<Research Article>

Effects of electroacupuncture stimulation on the expression levels of clock genes in the skeletal muscle of mice

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Summary The circadian rhythm of skeletal muscle is influenced by exercise and muscle contractions induced by electrical stimulation. Since electroacupuncture can induce muscle contractions, it is hypothesized that it may alter the expression levels of skeletal muscle clock genes; however, no studies have yet provided evidence to support this hypothesis. Thus, this study aimed to investigate the impact of the timing of electroacupuncture stimulation on the mRNA levels of skeletal muscle clock genes and to examine the temporal changes in the expression levels of these genes. Additionally, we aimed to elucidate the effects of electroacupuncture stimulation on related tissues. Electroacupuncture was administered to the bilateral anterior tibialis muscles of male C57BL/6J mice for 20 min at 1 Hz and 0.02 mA. Following stimulation at Zeitgeber time 6, the anterior tibialis muscle, gastrocnemius muscle, soleus muscle, and liver were harvested every 4 h and analyzed using quantitative PCR. In the anterior tibialis muscle, the expression of *Bmal1* was significantly elevated, peaking 10 h after electroacupuncture stimulation, with a 4.6-fold increase compared with the control group. Significant differences between the control and electroacupuncture groups were also observed in the expression levels of other major clock genes in the anterior tibialis muscle. Furthermore, notable differences in the expression levels of some major clock genes were detected in the gastrocnemius muscle, soleus muscle, and liver compared with the control group. These findings constitute the first report on the effects of electroacupuncture stimulation on circadian clock genes.

Key words: Electroacupuncture, Circadian rhythm, Clock gene, Bmal1, Skeletal muscle

1. Introduction

Circadian rhythms are oscillatory systems that synchronize physiological functions with the 24-h cycle of the Earth[1]. These circadian rhythms are

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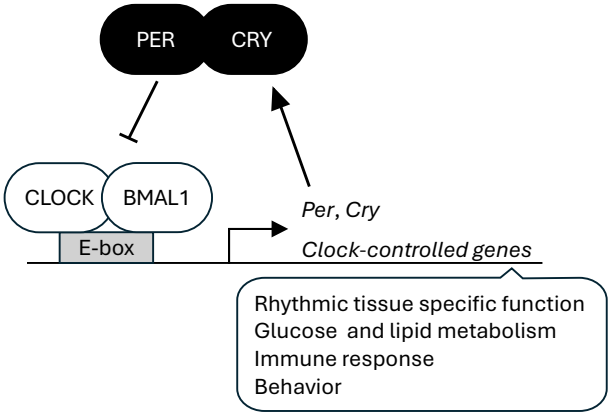

Fig. 1. Schematic diagram of the transcription–translation feedback loop of clock genes. CLOCK and BMAL1 dimerize and bind to the E-boxes in the promoter regions of *Per* and *Cry*, promoting the transcription of *Per*, *Cry*, and various clock-controlled genes involved in biological processes. PER and CRY further dimerize and bind to CLOCK:BMAL1, thereby inhibiting the binding to their own E-boxes and suppressing their transcription.

present in nearly all cells and drive daily fluctuations in fundamental physiological processes, including physical activity, immune response, metabolism, body temperature, and hormone secretion[2-6]. Disruptions to circadian rhythms are associated with increased risks of cardiovascular disease, metabolic disorders, mental health conditions, and cancer, making the stability of circadian rhythms crucial for maintaining health[7-9].

Research into the molecular mechanisms underlying circadian rhythms has advanced substantially, leading to the identification of clock genes that are conserved across species from *Drosophila* to humans[10-12]. Core clock genes involved in the regulation of circadian rhythms include the clock circadian regulator (*Clock*), basic helix-loop-helix ARNT-like 1 (*Bmal1*), period circadian regulator (*Per*), and cryptochrome circadian regulator (*Cry*), which together form a transcription–translation feedback loop[13]. CLOCK and BMAL1 form dimers that bind to the E-boxes in the promoter regions of *Per* and *Cry*, thereby enhancing their transcription[14, 15]. The resulting increase in PER and CRY protein levels leads to the formation of dimers that bind to CLOCK and inhibit their binding to the E-boxes, thereby suppressing their transcriptional activity (Fig. 1). Therefore, circadian rhythms are maintained by the expression of positive regulators *Clock* and *Bmal1* and negative regulators *Per* and *Cry*, which function in opposite phases in approximately 24-h cycles[13].

