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Mildly Increased Mechanical Nociceptive Sensitivity in REV-ERBα Knock-out Mice

Jaehyun Lee[1], Hyoung-Gon Ko[1], Kyungjin Kim[2] and Bong-Kiun Kaang[1]*

[1]Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 08826,
[2]Department of Brain and Cognitive Science, DGIST, Daegu 42988, Korea

Nociception is one of the most complex senses that is affected not only by external stimulation but also internal conditions. Previous studies have suggested that circadian rhythm is important in modulating nociception. REV-ERBα knock-out (KO) mice have disrupted circadian rhythm and altered mood-related phenotypes. In this study, we examined the role of REV-ERBα in inflammatory nociception. We found that the nociceptive sensitivity of KO mice was partially enhanced in mechanical nociception. However, this partial alteration was independent of the circadian rhythm. Taken together, deletion of REV-ERBα induced a mild change in mechanical nociceptive sensitivity but this alteration was not dependent on the circadian rhythm.

Key words: Circadian rhythm, REV-ERBα, Nociception, Inflammatory pain

INTRODUCTION

The circadian rhythm is an endogenous clock that has an approximately 24 hour cycle. Clinical trials have demonstrated significant differences in nociceptive sensitivities depending on the circadian rhythm in various diseases, such as burning mouth syndrome [1] and symptomatic knee osteoarthritis [2]. In animal studies, the thermal nociceptive sensitivity of male mice shows daily variation [3]. These findings suggest that pain perception could be affected by circadian rhythm.

Accumulating data from many studies imply that a nuclear receptor, REV-ERBα, regulates circadian rhythm. REV-ERBα is an orphan nuclear receptor, which is encoded by the *NR1D1* gene [4, 5]. REV-ERBα not only regulates cyclic *Bmal1* expression but also controls *Clock* and *Cry1* mRNA expression [6]. BMAL1 and CLOCK are transcription factors, which play a central role in the circadian rhythm by forming a BMAL1/CLOCK heterodimer [7]. Moreover, abnormal phase-shifting of the circadian system was observed in KO mice [6]. However, the role of REV-ERBα in nociceptive sensitivity is still unknown. In this study, we examined acute and chronic pain sensitivities in KO mice.

MATERIALS AND METHODS

Animals

REV-ERBα knock-out (KO) (n=8) and wildtype (WT) (n=12) mice with a C57BL/6J background were used. Both male and female mice were used and all experiments were performed as blind tests. All animals were single-housed in temperature-controlled (approximately 24°C) conditions, with food and water provided *ad libitum* under a 12-hour light-dark cycle (lights on 9:00 a.m., lights off 9:00 p.m.). After the experiment, mice were returned to their own home cage. All experimental procedures were approved by the Institutional Animal Care and Use

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*To whom correspondence should be addressed.
TEL: 82-2-880-7525, FAX: 82-2-884-9577
e-mail: kaang@ snu.ac.kr

Committee of Seoul National University.

Behavioral experiments

All behavioral tests were performed at circadian time (CT) 08-12 and CT 22-02. Mice were separated into two groups as a counterbalance for the time of the first exposure to the experiment. The investigators performing the tests were blind to the genotype.

Hot plate test

The hot plate test was performed as previously described [8]. Briefly, mice were placed in a behavior room for at least 2 hr to adapt to the experimental environment. The mice were then placed onto a hot plate at 55°C and latency of the first reaction (e.g., licking, shaking, jumping, and lifting of a hind paw) was recorded manually. To prevent tissue damage, if mice did not show any response within 20 s, the test was terminated and 20 s was recorded as the latency. The experiment was performed three times with 10 min intervals. The average of the three values was used as the latency to respond.

Tail flick test

The tail flick test was performed as previously described [8]. Briefly, mice were placed in a behavior room for at least 2 hr to acclimate to the experimental environment. Mice were then placed under the heat source. Heat was applied to the tail, approximately 1 cm from the tip, and the latency of the reflex withdrawal of the tail from the heat source was recorded manually. To avoid tissue damage, if mice did not show any response within 20 s, the test was terminated and 20 s was recorded as the latency. The experiment was performed three times with 10 min intervals. The average of the three values was used as the latency to respond.

