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Suprachiasmatic nucleus as the site of androgen action on circadian rhythms

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ABSTRACT

Androgens act widely in the body in both central and peripheral sites. Prior studies indicate that in the mouse, suprachiasmatic nucleus (SCN) cells bear androgen receptors (ARs). The SCN of the hypothalamus in mammals is the locus of a brain clock that regulates circadian rhythms in physiology and behavior. Gonadectomy results in reduced AR expression in the SCN and in marked lengthening of the period of free-running activity rhythms. Both responses are restored by systemic administration of androgens, but the site of action remains unknown. Our goal was to determine whether intracranial androgen implants targeted to the SCN are sufficient to restore the characteristic free-running period in gonadectomized male mice. The results indicate that hypothalamic implants of testosterone propionate in or very near the SCN produce both anatomical and behavioral effects, namely increased AR expression in the SCN and restored period of free-running locomotor activity. The effect of the implant on the period of the free-running locomotor rhythm is positively correlated with the amount of AR expression in the SCN. There is no such correlation of period change with amount of AR expression in other brain regions examined, namely the preoptic area, bed nucleus of the stria terminalis and premammillary nucleus. We conclude that the SCN is the site of action of androgen effects on the period of circadian activity rhythmicity.
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Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the locus of a master circadian clock controlling behavioral and physiological rhythms (Klein et al., 1991). Among other rhythms, the SCN regulates circadian rhythms in gonadal hormone secretion, and in turn hormones feedback to influence SCN functions (Fernandez-Guasti et al., 2000; Karatsoreos et al., 2007; Kashon et al., 1996). A role for gonadal hormones in the maturation of circadian rhythmicity has been suggested in rodents and in non-human primates (Hagenauer et al., 2011a,b; Hagenauer and Lee, 2011; Hummer et al., 2012; Melo et al., 2010; Sellix et al., 2013). In humans, there is a correlation between chronotype (morningness or eveningness) and circulating hormone concentrations during aging (Harman et al., 2001; Roenneberg et al., 2004).

In male mice, lack of testosterone dramatically affects locomotor activity rhythms. Gonadectomy results in a marked alteration of circadian behaviors, including a longer free-running circadian period, reduced precision of the activity onset, less early night activity, reduced overall activity levels, and a large decrease in androgen receptor (AR) expression in brain regions typically expressing AR. Systemic replacement of androgens by slow release silastic capsules restores these responses to those of the intact animal (Butler et al., 2012; Daan et al., 1975; Iwahana et al., 2008; Karatsoreos et al., 2007). Circadian period and precision are both rescued in gonadectomized (GDX) mice by testosterone propionate (TP), as well as by the non-aromatizable androgen, dihydrotestosterone (Karatsoreos et al., 2007), indicating mediation by the androgen receptor. In contrast, total daily activity is restored fully by TP but only partially by dihydrotestosterone, suggesting that both ARs and estrogen receptors mediate this aspect of behavior.

AR-containing cells are localized to numerous brain regions and to peripheral tissues (Dart et al., 2013), but the precise locus at which androgens affect circadian behavior is not known. ARs have been observed in the SCN of several species, including mouse, ferret, and human (Hagenauer and Lee, 2011; Karatsoreos et al., 2007), though there are species differences in their concentration and distribution (Jahan et al., 2015). In the mouse, these ARs are specifically localized in retinorecipient cells of the SCN core subregion (Iwahana et al., 2008; Karatsoreos et al., 2007). Hormone removal/replacement studies show specific effects on circadian behavior (Karatsoreos et al., 2007), light responsiveness of the circadian clock (Butler et al., 2012), and structure of the SCN (Karatsoreos et al., 2011). Therefore, the aim of the present

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study was to determine whether androgen treatment directed specifically to the SCN would suffice to alter the period of free-running circadian locomotor rhythms.

Materials and methods

Animals and housing

Male C57BL/6J mice (Charles River Laboratories, Kingston, NY, n = 24) aged 7 weeks were housed individually on pine shavings in clear polycarbonate cages (32 × 14 × 13 cm) equipped with running wheels (13 cm diameter). Cages were placed in light-tight chambers with independent lighting control and ventilation (Phenome Technologies, Inc., Skokie, IL). Animals were provided with *ad libitum* access to food and water, and maintained in constant dim red light (peak wavelength 639 nm, half-maximal width 18 nm, Avago Techologies, San Jose, CA; Butler and Silver, 2011). Illuminance was 0.3 lux at the cage floor (ILT1700, International Light Technologies, Peabody, MA, USA). All animal maintenance and experimental protocols were approved by Columbia University's Institutional Animal Care and Use Committee.

Experimental groups

After 2 weeks of baseline behavioral monitoring, mice (n = 24) were assigned randomly to one of the following groups: Intact (no manipulations, n = 4), Gonadectomized (GDX, n = 3), and GDX-TP Implanted (GDX-TP; n = 17). The Intact and GDX groups served as immunohistochemical controls to confirm previous work on AR expression in the SCN (Butler et al., 2012; Iwahana et al., 2008; Karatsoreos et al., 2007). All mice were first tested in the running wheels when they were intact. At that point, the GDX and GDX-TP groups continued in the testing apparatus for two more weeks. Two weeks after gonadectomy, mice in the GDX-TP group received an intracranial implant containing androgen (details below), and monitored for an additional 12 days.