Furthermore, circadian rhythms are regulated by the central suprachiasmatic nucleus and peripheral tissues such as the heart, liver, stomach, and skeletal muscles[16-18]. Knockout of the core clock gene, *Bmal1*, in skeletal muscles results in impaired glucose tolerance, reduced expression of the glucose transporter GLUT4, and heterogeneous sarcomere lengths[19, 20]. Additionally, exercise influences both the amplitude (expression level) and phase (rhythmic timing) of skeletal muscle clock genes, with different exercise timings inducing distinct phase shifts in the skeletal muscle clock[21]. Moreover, the skeletal muscle clock affects daily variations in exercise capacity, and the training timing can impact subsequent endurance performance[22]. These findings suggest that circadian rhythms in skeletal muscle are vital for maintaining muscle structure and metabolic health.

Acupuncture is a traditional therapeutic practice established in China around 100 BC that has evolved into electroacupuncture (EA) in clinical settings[23]. EA combines Eastern acupuncture techniques with Western electrotherapy to provide direct electrical stimulation to skeletal muscle[24, 25]. However, there is limited research on the specific effects of EA on skeletal muscle circadian rhythms. A previous study reported increased *Bmal1* and *Per2* mRNA levels in the soleus muscles 60 min after electrical stimulation[26]. In contrast, another study reported decreased *Bmal1* and *Per2* mRNA levels under various

electrical stimulation conditions[21]. Another study revealed decreased expression of *Clock*, which regulates the circadian rhythms with *Bmal1*, 12 h after endurance exercise[27]. These studies suggest that electrical stimulation affects the circadian rhythms. However, they were conducted using cultured cells primarily focusing on specific time points, thus only serving as snapshots of the studied time points.

The present study aimed to investigate the effects of EA on the mRNA levels of skeletal muscle clock genes over 24 h. To achieve this, we analyzed the relationship between the timing of EA stimulation and the expression levels of skeletal muscle clock genes, as well as the temporal effects of EA stimulation on skeletal muscle and liver tissues.

2. Materials and Methods

Animals

The animal experiments in this study were approved by the Tsukuba University Animal Resource Center (approval number 23-460). Male C57BL/6J mice were purchased from CLEA Japan, Inc. and housed in a specific pathogen-free facility. The mice had ad libitum access to food and water and were maintained in an environment with an 8:00–20:00 light cycle, a room temperature of 23.5 ± 2.5°C, humidity of 52.5 ± 12.5%, and illuminance of 200 lux. The lighting period was from Zeitgeber time (ZT) 0 to ZT12. Following a 2-week acclimation period, 11–12-week-old mice were randomly assigned to either the control (CON) group or the EA group. Experiments conducted during the dark period (ZT12–24) were conducted under dim red light (635 nm) to minimize light exposure to the mice.

EA stimulation

In the EA group, EA stimulation was administered under inhalation anesthesia with isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) using a NARCOBIT-E unit (Natsume Seisakusho, Bunkyo, Tokyo, Japan). A sterilized stainless-steel needle (Seirin, Shizuoka, Japan) with a diameter of 0.20 mm and length of 30 mm was inserted into the anterior tibialis muscle of both hindlimbs to a depth of approximately 3 mm. The acupuncture needles were connected to an Ohm Pulsar LFP-2000e (Zen Iryoki, Fukuoka, Japan), and low-frequency electrical stimulation was administered at a frequency of 1 Hz and an intensity of 0.02 mA for 20 min. This facilitated the stable and repeated contraction of the tibialis anterior muscles while ensuring that the circadian rhythms were unaffected by anesthesia[28]. The CON group were maintained in a resting state for 20 min under isoflurane inhalation anesthesia without receiving acupuncture.