Electronic von Frey test

The electronic von Frey test was performed as previously described [9]. Briefly, the electronic von Frey test was performed on Day 0, Day 3, and Day 7. After the Day 0 test, 10 μl 50% complete Freund's adjuvant (CFA) (diluted with saline) was injected in the right hind paw. Mice were placed in a behavior room for at least 1 hr to acclimate to the experimental environment. Electronic von Frey stimuli were given to the right hind paw with at least 1 min intervals. The paw withdrawal (flinching, licking, and shaking) threshold was measured automatically. Five values were acquired per mouse. The highest and lowest values were excluded, and the average of the remaining values was recorded as the paw withdrawal threshold.

Data analysis

Two-way repeated measures ANOVA and one-way repeated measures ANOVA were used to compare values between the KO and WT groups. All data are presented as mean±SEM.

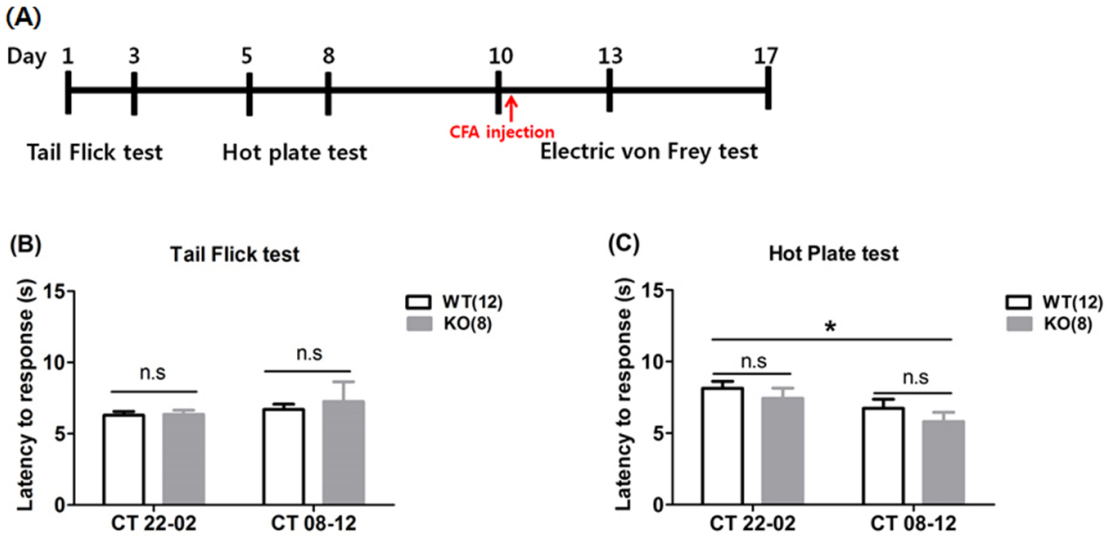

Fig. 1. Thermal nociceptive sensitivity measured by latency to respond to heat stimulus. (A) Behavioral test scheme. (B) Tail flick test. Thermal nociceptive sensitivity was assessed by the latency of tail withdrawal response (two-way repeated measures ANOVA analysis: interaction p=0.6902, CT p=0.3051, genotype p=0.6312). (C) Hot plate test. Thermal nociceptive sensitivity evaluated by the latency of paw withdrawal response (two-way repeated measures ANOVA analysis: interaction p=0.8287, CT p=0.0112, genotype p=0.2640, *p<0.05). All data are displayed as mean±SEM (WT mice: n=12; KO mice: n=8).

RESULTS

To investigate the nociceptive sensitivity of KO mice, we measured acute and chronic pain sensitivities. In addition, we examined whether nociceptive sensitivity of KO mice is affected by circadian rhythm. Both male and female mice were used in each pain test. There are no significant differences between both groups (Data not shown). The acute pain test and chronic pain test were performed at CT 08-12 and CT 22-02 because KO mice had previously been shown to have abnormal mood and anxiety-like behavior at CT 08-12 but not CT 22-02 [10]. First, we examined acute nociceptive sensitivity by measuring their basal thermal perception. In the tail flick test, KO mice and WT mice did not show any significant difference in their latency to respond (Fig. 1A). However, in the hot plate test, the latency to respond to thermal stimuli was decreased in both KO mice and WT mice at CT 08-12 (Fig. 1B). There was no difference in acute nociceptive sensitivity depending on the genotype.

