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Gonadectomy reveals sex differences in circadian rhythms and suprachiasmatic nucleus androgen receptors in mice

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Abstract

In mammals, it is well established that circadian rhythms in physiology and behavior, including the rhythmic secretion of hormones, are regulated by a brain clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. While SCN regulation of gonadal hormone secretion has been amply studied, the mechanisms whereby steroid hormones affect circadian functions are less well known. This is surprising considering substantial evidence that sex hormones affect many aspects of circadian responses, and that there are significant sex differences in rhythmicity. Our previous finding that "core" and "shell" regions of the SCN differ in their expression of clock genes prompted us to examine the possibility that steroid receptors are localized to a specific compartment of the brain clock, with the discovery that the androgen receptor (AR) is concentrated in the SCN core in male mice. In the present study, we compare AR expression in female and male mice using Western blots and immunochemistry. Both of these methods indicate that ARs are more highly expressed in males than in females; gonadectomy eliminates and androgen treatment restores these sex differences. At the behavioral level, gonadectomy produces a dramatic loss of the evening activity onset bout in males, but has no such effect in females. Treatment with testosterone, or with the non-aromatizable androgen dihydrotestosterone, restores male locomotor activity and eliminates sex differences in the behavioral response. The results indicate that androgenic hormones regulate circadian responses, and suggest an SCN site of action.

Keywords: Circadian rhythms; Mouse; Gonadal hormones; Dihydrotestosterone; Testosterone; Androgen receptor; Locomotor activity

Introduction

In all species, appropriate timing of physiological and behavioral processes is necessary for optimal functioning in the environment. The circadian (daily) timing system ensures that incompatible biochemical and behavioral rhythms are temporally segregated within individual cells and tissues, as well as in the organism as a whole. In mammals, circadian timing is regulated by a master clock within the suprachiasmatic nucleus (SCN) of the hypothalamus, and this nucleus is necessary for the generation and maintenance of circadian rhythms (Klein et al., 1991). In proof, lesions of the SCN abolish circadian rhythms, and neural transplantation of the SCN from a donor to a lesioned host restores circadian rhythms in the host (Lehman et al., 1987; Ralph et al., 1990).

In addition to controlling daily rhythms, the SCN is also essential for the synchronization (or entrainment) of an organism's internal circadian time to the external environmental world, integrating internal and external signals. Both environmental and internal stimuli can set the phase of SCN oscillators. The most salient environmental timing cue is the daily cycle of light and darkness (Morin and Allen, 2006). Photic input from the retina reaches the SCN via a direct projection via the retinohypothalamic tract (RHT), which terminates with greatest density within the core region of the SCN (Abrahamson and Moore, 2001; Antle and Silver, 2005). The core compartment also receives afferent input from the intergeniculate leaflet (IGL) and the raphe nucleus (RN) (Kriegsfeld et al., 2004; Moga and Moore, 1997; Zhang and Rusak, 1989). As discussed below, internal cues

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such as hormonal signals, many of which are secreted on a circadian basis, also target the core SCN compartment.

Much experimental work done in laboratory rodents indicates that the gonadal hormones estrogen (E) and testosterone (T) modulate circadian activity rhythms (Jechura et al., 2000; Morin, 1986). Additionally, field studies demonstrate hormone-dependent effects, with some species even changing seasonally from diurnality to nocturnality (Yokota and Oishi, 1992). Though few studies have been performed in mice, they suggest that circadian period and precision, as well as the organization of daily activity bouts, are dramatically affected by gonadal hormones (Daan et al., 1975; Karatsoreos et al., 2007). Thus, following gonadectomy (GDX) of males there is a loss of the onset component of daily activity, lengthening of free running period, a decrease in precision, a reduction in duration of daily activity bouts, and a decrease of total daily activity. In contrast, in female rats and hamsters, ovariectomy (OVX) produces an overall reduction of total daily activity levels, and hormone replacement shortens the free-running period (Albers, 1981; Morin, 1980; Morin et al., 1977; Zucker et al., 1980).

Many of the circadian effects of E and T are thought to occur outside of the SCN, either on SCN afferents or efferents (de la Iglesia et al., 1995, 1999). This assumption has been supported, in part, by reports of sparse steroid receptor localization in the SCN (Mitra et al., 2003; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001). In contrast, we found that androgen receptors (AR) in male mice are highly localized to the mid-caudal region of the SCN, termed the "core" (Karatsoreos et al., 2007). This distribution of AR may have been missed in earlier studies performed when intra-SCN regional differences were unknown, as it was thought that all SCN cells were equivalent oscillators (Reppert and Weaver, 2001). Today, there is general agreement that functional specializations map to these anatomically distinct ventral core and dorsal shell regions (reviewed in: Antle and Silver, 2005). In the present study, we explore sex differences in AR expression and in circadian locomotor activity. Because SCN AR expression in castrated males resembled that of females, we studied the effect of testosterone replacement on the androgen-dependent responses.

