response of women showed alterations in nociception?? during various phases of the menstrual cycle (Goldstein et al., 2010). Several studies in rats have also found a low threshold and a high sensitivity of nociception??? in the proestrus phase (Moloney et al., 2016, Kaur et al., 2018); however, another study suggested that there is no difference in nociception during the women's menstrual cycle (Balter et al., 2013). Chronic unpredictable mild stress (CUMS) causes significant changes in female rats during the diestrus phase in behavior response and stress-related molecules activation (Lu et al., 2015). However, it is still not clear, whether female animals have different stress-related pain responses in different estrous phases. Given the influence of gonadal hormones on the activity of the HPA axis, we

hypothesize that the effect of chronic stress on pain differ depending on the phase of the estrous cycle. This study aimed to investigate the changes in different nociception (thermal, mechanical, formalin-evoked acute and inflammatory pain) of female rats after chronic stress treatments.

Methods

Animals

Female Sprague–Dawley rats (Animal Centre of the Second Affiliated Hospital, Harbin Medical University, Certificate No.09-2-1) weighing between 150-170 g on arrival were used in this study. Rats were individually housed in cages during the five weeks of the study. The rats were maintained at 22 ± 2 °C with 12:12 light dark cycle. Food and water were available ad libitum. All the experimental procedures were approved by the Institutional Animal Care and Use Committee, Harbin Medical University, PR China.

Rats were randomly assigned into the following two groups: (1) Control rats, n=15; (2) CUMS rats, n=24. Control rats were maintained in normal condition, and CUMS rats were exposed to chronic stressors according to the CUMS procedures protocols as described by Liu et al., (2014) and Lian et al., (2017). The estrous phase was determined by the examination of vaginal changes. Vaginal cytology samples were collected daily in the morning. The phase of the estrous cycle (metestrus, diestrus, proestrus or estrus) was determined by microscopic examination based on the types of cells (leukocytes, nucleated epithelial or cornfield epithelial cells) (McLean et al., 2012). According to the phase of the estrous cycle of each rat, Control and CUMS rats were further divided to eight subgroups: (1) control in the proestrus phase (P control rats); (2) control in the estrus phase (E control rats); (3) control in the metestrus phase (M control rats); (4) control in the diestrus phase (D control rats); (5) CUMS in the proestrus phase (P

CUMS rats); (6) CUMS in the estrus phase (E CUMS rats); (7) CUMS in the metestrus phase (M CUMS rats); and (8) CUMS in the diestrus phase (D CUMS rats).

CUMS female rats' model

The CUMS procedures were performed according to the previously reported CUMS protocols (Liu et al., 2014, Lian et al., 2017). All CUMS rats were treated by one stressor each day for 35 days. The stressors included: damp bedding overnight; high platform; restraint stress; food deprivation (24 hours); swimming in 4 ℃ cold water for 5 minutes; water deprivation (24 hours). Stressors were scheduled randomly and administered at any time of day throughout the 5-week's experiment. The stress sequence was changed every week for unpredictable stress procedure. Control animals had no contact with CUMS rats. During and after the CUMS exposure, nociception behavioural analyses were performed by an observer blind to the experimental conditions.

Tail flick test

Tail-flick test (Nazeri et al., 2014) was performed after vaginal smear in the morning on the 3rd day of every week and used to measure the pain response to acute thermal noxious stimuli. The rats were maintained in a tube and placed on the apparatus (PL-200, Taimeng, Chengdu, China). Their tails were allowed to hang freely. A beam of light with 55% intensity was focused at a 5 cm distant on the rat's tail. The tail-flick latency was defined as the time from turning on the light to tail flick to the side. To avoid tissue hot damage, a cut-off time of 10 seconds was defined as the maximal thermal pain latency. The average tail flick latency time was calculated from three consecutive tests with an interval of about five minutes.

von Frey test

Mechanical paw-withdrawal thresholds were tested after vaginal smear in the morning on the 4th day of every week using the up-and-down method as described by

