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Changes in estrogen receptor signaling alters the timekeeping system in male mice

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HIGHLIGHTS

- This novel research explores non-genomic estrogenic signaling in circadian rhythms.
- Disruptions in ESR1 signaling alters amount and timing of activity in males.
- Use of a series of transgenic mice with differential ability to respond to estrogens.
- Estrogen signaling plays a role in male timekeeping system.

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ABSTRACT

Circadian rhythms are modulated by steroid hormones; however, the mechanisms of this action are not fully understood, particularly in males. In females estradiol regulates activity level, pattern of expression, and free running period (tau). We tested the hypothesis that activity level and distribution in male mice includes both classical and "non-classical" actions of estrogens at the estrogen receptor subtype 1 (ESR1). We used transgenic mice with mutations in their estrogen response pathways: ESR1 knock-out (ERKO) mice lack the ability to respond to estrogens via ESR1. "Non-classical" estrogen receptor knock-in (NERKI) mice have an inserted ESR1 receptor with a mutation in the estrogen-response-element binding domain, allowing activation via non-genomic and second messenger pathways. Gonadectomized male NERKI, ERKO, and wildtype (WT) littermates were given oil, or low or high dose estradiol and daily activity parameters were quantified. Estradiol shortened the ratio of activity in the light relative to dark (LD ratio), shortened tau, advanced the time of activity onset, and altered responsiveness to light cues administered in the late subjective night, suggesting modulation by an ESR1-independent mechanism. Estradiol treatment in NERKI but not WT males altered the timing of activity onset, LD ratio, and the behavioral response to light cues. These results may represent disruptions in the balance of genomic/nongenomic or ESR1/ESR2 signaling pathways. We also found a significant genotype effect on total activity, LD ratio, tau, and activity duration. These data provide new information about the role of ESR1-dependent and independent signaling pathways on the timekeeping system in male mice.

1. Introduction

Gonadal hormones influence circadian rhythms and activity patterns by modifying the amount and distribution of daily activity, the intrinsic free-running period (tau) in constant conditions, and the phase response to light pulse [1]. Yet how these hormones exert their effects remains to be determined. Secondly, how these hormones regulate circadian rhythms and activity differentially in males and females is relatively unknown [2]. Hormones are traditionally described as having activational and organizational effects. Acute responses to the presence of hormones are activational;

Abbreviations: ArKO, aromatase knockout; DD, constant darkness; E, estradiol; ERKO, estrogen receptor type 1 knockout; ESR1, estrogen receptor type 1; ESR2, estrogen receptor type 2; LD, light:dark; NERKI, non-traditional estrogen receptor knock-in; ZT, Zeitgeber time.

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hormones cause transient changes in the brain and behavior and these changes do not persist in the absence of that hormone [3]. In many rodent species there is a well-established effect of female reproductive cycle stage on circadian rhythms and activity level; on days of high circulating estrogens, there is increased activity, advanced activity onset, and increased duration of activity [4–6]. Estradiol replacement to gonadectomized animals confirms that these changes over the reproductive cycle are due to circulating estrogens [5,7–9]. Estradiol may have differential roles in males and females. Estradiol treatment following gonadectomy decreases the free-running period in female rats; however, in male rats estradiol either increases or decreases the length of tau as a function of the individual pre-treatment tau [7].

Organizational effects of hormones are permanent changes in the brain and behavior caused by the presence of hormones during development or at specific critical periods. There is evidence for an organizational role of estrogens during development on the expression of circadian rhythms in adults. In male and female rats, the change in free-running period that results from gonadectomy and hormone replacement in adulthood is affected by perinatal exposure to steroid hormones [7]. Other circadian parameters are modified by organizational activity of gonadal hormones. In degus, the timing of activity onset relative to the time of lights on (phase angle) changes during adolescence and depends on the presence of gonadal hormones during development [10].

In male mice, androgens have a regulatory role on activity and circadian rhythms; gonadectomy increases free-running period, decreases daily activity, and alters the distribution of wheel-running across the day and androgen administration following gonadectomy restores these patterns to that of intact animals [11–13]. However, there is evidence that estrogens also modify circadian behaviors in males. Aromatase knock-out mice (ArKO) lack endogenous circulating estrogens and intact and gonadectomized male ArKO mice have decreased total activity and altered distribution of activity throughout the light:dark (LD) cycle relative to wildtype (WT) counterparts [14]. Differences in circadian behavior between ArKO mice and WT littermates that persist following gonadectomy indicate an organizational role for estrogenic modification of circadian rhythms in adulthood. Estradiol exposure and estrogen receptor expression modulate both reproductive and non-reproductive behaviors in male rodents including mating, aggression and anxiety [15,16]. To date, relatively little is known about the role of estrogen signaling pathways in the expression of biological rhythms in male rodents.