Wheel-running behavior

Free-running behavior was recorded continuously. Wheel revolutions per 10 min bin were stored on a computerized data acquisition system (VitalView, Respironics, Inc., Murraysville, PA: currently Starr Life Sciences, Oakmont, PA). Period and daily onset of activity bouts were calculated using Clocklab (Actimetrics, Wilmette, IL, USA). To calculate the precision of onset of activity, the daily difference between the actual and the projected free-running onset time was tracked, using Clocklab. The standard deviation of these daily differences is reported as the precision. Thus, the smaller the standard deviation, the more precise is the animal's onset from day to day.

Gonadectomy

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. GDX was performed by abdominal incision and removal of both testes. Muscle and fascia were closed using surgical silk, and the overlying skin was sutured.

Steroid implants

Implants were made by mixing TP (Steraloids, Inc., Newport, RI) in beeswax at a ratio of 1:5 TP:wax (Veney and Rissman, 2000). The mixture was spread in a thin layer, and a 26-gauge, blunt-ended stainless steel guide cannula (C315GA/SPC; Plastics One, Roanoke, VA) was tamped to create a pellet 400 μm in length and 260 μm in diameter. Intracranial implantation was performed using a stereotaxic instrument. The cannula containing the TP pellet was directed at a point above the SCN, using the Franklin and Paxinos Atlas of the Mouse (1997) with the following coordinates in relation to bregma: AP = −0.5 mm ML = −0.5 mm DV = −5.3 mm from skull surface. The guide cannula was then raised 0.4 mm and the pellet was expelled using a fitted dummy cannula through the guide. The spread of steroid from the source was assessed by expression of AR in the hypothalamus of the castrated animals.

Perfusion and immunohistochemistry

To explore the effects of androgens on AR expression at the end of the behavioral study, the brains from 4 intact, 3 castrated, and 17 GDX-TP-implanted animals were collected (only 4 Intact and 3 GDX animals were used here as both their behavior and their SCN AR expression have been reported previously) (Butler et al., 2012; Daan et al., 1975; Iwahana et al., 2008; Karatsoreos et al., 2007). Animals were deeply anesthetized (pentobarbital: 200 mg/kg i.p.) and perfused intracardially with 50 ml saline followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Brains were post-fixed for 4 h at 4 °C, cryoprotected in 20% sucrose in 0.1 M PB overnight, and sliced at 50 μm on a cryostat. Free-floating sections were blocked in normal donkey serum for 1 h in PB with 0.1% Triton X (PBT), and then incubated for 48 h in AR primary antibody made in rabbit (Santa Cruz, CA; 1:1000) in PB with 0.3% Triton-X. Following the primary incubation, sections were washed 3 × 10 min with PBT, and then placed into a donkey anti-rabbit secondary conjugated to CY3 (1:200, Jackson ImmunoResearch, West Grove, PA) for 2 h. Sections were washed in PB, mounted onto gel-coated slides, and dehydrated in a graded series of alcohols (50–100%) and cleared in xylenes. Coverslips were applied with Krystalon (EM Science, Gibbstown, NJ).

Quantification of AR

Images of the brain nuclei that showed high AR expression in intact animals [SCN, preoptic area (POA), bed nucleus of the stria terminalis (BNST) and premammillary nucleus (PMN)] were captured with a Nikon Eclipse E800 epifluorescent microscope (Nikon, Tokyo, Japan) equipped with a cooled CCD camera (Retiga Exi; Q-Imaging, Surrey, Canada), using Q-capture Pro software (Q-Imaging). Sections were excited and emission filtered using filter cubes for Cy3. Images were transferred to ImageJ (NIH, Bethesda, MD). The freehand drawing tool was used to measure the optical density (OD) of these nuclei and of non-stained areas (background) in the same images. Relative optical density (ROD) was calculated by subtracting background OD from nuclei OD. Distance between implant and SCN was measured using the straight line tool in Image J. If the implant was not on the same section as the SCN, the location of the implant was projected onto the slide bearing the SCN, and a line was drawn from SCN border to the projected implant border (distance A). The number of sections separating SCN and implant was counted and multiplied by 50 μm (distance B). Distance was the length of the hypotenuse of the right triangle formed by sides A and B. Distances between implant and the other three regions were calculated similarly.

Statistical analysis

For comparison of animals when intact, GDX, and GDX-TP implanted, differences in AR-immunoreactivity (AR-ir) in various brain regions and period of locomotor activity period were analyzed using a two-way ANOVA, followed by Tukey HSD tests, and were considered statistically significant at the $p < 0.05$ level. Period comparison between animals when intact, GDX, and GDX-TP implanted was done using a repeated measures ANOVA. The effect size is indicated (η^2, Eta squared). Linear regression analysis was used to establish the correlation between

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