Clock gene expressions

In Experiment 1, we examined the effect of the timing of EA stimulation on the expression levels of clock genes in skeletal muscle. EA was administered to the anterior tibialis muscles at ZT6 (midpoint of the light phase), ZT12 (light/dark transition), and ZT18 (midpoint of the dark phase), with n=4 mice per time point. The anterior tibialis muscle was harvested 8 h after EA stimulation (i.e., at ZT14, ZT20, and ZT2), immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. In Experiment 2, we examined the effects of EA stimulation on the expression rhythms of the core clock genes *Bmal1*, *Clock, Per1*, and *Per2* in the tibialis anterior muscle. EA stimulation was administered to mice for 20 min, and the anterior tibialis muscle, gastrocnemius muscle, soleus muscle, and liver were collected at 4-h intervals for quantitative PCR (qPCR) analysis, with n=4 mice per group at each time point.

cDNA synthesis and quantitative PCR

Mouse skeletal muscle and liver tissues were homogenized in RNAiso Plus (Takara Bio, Kusatsu, Shiga, Japan), and total RNA was extracted following the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan). The resulting cDNA was diluted 10-fold with Milli-Q water and used for qPCR.

qPCR primers were designed using Primer-BLAST (NCBI) (Table 1). qPCR was performed with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Kusatsu, Shiga, Japan), with a 10 μL

PCR reaction mixture per reaction. The amplification was conducted using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) under the following cycling conditions: 40 cycles of 95°C for 30 seconds, 95°C for 3 seconds, and 60°C for 30 seconds. Relative gene expression levels were calculated using the ΔΔCt method. Ribosomal protein L26 (*Rpl26*) was used as an internal reference gene for normalization, as previously reported[21].

Table 1 Primer sequences used in this study

Target gene	Primer sequences (5' to 3')		Amplicon size (bp)
Clock	Forward	CAATCACGGACTCGGCTGG	110
	Reverse	GCTTTTCTTTCACGGGAGCG	
Bmal1	Forward	AGGCCTTCATTGCACCTTCC	139
	Reverse	CTTGCGATTGCAGTCCACAC	
Per1	Forward	CGGATTGTCTATATTTCGGAGCA	142
	Reverse	TGGGCAGTCGAGATGGTGTA	
Per2	Forward	GCATGACACCCTACCTGGTC	115
	Reverse	GGAGGGATTCTAGGCGCTTC	
Rpl26	Forward	CGAGTCCAGCGAGAGAAGG	115
	Reverse	GCAGTCTTTAATGAAAGCCGTG	

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). Experiment 1 analyzed the effect of the timing of EA stimulation on *Bmal1* mRNA levels in skeletal muscle. Data normality was first assessed using the Shapiro–Wilk test, followed by an unpaired t-test for group comparisons. In Experiment 2, the influence of EA stimulation on the rhythmic expression levels of core clock gene mRNAs was evaluated using the Mann–Whitney U test. *P*-values of < 0.05 were considered indicative of statistical significance.

3. Results

Effect of electroacupuncture stimulation timing on *Bmal1* mRNA levels in skeletal muscle

We used qPCR to investigate the impact of the timing of EA stimulation on the levels of *Bmal1* mRNA in the anterior tibialis muscle compared with the CON group (Fig. 2A). EA stimulation at ZT6 resulted in a significant 1.89-fold increase in *Bmal1* expression in the EA group compared with the CON group (Fig. 2B). Conversely, EA stimulation at ZT12 and ZT18 yielded 1.04-fold and 1.07-fold greater *Bmal1* expression levels, respectively, compared with the CON group, although these differences at these timepoints were not significantly different. Based on these findings, we selected ZT6 for subsequent EA stimulation and further analysis of clock gene expressions.

Effect of electroacupuncture stimulation on the circadian rhythms of core clock gene mRNA levels