Chaplan et al., (1999). The rats were placed in a transparent cage with a mesh floor. Ten von Frey filaments were applied and the test was initiated with a 2.0 g filament to the plantar surface of the paw through the mesh floor. Depending on whether rats showed positive (a brief paw withdrawal) or negative responses (without any paw withdrawal), the next weaker or stronger filament was chosen. Counting of six consecutive points did not begin until the first positive response occurred. The 50% mechanical withdrawal threshold (50% MWT) was then calculated using the formula proposed by Chaplan et al., (1994).

Formalin test

Inflammatory pain thresholds were measured in the morning on the 36th day using the formalin test (Roche et al., 1996). Fifty μl of formalin 5% was injected subcutaneously into the right hind paw pad. Then rats were immediately placed in a transparent chamber with an open roof to observe spontaneous pain responses of the injected paw. The licking time of Phase I (during 0 - 10 minute after injection) and Phase II (during 11 - 60 minute after injection) were recorded.

Statistical analyses

Analysis of data was performed using SPSS 19.0 software (IBM). All data were expressed as the mean ± standard error of mean (S.E.M). Friedman measure analysis of variance was performed to test the differences in 50% mechanical withdrawal thresholds between CUMS female rats and controls. One-way repeated measures ANOVA followed by Bonferroni's post-hoc test was performed to determine the differences in the tail flick latency, and the body weight between CUMS female rats and controls. Independent t-test was performed to determine the differences in the licking time of the formalin test between the group analyses. Due to the small number

in each subgroup, Mann-Whitney U was used to determine differences in the 50% mechanical withdrawal thresholds, the licking time in the formalin test, the tail flick latency between CUMS subgroup and its corresponding control subgroup at a specific phase of the estrous cycle. Spearman rank correlation was used to test the association. Statistical significance was determined as $p < 0.05$.

Results

Mechanical pain sensitivity of the estrous cycle after exposure to chronic stress

Results of von Frey tests showed that 50% mechanical withdrawal threshold (MWT) was significantly (χ^2 (9) = 21.89, p = 0.009) decreased in the female CUMS rats compared to the control group, during the 3rd week (2.48 ± 1.7 g vs. 5.31 ± 4.1 g) and the 5th week (2.54 ± 1.5 g vs. 5.48 ± 4.3 g) after the stress exposure. This indicated that the stress-induced mechanical hyperalgesia occurred from the 3rd week (Figure 1A).

The MWT decreased significantly in the P CUMS rats compared to the P controls during the 5th week (2.05 ± 1.4 g vs. 7.46 ± 4.1 g, U = 2.00, p = 0.023) (Figure 1B). However, no statistical significant difference in the mean MWT was observed among the other CUMS subgroups (estrus, metestrus and diestrus) when correspondingly compared to their control subgroups. These results indicated that CUMS rats in the proestrus stage showed more sensitive to mechanical stimulus than the control rats in the proestrus. There was no difference between the subgroups of the control rats.

At the 3rd week the rat number of the control subgroups in the proestrus was only 1, so was the number of CUMS subgroups in the proestrus at the 4th week. The number of rats left over as at the end of the 3rd week in the proestrus control subgroups as well as the 4th week in the proestrus CUMS subgroups were 1 each, respectively. Therefore, the rats in the proestrus and estrus were combined into one group (P/E subgroup with high levels of gonadal hormones), while the rats in the metestrus and diestrus were

combined into one group as well (M/D subgroup with low levels of gonadal hormones for subgroup statistical analyses) (Egan et al., 2018). During the 3rd (2.57 ± 1.44 g vs. 6.01 ± 4.03 g, U = 33.00, p = 0.005) and the 4th (2.76 ± 2.05 g vs. 5.28 ± 4.00 g, U = 45.00, p = 0.033) weeks, the mean MWTs were significantly reduced in P/E CUMS rats compared to the controls (Figure 1C, D). During the 1st and 2nd weeks, there were no difference in the mean MWTs between the CUMS subgroups and their corresponding control subgroups (Figure 1E, F).