Next, we decided to examine whether KO mice showed any change in chronic pain depending on circadian rhythm. Baseline mechanical nociceptive sensitivity on Day 0 did not show any significant difference between both genotypes (Fig. 2A). However, paw withdrawal thresholds were significantly reduced on Day 3 and Day 7 in both genotypes compared with Day 0. One-way repeated measures ANOVA analysis suggests that mechanical nociceptive sensitivity was increased by injection of CFA and maintained until Day 7 in all groups (Fig. 2A). Interestingly, the withdrawal threshold in KO mice decreased on Day 3 (genotype p=0.0034) compared with WT mice, but not Day 7 (genotype p=0.7066) (Fig. 2B~D). Overall, all groups did not show any alteration of mechanical nociceptive sensitivity depending on circadian rhythm. Taken together, our results suggest that the KO of REV-ERBα partially increased mechanical nociceptive sensitivity but not thermal nociceptive sensitivity.

DISCUSSION

In the present study, we showed that KO mice exhibit increased mechanical nociceptive sensitivity in electronic von Frey test, and this phenomenon was not dependent on the circadian rhythm. Both WT and KO mice showed altered acute nociceptive sensitivity in accordance with circadian rhythm in the hot plate test. Therefore, our hot plate test results support a previous study showing that thermal nociceptive sensitivity of male mice