We assessed the mRNA levels using qPCR to

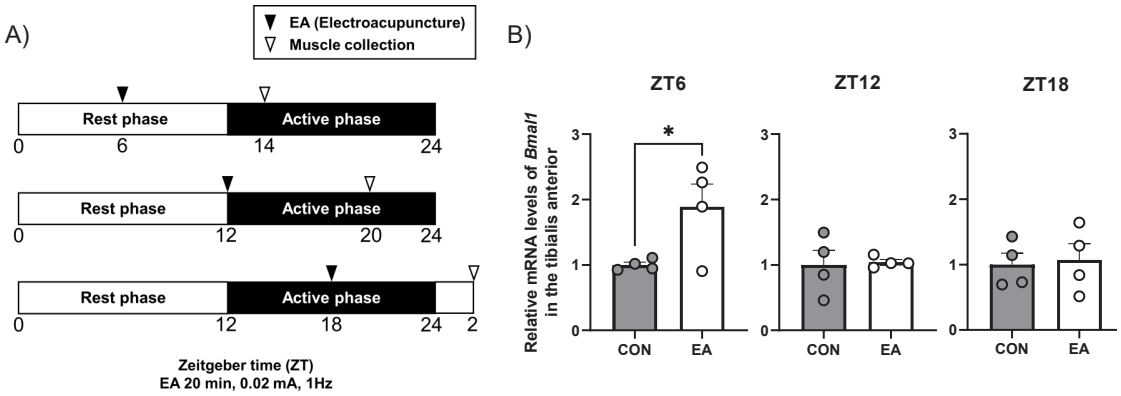

Fig. 2. Effect of EA stimulation timing on mRNA levels of *Bmal1* in skeletal muscle. (A) Schedule for Experiment 1. Mice underwent 20 min of EA stimulation (indicated by black arrowheads) at ZT6, ZT12, and ZT18. Skeletal muscle samples were collected 8 h post-stimulation (indicated by white arrowheads). White bars represent the resting period (light phase), while black bars denote the active period (dark phase). (B) mRNA levels of *Bmal1* in the tibialis anterior muscle of mice at ZT6, ZT12, and ZT18. *Rpl26* was used as the internal standard gene. Data are presented as mean ± SEM (n=4 mice per group). *P < 0.05 versus the control group (t-test)

evaluate the impact of EA stimulation on the circadian rhythms of the core clock genes *Bmal1*, *Clock*, *Per1*, and *Per2* (Fig. 3A).

Initially, we examined the mRNA fluctuations of clock genes in the anterior tibialis muscle. The expression of *Bmal1* in the EA group was significantly increased by 4.6-fold at 10 h post-stimulation, 2.3-fold at 14 h post-stimulation, and 1.8-fold at 22 h post-stimulation compared with the CON group (Fig. 3B). The expression of *Clock* in the anterior tibialis muscle was significantly higher in the EA group than the CON group by 1.3-fold at 10 h and 26 h post-stimulation. The *Per1* expression levels were significantly elevated in the EA group by 1.4-fold at 18 h post-stimulation compared with the CON group. Conversely, the expression of *Per2* in the anterior tibialis muscle was significantly lower in the EA group by 0.6-fold at 2 h post-stimulation, 0.8-fold at 10 h post-stimulation, and 0.7-fold at 22 h post-stimulation compared with the CON group.

In the gastrocnemius muscle, the *Bmal1* expression was significantly increased by 1.9-fold in the EA group compared with the CON group at 14 h post-stimulation (Fig. 4A). The *Clock* expression in the gastrocnemius muscle was significantly higher in the EA group than the CON group by 1.7-fold at 10 h post-stimulation. The *Per1* levels in the EA group were significantly decreased by 0.7-fold at 6 h post-stimulation but significantly increased by 1.8-fold at 22 h post-stimulation compared with the CON group.