CUMS induced acute hyperalgesia in M/D female subgroup in the formalin test

Chronic stress exposure did not change the average licking time in formalin induced pain in both phases I (t= -1.44, p = 0.16) and phase II (t= -0.60, p = 0.55) (Figure 2A). However, there was a significant increase in the average licking time among the M/D CUMS rats when compared to the M/D control group (9.00 ± 2.79 sec vs. 5.19 ± 2.91 sec, U=10.00, p = 0.045) in phase I (Figure 2B) but not in phase II (Figure 2C). These results suggested that formalin-evoked acute but not inflammatory hyperalgesia in female CUMS rats in the metestrus and diestrus stages. There was no difference in the licking time among the control subgroups. In the CUMS rats, a positive correlation was found between MWTs and the average licking time in phase I of the formalin test (r_s (22) = 0.412, p = 0.045). No correlation was found between MWTs and the average licking time in phase I of the formalin test in the control rats.

Thermal pain sensitivities of female rats did not vary after exposure to chronic stress

Tail-flick tests were performed every week to study the changes in thermal nociception during the stress exposure. Chronic stress treatment did not change the tail-flick latency ($F_{(4,148)}$ = 0.991, p = 0.259, at the 5th week 5.53 ± 1.44 sec vs. 6.01 ± 1.31 sec, at the 4th week 6.34 ± 1.92 sec vs. 5.48 ± 1.22 sec) (Figure 3A). Furthermore, there was no difference between CUMS subgroups compared to their corresponding control

subgroups (at the 5th week for P/E 5.63 ± 1.6 sec vs. 6.15 ± 1.37 sec, for M/D 5.41 ± 1.23 sec vs. 5.13 ± 0.09 sec, $\chi^2(3) = 3.52$, $p = 0.32$; at the 4th week for P/E 6.05 ± 2.07 sec vs. 5.19 ± 1.27 sec, for M/D 6.6 ± 1.81 sec vs. 6.3 ± 0.6 sec, $\chi^2(3) = 6.63$, $p = 0.085$) (Figure 3B).

Rats in the CUMS group exhibited a retardation in body weight gain

There was no significant difference in basic body weight between CUMS rats and control rats prior to initiation of the CUMS treatment. Rats in CUMS group exhibited a retardation in body weight gain from the 7th day until the end of the CUMS procedure, compared to the rats in the control group (Figure. 4A; the 7th day: 161.75 ± 20.0 g vs.193.98 ± 14.7 g; the 35th day: 233.26 ± 29.52 g vs.267.5 ± 9.3 g, $F_{(2.997,110.887)} = 144.864$, $p = 0.002$). In both the CUMS rat (Figure. 4B; rs (22) = - 0.47, $p = 0.021$) and the control rat groups (rs (13) = - 0.675, $p = 0.006$), weight gain fraction (body weight at 35th day-basic body weight)/ basic body weight) was negatively correlated with 50% MWT in the von Frey test.

Discussion

Understanding stress related hyperalgesia mechanisms which underlie the prevalence of persistent pain conditions in women is important for improving women's health. Even though confirmed conclusion has not yet been achieved on chronic mild stress-induced hyperalgesia in female animals, we designed an experiment to characterize the change of nociception sensitivity among female rats across the estrous cycle. Our findings indicated that (1) chronic stress induced mechanical hyperalgesia in proestrus and estrus females, whereas formalin-evoked acute hyperalgesia but not thermal hyperalgesia in metestrus and diestrus rats; (2) there were positive correlations between the mechanical withdrawal thresholds (MWTs) and formalin-evoked acute hyperalgesia after chronic stress exposure, but a negative correlations between

mechanical withdrawal thresholds and the body weight gain. We noticed that the von Frey MWTs in the control rats were lower than what was usually reported for rats of the size used in a previous experimental study (Chaplan et al., 1994). Since the vaginal smear procedure is also a known stressor, it should be considered to play a potential role in affecting the nociception responses.