Estrogen signaling is mediated through two distinct nuclear receptors: estrogen receptor subtype 1 (ESR1) and estrogen receptor subtype 2 (ESR2), as well as more recently characterized "non-classical" pathways [17,18]. To exert action via nuclear receptor-mediated transcriptional activation, estrogens bind to receptors and the ligand–receptor complexes form dimers that the bind transcription factors. In the "classical" pathway, the hormone-receptor dimer binds to the estrogen response element (ERE) on regulatory regions of target genes to alter gene expression. "Non-classical" mechanisms of estrogen signaling include the action of dimers at non-ERE transcription factors, membrane-initiated action via protein kinases, and ligand-independent estrogenic signaling via second messenger pathways [19].

In this study, our goal was to investigate the role of estrogens in modifying the expression of behavioral rhythms in male mice. We hypothesized that estradiol influences activity level and distribution, free-running rhythms, and response to light pulse via activational effects of the hormone at both ERE-dependent and independent pathways. To test this hypothesis, we used ESR1 knock-out (ERKO, formerly ERα knockout) and "non-classical" estrogen receptor knock-in (NERKI) mice. ERKO mice lack ESR1, and NERKI mice have a mutation in the ERE-binding domain of a knocked-in ESR1. NERKI mice can still respond to estrogens binding ESR1 via non-ERE-mediated mechanisms. Both transgenic strains retain intact ESR2 (formerly ERβ) receptors. To characterize the activational effects of estrogens on circadian activity we used gonadectomized NERKI, ERKO, and their WT littermates, with and without estradiol replacement.

2. Methods

2.1. Animal breeding and care

Adult male NERKI and ERKO mice and their WT littermates were used for these experiments (total $n = 71$). All mice were obtained from a breeding colony established at the University of Illinois. ERKO mice (Dr. Pierre Chambon, IGBMC, France) were obtained by mating males and females heterozygous for the mutation. NERKI mice (Dr. J. Larry Jameson, University of Pennsylvania, Philadelphia, PA) were obtained from breeding heterozygous males containing one copy of the mutated ESR1 allele with females heterozygous for the ERKO mutation, as NERKI females are infertile. The non-classical estrogen receptor knock-in is thus on an ESR1 knock-out background. All genotypes were confirmed using PCR. Control mice are wildtype littermates of NERKI and ERKO mice.

Food and water were given ad libitum. Breeding animals and litters were maintained on Teklad 8626 rodent diet. Prior to activity monitoring, animals were given Teklad 2016 diet, which contains low soy estrogens (isoflavones) in the range of non-detectable to 20 mg/kg, and remained on this diet for the remainder of the studies. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

2.2. Gonadectomy and estradiol replacement

Adult males were gonadectomized at 2 months old and given one of the following treatments at time of gonadectomy: no hormone replacement, one silastic capsule containing estradiol benzoate in sesame oil, or three capsules. Silastic capsules (1.02 mm inner diameter; 10 mm in length) contained 1 mg/mL estradiol benzoate in sesame oil. This dose was selected based on previous reports from ours and other labs that it is sufficient to elicit activity changes [20,21]. Serum concentrations of estradiol (E) were determined for a subset of mice 10 days following capsule implantation and were less than the detectable level of the assay (59 pg/mL, DRG International, Mountainside, NJ) even for the high estradiol replacement group

2.3. Determination of daily activity patterns and circadian parameters

Mice were maintained in 12 h light: 12 h dark (LD) cycles unless otherwise indicated, and the light intensity in the cages ranged from 220 to 360 lux (average 290 lux). Mice were individually housed in cages ($28 \times 16 \times 12$ cm) equipped with a metal wheel affixed to the top of the cage. Wheel revolutions were registered by a magnetic switch and recorded in 10-min bins of activity. Wheel running activity was recorded and visualized using VitalView and ActiView (Starr Life Sciences Corp.). The following variables were quantified: average daily wheel revolutions, light:dark ratio of activity (LD ratio), phase angle of activity onset relative to lights-off, duration of free-running period (tau), and length of active phase (alpha) constant dark (DD) conditions. All mice underwent these series of experiments in randomized order with a period of 10 days re-entrainment to standard 12:12 LD between experiments to minimize any effect of previous light environment. For all parameters