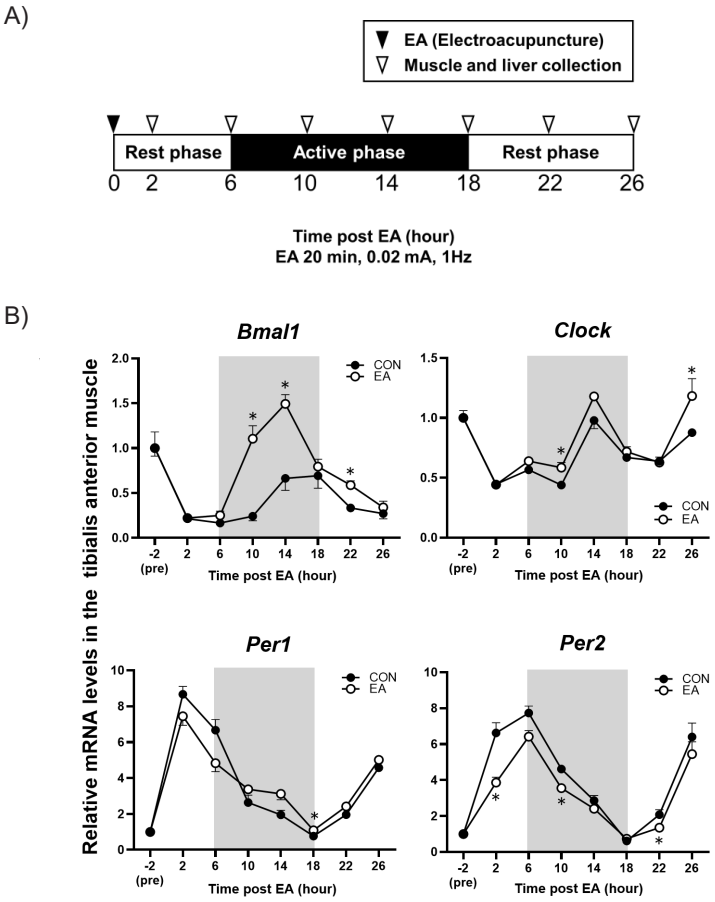

Fig. 3. Effect of EA stimulation on the rhythmic expression of clock gene mRNA in the anterior tibialis muscle. (A) Schedule for Experiment 2. White bars represent the resting period (light phase), while black bars indicate the active period (dark phase). Tissue samples from mice were collected at seven 4-h intervals (indicated by white arrowheads), with ZT6, the time of EA stimulation, serving as the reference point (indicated by the black arrowhead). (B) Rhythms of clock gene mRNA expression levels in the anterior tibialis muscle. *Rpl26* was used as the internal standard gene. The gray shading denotes the active period (dark phase). Data are presented as mean ± SEM. *P < 0.05 versus the control group (Mann–Whitney U test).

The expression of *Per2* in the gastrocnemius muscle was significantly higher in the EA group than the CON group by 1.8-fold at 18 h post-stimulation and 1.3-fold at 22 h post-stimulation.

There were no significant differences between the EA and CON groups in the expression levels of *Bmal1* and *Clock* in the soleus muscle (Fig. 4B). The expression of *Per1* in the soleus muscle was significantly decreased in the EA group compared with the CON group by 0.6-fold at 6 h post-stimulation but was significantly increased by 1.9-fold at 22 h post-stimulation. The *Per2* expression level was significantly decreased in the EA group compared with the CON group by 0.6-fold at 6 h post-stimulation and 0.4-fold at 10 h post-stimulation.

In the liver, there were no significant differences between the EA and CON groups in the expression levels of *Bmal1* and *Per2* (Fig. 5). The expression level of *Clock* in the liver was significantly lower in the EA group by 0.7-fold at 14 h post-stimulation, 0.8-fold at 18 h and 22 h post-stimulation, and 0.8-fold at 26 h post-stimulation compared with the CON group. However, the *Per1* expression was significantly increased by 2.4-fold in the EA group at 22 h post-stimulation compared with the CON group.