In the present experiment, a positive correlations were found between mechanical withdrawal thresholds and the average licking time in phase I of the formalin test in CUMS rats but not in the controls. This result indicated that the correlation between the mechanical nociception and formalin induced acute nociception depends on the chronic stress condition. Also, the negative correlations found between the weight gain fraction and 50% MWT in both CUMS and control rats, indicated that the weight gaining rats are associated with a lower mechanical pain threshold and are also susceptible to stress-induced mechanical hyperalgesia.

Although these mechanisms are not fully understood, there might be sex differences contributing to the changes of nociception induced by chronic stress exposure. Previous studies have reported that women are at increased risk for many chronic pain conditions compared to men (Fillingim et al., 2009, Mogil, 2012). In our preliminary study after the exposure of male rats to chronic stress, we found that the male rats showed a transient mechanical hyperalgesia, but demonstrated thermal and formalin-induced acute and inflammatory hypoalgesia, suggesting that male rats could have distinct pain response depending on the different types of pain after chronic stress (Lian et al., 2017). Results in the present study showed that female rats were highly susceptible to mechanical and formalin-induced acute nociception but not to thermal pain under chronic stress condition, evidencing that female rats exhibited the different changes of nociception compared to male rats after chronic stress exposure (Lian et al.,

2017). The difference in response to pain in relation to sex might be explained by the differences in sex hormones and microglia activity (Sorge and Totsch, 2017).

Responses of the hypothalamic–pituitary–gonadal axis (HPA) axis to stress have been associated with the female estrous cycle (Stephens et al., 2016). After restrictive stress treatment, the deprived females at the proestrus phase had a higher corticosterone level compared to the normal females (Mourlon et al., 2011). Corticosterone has been shown to decrease the expression of cannabinoid receptor 1 in dorsal root ganglion neurons of rats with chronic stress induced visceral hyperalgesia. (Hong et al., 2011). Stress sensitivity in women also seems to be linked with the variations in ovarian hormones during the menstrual cycle (Handa and Weiser, 2014). Fluctuations in gonadal hormones have been found to modulate the way males and females react to stress and nociception (Oyola and Handa, 2017, Rosen et al., 2017), although the exact mechanism is still unclear. A previous study conducted among females suggested that estrogen and its receptors produce antinociceptive and antihyperalgesic effects (Robinson et al., 2016) whereas another study reported a contradictory results (Nag and Mokha, 2016).

The data in this current study confirmed that estrous cycle and chronic stress are critical factors in mechanical pain and formalin-evoked acute nociception in female rodents. Mechanical hyperalgesia occurred in CUMS rats in proestrus and estrus, but in contrast, formalin-evoked acute hyperalgesia was observed in metestrus and diestrus; and positive correlations between mechanical thresholds in von Frey tests and the licking time in phase I of the formalin test. Several studies, however, have reported different conclusions regarding how the effect of stress on the estrous cycle induces nociception (Devall et al., 2011, Moloney et al., 2016). Research conducted among rats who underwent maternal separation in early life showed a decreased pain thresholds

and an increased pain behaviours to colorectal distension (visceral pain) across all phases of the estrous cycle (Moloney et al., 2016). Exposure to mild stress induced a decrease in tail flick latency and hyperalgesia in animals in the late diestrus phase (Devall et al., 2011). These discrepancies in rats stress models may be due to stressor diversity and nature of pain.

Conclusion

In conclusion, our studies showed that chronic stress influences nociception sensitivity of female rats in an estrous cycle-dependent manner. Future studies are warranted to elucidate these potential mechanisms which underlie the hypothalamic–pituitary-gonadal axis modulation of stress-induced hyperalgesia in females.

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