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Reversing song behavior phenotype: Testosterone driven induction of singing and measures of song quality in adult male and female canaries (*Serinus canaria*)

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ABSTRACT

In songbirds, such as canaries (*Serinus canaria*), the song control circuit has been shown to undergo a remarkable change in morphology in response to exogenous testosterone (T). It is also well established that HVC, a telencephalic nucleus involved in song production, is significantly larger in males than in females. T regulates seasonal changes in HVC volume in males, and exposure to exogenous T in adult females increases HVC volume and singing activity such that their song becomes more male-like in frequency and structure. However, whether there are sex differences in the ability of T to modulate changes in the song system and song behavior has not been investigated in canaries. In this study, we compared the effects of increasing doses of T on singing and song control nuclei volumes in adult male and female American Singer canaries exposed to identical environmental conditions. Males were castrated and all birds were placed on short days (8L:16D) for 8 weeks. Males and females were implanted either with a 2, 6 or 12 mm long Silastic™ implant filled with crystalline T or an empty 12 mm implant as control. Birds were then housed individually in sound-attenuated chambers. Brains were collected from six birds from each group after 1 week or 3 weeks of treatment. Testosterone was not equally effective in increasing singing activity in both males and females. Changes in song quality and occurrence rate took place after a shorter latency in males than in females; however, females did undergo marked changes in a number of measures of song behavior if given sufficient time. Males responded with an increase in HVC volume at all three doses. In females, T-induced changes in HVC volume only had limited amplitude and these volumes never reached male-typical levels, suggesting that there are sex differences in the neural substrate that responds to T.

1. Introduction

In temperate zone songbird species such as canaries (*Serinus canaria*), photoperiodic changes, along with a variety of supplementary cues, regulate the timing of the breeding season and modulate the associated anatomical, physiological, and behavioral changes (e.g., Nottebohm 1981; Wingfield and Kenagy 1991; Wingfield et al., 1993; Leitner et al., 2001; Hurley et al., 2008). In spring, gonadal volume and blood concentrations of testosterone increase in parallel with increases in day length. This change in physiological state is sufficient, although not necessary (Ball et al., 1999), to facilitate an increase in song behavior (Schlinger 1997; Ball et al., 2003; Harding 2004). Such a change in song behavior during the breeding season is thought to serve two functions: mate acquisition and territory defense (Baker et al., 1981; Catchpole, 1980, 1982; Darwin, 1871; King and West 1977; Krebs, 1977; Kroodsma, 1976; Marler, 1956).

In many songbird species, males and females differ in the rate and quality of song as well as in the morphology of the controlling neural substrate (Nottebohm and Arnold, 1976; Arai et al., 1989; Kirn et al., 1989; Brenowitz et al., 1991; Whitfield-Rucker and Cassone, 1996; see Ball et al., 2008 for a review). Male temperate zone songbirds tend to sing songs that are longer in duration, acoustically more complex, and produced at higher rates compared to females (see Ball et al., 2008; Catchpole and Slater 2008 for reviews). In wild and domesticated canaries, *S. canaria*, this sex difference in the production of song is also observed (e.g., Poulsen, 1959; Nottebohm et al., 1986; Leitner et al., 2001 Leitner and

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Catchpole, 2002). However, there is wide variation in the naturally occurring behavior of female temperate zone songbirds and in the ability of exogenous administration of testosterone (T) to pharmacologically induce in females male-like patterns of brain and behavior (Riebel, 2003; Harding, 2004). Adult female canaries, which normally sing less frequent and simpler songs than males, sing with greater frequency and quality in response to T-treatment (Gahr and Garcia-Segura, 1996; Hartog et al., 2009). Likewise, the volumes of song control nuclei in T-treated adult female canaries tend to be larger than those of non-treated adult females (Nottebohm, 1980). T-treatment in adult female songbirds induces a cascade of molecular, morphological, and behavioral changes that appear to result in the recapitulation of the sensorimotor phase of song learning so that the ensuing song sounds more male-like (Nottebohm, 1980). The activation of male-like song in female canaries seems to recapitulate a developmental learning stage typically observed in males (Nottebohm et al., 1986, 1987), and is also associated with the partial masculinization of the brain of T-treated females (Nottebohm 1980).