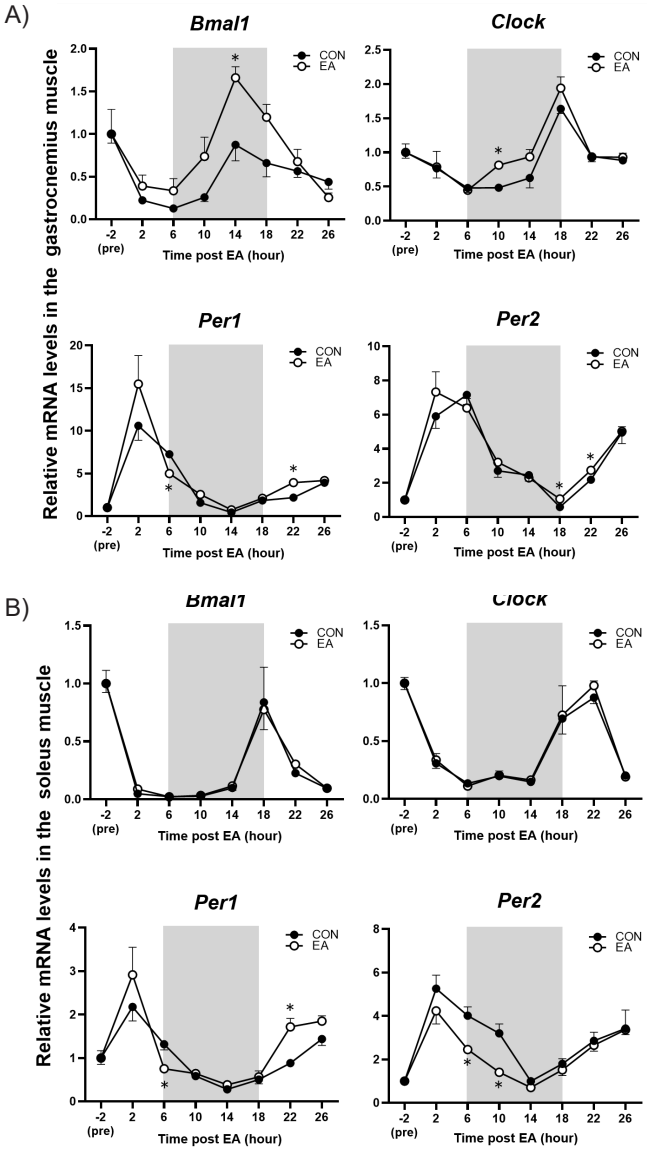

Fig. 4. Effect of EA stimulation on the expression rhythms of clock gene mRNA in the muscles of the posterior lower leg. Rhythms of clock gene mRNA expression levels in the (A) gastrocnemius muscle and (B) soleus muscle. *Rpl26* was used as the internal standard gene. The gray shading denotes the active period (dark phase). Data are presented as mean ± SEM. *P < 0.05 versus control group (Mann–Whitney U test).

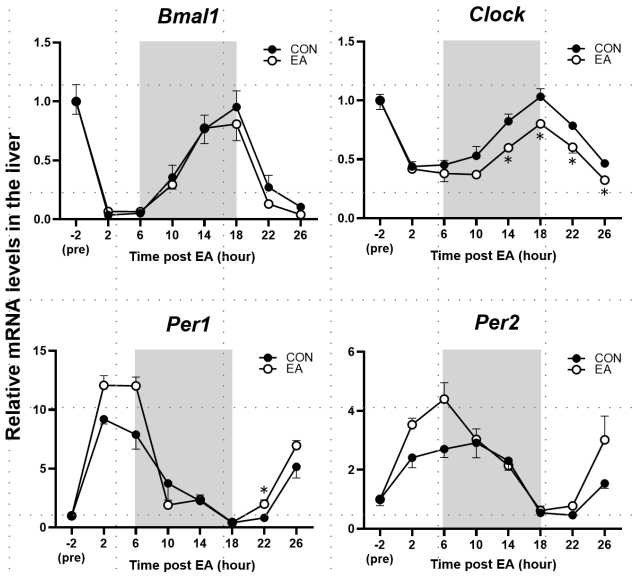

Fig. 5. Effect of EA stimulation on the expression rhythms of clock gene mRNA in the muscles of the liver. Rhythms of clock gene mRNA expression levels in the liver. *Rpl26* was used as the internal standard gene. The gray shading denotes the active period (dark phase). Data are presented as mean ± SEM. *P < 0.05 versus the control group (Mann–Whitney U test).

4. Discussion

The present study investigated the effects of EA stimulation on the skeletal muscle clock. In Experiment 1, EA stimulation was administered to the tibialis anterior muscle at ZT6, ZT12, or ZT18. In Experiment 2, we examined the impact of EA stimulation at ZT6 on the temporal expressions of the core clock genes *Bmal1*, *Clock*, *Per1*, and *Per2* in the lower leg skeletal muscle and liver.

In Experiment 1, only EA stimulation at ZT6 affected the expression of *Bmal1*. Prior studies have reported that EA stimulation suppresses the mitogen-activated protein kinase pathway and induces the expression of proliferator-activated receptor-gamma coactivator-1alpha. These factors are known to influence the expressions of *Bmal1* and other core clock genes and affect the maintenance of circadian rhythms[29, 30]. In the present study, EA stimulation at ZT6 might cause *Bmal1* expression throughout such kinase pathways or receptors in the tibialis anterior muscle[31, 32].