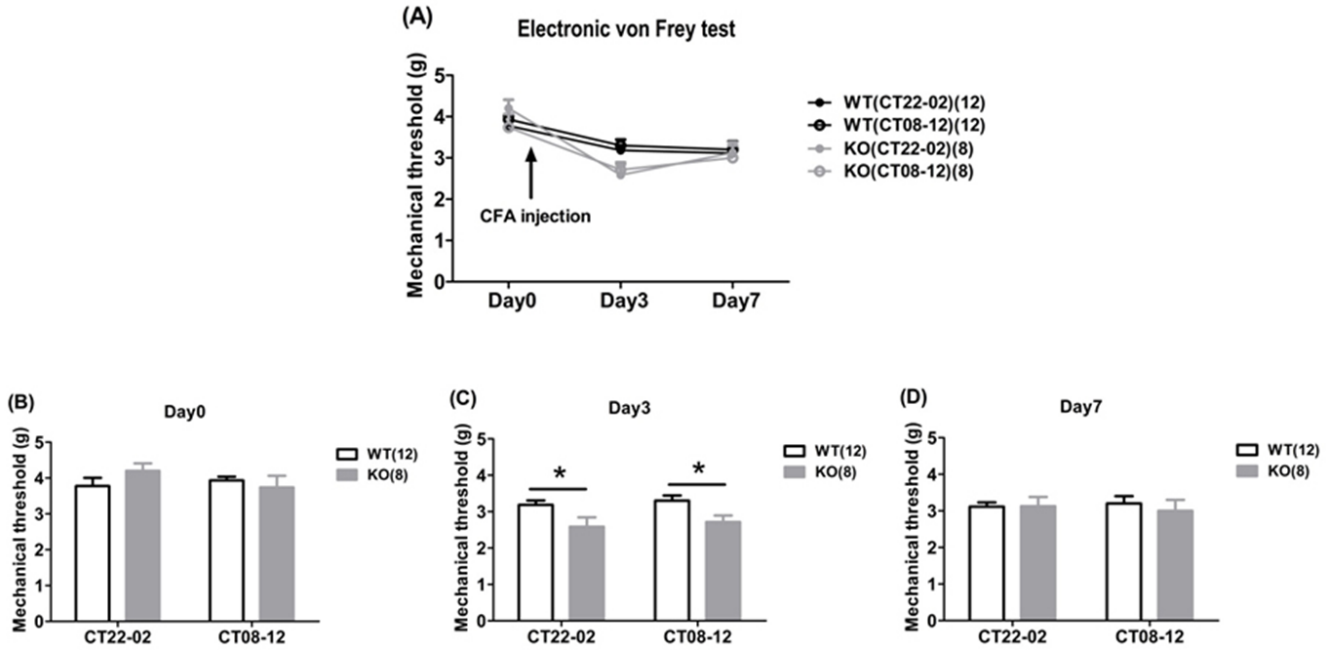

Fig. 2. Mechanical nociceptive sensitivity estimated by paw withdrawal threshold on all test days. (A) CFA treatment significantly decreased paw withdrawal latency on Day 3 and Day 7. Daily variation of mechanical nociceptive sensitivity was not observed on all test days. All data are displayed as mean±SEM (one-way repeated measures ANOVA analysis: WT mice (CT 22-02) p=0.0142; WT mice (CT08-12) p=0.002; KO mice (CT22-02) p=0.0022; KO mice (CT08-12) p=0.0116, *p<0.05, WT mice: n=12; KO mice: n=8). (B) No significant difference in Day 0 baseline between both genotypes (two-way repeated measures ANOVA analysis: interaction p=0.1888; CT p=0.5118; genotype p=0.6023). (C-D) Significant difference in mechanical nociceptive sensitivity on Day 3 (two-way repeated measures ANOVA analysis: interaction p=0.9804; CT p=0.4798; genotype p=0.0034, *p<0.05) but not on Day 7 (two-way repeated measures ANOVA analysis:p=0.5458; CT p=0.9034; genotype p=0.7066).

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undergoes daily variation [3]. On the other hand, in the tail flick test, both WT and KO mice did not show altered acute nociceptive sensitivity in relation to the circadian rhythm, and this discrepancy may stem from the differences between the methods.

Deletion of REV-ERBα increased mechanical nociceptive sensitivity but the increase was not maintained. This suggests that the deletion of REV-ERBα only transiently increases the mechanical nociceptive sensitivity. Although the transient nature of the increased nociception is yet to be explained, we propose several explanations to account for the enhanced mechanical nociceptive sensitivity in KO mice. A previous study has shown that KO mice have elevated dopamine levels in the midbrain and that this dopaminergic hyperactivity induces mood-related behavioral changes [10]. Accumulating evidence implies that pain perception is affected by both dopamine activity and mood differences. For example, it is well known that elevated dopamine activity produces anti-nociceptive effects [11-13]. In spite of elevated dopamine activity, KO mice showed increased mechanical nociceptive sensitivity. Dopamine is known to contribute to mood regulation [14, 15]. A number of studies reported that mood alteration and pain are reciprocally associated [16-18]. Chung et al. reported that KO mice showed abnormal mood-related behavior observed in CT12 [10]. However, our results revealed that KO mice exhibited mechanical nociceptive sensitivity changes in both CT0 and CT12. Therefore, our results support the point that mechanical nociceptive sensitivity changes are not coincident with mood alteration in KO mice. Taken together, our results suggest that enhanced dopamine levels and mood alteration may not directly modulate mechanical nociceptive sensitivity in KO mice. The detailed mechanisms should be further investigated.

One possible explanation for the enhanced nociceptive sensitivity in KO mice is the increased level of pro-inflammatory cytokines [19]. Gibbs et al. reported that KO mice have elevated expression of pro-inflammatory cytokines, such as IL-6, CXCL1, CCL5 and CCL2, with a significant increase of IL-6 expression in particular [20]. Previous findings provide evidence that injection of IL-6 to rodent hind paws induces hyperalgesia in a dose-dependent manner [21]. In contrast, IL-6 deficiency in mice leads to lowered nociceptive responses to mechanical and thermal stimulation compared to WT controls [22]. Therefore, increased levels of IL-6 and pro-inflammatory cytokines may account for hyperalgesia observed in KO mice.

Another interpretation of the increased mechanical nociceptive sensitivity of KO mice may be that fatty acid binding proteins (FABP) are dysfunctional in pain perception. FABPs act as intracellular transporters and bind to endocannabinoids such as anandamide (AEA) [23] and N-acylethanolamines (NAEs) [24]. The endocannabinoid ligands activate cannabinoid receptor 1 (CB1) and produce anti-nociceptive effects [25, 26]. Overexpression of FABPs enhances AEA uptake and, subsequently, hydrolysis of AEA, whereas inhibition of FABPs attenuates inactivation of AEA [23]. Therefore, inhibition of FABPs has caused analgesia in various pain models [27]. FABP5- and FABP7-deficient mice have a high level of AEA and NAEs in the brain and show analgesia in inflammatory pain models [28]. *FABP7* mRNA expression is known to be regulated by REV-ERBα, and FABP7 is highly expressed in KO mice [29]. Therefore, the excessive expression of FABP7 may have contributed to causing the mechanical nociceptive sensitivity change in the KO mice in the present study.

In conclusion, we found that KO mice exhibit temporally increased sensitivity in mechanical nociception, and this transient change in mechanical nociceptive sensitivity was not dependent on circadian rhythm. Further studies are required to investigate the molecular mechanisms of nociceptive sensitivity changes in KO mice and examine the nociceptive sensitivity of KO mice in other pain models.

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