Methods

Animals and housing

Male and female C57BL/6J mice (Charles River Laboratories, Kingston, NY) aged 8–10 weeks were group housed for at least 2 weeks following arrival. Animals used for the behavioral experiments were placed in individual cages equipped with running wheels, as described below. All other animals were group housed. To determine the phase of the estrus cycle, vaginal smears were taken every morning (at lights on) for at least two consecutive cycles.

All animals were provided with *ad libitum* access to food and water, and maintained in a 12:12-h light:dark (LD) cycle (unless otherwise noted) in accordance with the guidelines of Columbia University's Institutional Animal Care and Use Committee.

Behavioral measurements

For assessment of wheel-running activity, mice were maintained in constant darkness (DD) and housed individually in translucent plastic cages (36×20×20 cm) equipped with a running wheel (13 cm diameter) with the number of wheel revolutions recorded by a computerized data acquisition system (VitalView, Respironics, Inc, Murraysville, PA). The free-running behavior of intact (INT) mice was recorded for at least 3 weeks (n=14 males, 26 females). Animals were then orchidectomized (GDX, n=14) or ovariectomized (OVX, n=24), with time of surgery performed during the animal's subjective day so as to avoid a light induced phase shift. The behavior of gonadectomized animals was monitored for 3 weeks, after which some of the mice were implanted with Silastic capsules containing either testosterone propionate (TP; males, n=8, females, n=9) or 5α-dihydrotestosterone (DHT; males, n=6, females, n=8), and returned to DD for an additional 3 weeks. The behavior of male mice was previously reported in Karatsoreos et al. (2007); for this report, a new analysis was performed on activity, period and precision to permit comparisons with the behavior of females maintained in the same housing conditions.

To evaluate sex differences and androgen effects on circadian behavior in males and females, analysis was carried out on at least 10 consecutive days of data, and at least 7 days following any manipulation. Data were analyzed using Clocklab (Actimetrics Inc., Wilmette, IL) for Matlab (The MathWorks Inc., Natick, MA). Free-running period was calculated using a fast Fourier periodogram, and is reported in hours±S.E.M. Amount of daily activity was calculated as the number of wheel revolutions per day. The precision of the daily onset of activity was calculated for each individual, as previously described (Karatsoreos et al., 2007). Briefly, the projected activity onset time was calculated for each day from the free-running period. Precision was assessed as the difference between the actual and projected activity onset time, with smaller values reflecting more precise timing.

To quantify the re-organization of circadian activity following GDX and hormone treatment, the circadian day of each animal was divided into four epochs of 6 h duration. Activity offset was used to define circadian time (CT) 24, as GDX male mice show highly inaccurate early night onsets, or no detectable onsets at all. The daily activity for each animal in each of the time epochs was calculated (activity within each epoch/total daily activity), averaged over 7 days, and reported as percent±S.E.M.

Gonadectomy and hormone replacement

Mice were deeply anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), and buprenorphine (0.5 mg/kg, s.c.) was used as an analgesic. In males, GDX was performed by abdominal incision and removal of both testes. Females were ovariectomized by bilateral flank incisions, and removal of both ovaries. In both cases, muscle and fascia were closed using surgical silk, and the overlying skin was sutured. For controls, animals were anesthetized but not surgically manipulated.

Steroid implants

Implants were prepared as previously described (Lindzey et al., 1998; Wersinger et al., 1999). Briefly, Silastic capsules (Dow Corning, I.D. 0.98 mm, O.D. 2.16 mm) were filled with crystalline TP (5 mm long) or DHT (10 mm long; Steraloids Inc., Newport, RI), sealed with Silastic glue and allowed to dry overnight. All capsules were primed in a 37 °C 0.9% saline bath for 36 h before implantation.

Western blotting

For Western blots, animals (n=6 males; 6 females) were euthanized with CO_2, and their brains removed and placed in ice-cold 0.9% saline. Using a Vibratome, 400 μm sections of hypothalamus were collected in ice-cold saline, and the SCN were harvested bilaterally with the aid of a dissecting microscope. To prepare SCN lysates, the tissues were homogenized by sonication in lysis buffer (1% SDS in dH_2O with Roche complete, Mini, EDTA-free protease inhibitor cocktail), and then incubated in a boiling water bath for 10 min. Lysates were spun in a microfuge (13,000 rpm for 30 s) to remove insoluble material. The protein concentration of the cleared supernatant was determined using the BCA method (Pierce, Rockford, IL). Lysates (5 μg/lane) were subjected to SDS gel electrophoresis, blotted to a nitrocellulose membrane and probed with the AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were