Conversely, the lack of effect of EA stimulation at ZT12 and ZT18 on *Bmal1* expression may be attributed to the influence of both the timing of EA stimulation and the timing of sampling. Previous studies have demonstrated that *Bmal1* expression fluctuates depending on the exercise pattern and sampling time post-exercise[27]. Additionally, *Bmal1* expression in myotube cells varies with contraction timing[21]. Future investigations should further explore the relationship between EA stimulation timing and *Bmal1* mRNA levels.

In Experiment 2, we observed changes in the expression levels of all four core clock genes in the anterior tibialis muscle within 24 h of EA stimulation. This indicates that EA stimulation influenced the expression levels of skeletal muscle clock genes over a period of approximately 24 h. The EA stimulation in the present study may have led to the fluctuations in the expression levels of clock genes by providing electrical stimulation and causing muscle contractions that were like exercise in the cells. Future research should investigate how variations in the intensity, frequency, and duration of EA stimulation affect the expression levels of clock genes.

Transcriptional and translational feedback loops governing clock gene expression involve *Clock* and *Bmal1*, which act as positive regulators, and *Per* and

Cry, which act as negative regulators, operating in opposite phases relative to one another. Notably, *Bmal1* and *Per* expression levels can be simultaneously upregulated or downregulated in response to transient stimuli[21, 33]. In this study, we observed fluctuations in clock gene expression levels independent of the transcription–translation feedback loop. Further investigations are necessary to verify the mechanisms by which EA stimulation affects clock gene expression.

In the EA group, the *Bmal1* and *Clock* mRNA levels were increased in the gastrocnemius muscle, located posterior to the tibialis anterior. In the soleus muscles posterior to the tibialis anterior muscles, only *Per1* and *Per2* expression levels were significantly altered in response to EA stimulation. The observed effects on adjacent skeletal muscle are hypothesized to result from cytokines, such as IL-6 and MCP-1, which are produced in response to skeletal muscle stimulation[34]. These molecules are known to play a role in the circadian rhythm of skeletal muscle and may be transported via the circulatory system to synchronize peripheral tissues[1, 34]. Electrical stimulation of myotube cells alters the blood cytokine levels, including the IL-6 and MCP-1 levels, suggesting that EA influences the production of these substances[35].

Interestingly, EA stimulation also influenced the expressions of clock genes in liver tissue. Circadian rhythms in the liver are instrumental in regulating the diurnal variation in bile acid synthesis as well as glucose and lipid metabolism, which are implicated in the pathogeneses of various metabolic diseases[36, 37]. Previous research has highlighted the significance of metabolic rhythm synchronization between muscle and liver[38]. Although this has not been statistically analyzed, one study indicated a slight trend toward increased expression of *Bmal1* in skeletal muscle when *Bmal1* is specifically deleted in the liver[39]. Peripheral tissues synchronize their circadian rhythms through neurotransmitters, circulating endocrine factors, and sleep and feeding cycles[1, 17]. Given that EA has known effects on local tissues as well as the nervous, endocrine, and immune systems, it is plausible that EA-induced changes in the expression levels of skeletal muscle clock genes may influence circadian rhythms in peripheral tissues, including the liver[40, 41].

The present study has limitations. We did not investigate the molecular mechanisms by which EA stimulation affects the expression levels of clock genes in local and distant organs, or the rhythms and functions of proteins translated from mRNA. Previous reports suggest that electrical stimulation can induce phase shifts in the skeletal muscle clock depending on the time of day, and EA may similarly affect these phase shifts[21]. Future research should explore how EA stimulation conditions, including intensity and duration, influence skeletal muscle circadian rhythms.

In conclusion, our study demonstrated that EA affects the mRNA levels of the skeletal muscle clock genes. The effects of EA stimulation vary with the time of day and extend to the gastrocnemius and soleus muscles and the liver. These findings provide foundational data for the potential use of EA in maintaining, restoring, and enhancing circadian rhythms and mitigating metabolic disease symptoms through the modulation of skeletal muscle circadian rhythms.

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Conflicts of interests

All authors declare that there is no conflict of interest regarding the publication of this article.

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