In captive male canaries, naturally occurring changes in T concentrations are related to the development of song progressing from subsong, to plastic song and crystallized song (Nottebohm et al., 1986; 1987). These changes in song behavior are causally related to T as T-treatment in adult male gonadectomized songbirds induces a cascade of molecular, morphological, and behavioral changes similar to changes that are observed in intact photostimulated males. Moreover, in gonadally intact adult male canaries song repertoire can vary across seasons with the addition and deletion of syllable types as a function of age. These changes parallel and are partially caused by seasonal changes in T concentration (Nottebohm et al., 1986, 1987; Leitner et al. 2001).

T thus appears to have powerful effects on song behavior and the underlying brain regions mediating song behavior in male and female canaries. However, it is not known if male and female songbirds respond to T in the same way. When adult sex differences are mediated by differences in circulating steroid concentration (i.e. activational effects rather than organizational effects early in ontogeny), it is often assumed that males and females have similar capacities to respond to T. This is not necessarily the case. It is not known if the pattern of vocal development and associated changes in neural morphology is the same in T-treated male and female songbirds. This study systematically investigates the activational effects of varying doses of T-treatment in adult male and female canaries housed under the same photoperiodic conditions.

2. Methods

2.1. Experimental animals

Forty-three male and forty-three female American singer canaries ranging from 12–18 months old were obtained from a local breeder (Maryland Exotic Birds) and housed in an indoor aviary on an 8L:16D (light:dark) light cycle for at least eight weeks. Birds were kept in 49 × 95 × 51 cm cages (six birds of the same sex per cage) at Johns Hopkins University, Baltimore, MD and fed canary food and provided water *ad libitum*. Care and handling of all animal subjects was in accordance with guidelines published by the National Research Council (2011) and all experimental procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. After eight weeks on 8L:16D, females' ovaries were regressed, as confirmed by laparotomy, and males with regressed testes were castrated under general anesthesia using isoflurane (mix of air gas and 3% isoflurane for induction and 2.5% during the surgery). The two testes were removed through a small incision in the flank, posterior to the last rib. The incision was sutured closed and lidocaine and antibiotic ointment were placed on the wound. Males were allowed to recover under warm light before being returned to their home cages. One week after castration, blood samples were taken from each bird and only male and female canaries with undetectable blood T levels were placed on study. Females in this study were not ovariectomized based on two factors. First, many studies of T-induced adult neuroplasticity have successfully used female canaries that were not ovariectomized but had photoregressed ovaries (e.g., Goldman and Nottebohm, 1983; Hartog et al., 2009). Second, there is a high vascularization of the ovary in female canaries even in photoregressed individuals. This vascularization increases mortality rates when removing this structure. There may be some concern that the short day photosensitive males experience a castration response (i.e. an increase in Gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) due to the removal of negative feedback from steroid hormones that will not occur in the photoregressed females thus introducing a bias in the sex comparison in the responses to T. We do not think this difference significantly influences our results as work by Storey and Nicholls (reviewed in Nicholls et al., 1998) showed that males on short days have an attenuated castration response as compared to males on long days and most males received implants of T that rapidly decreases GnRH and LH release. The castrated males that received empty implants did not produce high levels of song or large song control nuclei, in agreement with the published literature suggesting that there are no known direct effects of GnRH or LH on song behavior and brain plasticity.

Males and females remained on an 8L:16D light cycle for at least eight additional weeks before they were implanted subcutaneously with one Silastic™ implant (Dow Corning, Midland, MI, USA, No. 602-175; 0.76 mm inner diameter [ID], 1.65 mm outer diameter [OD]) of either 2 mm, 6 mm, 12 mm length filled with crystalline T or a 12 mm implant left empty as a negative control. We defined the T treatments as low, medium, and high dose respectively. Implant length and diameter were selected based on previously published studies in canaries indicating that these implant sizes are sufficient to activate singing and to increase song control nuclei size to levels characteristic of gonadally-intact males (Appeltants et al., 2003; Nottebohm, 1980; Sartor et al., 2005).

Final numbers of males (M) and females (F) in each treatment that were analyzed for behavioral and histological variables were as follows: week 1, control: 5M/6F, low: 6M/5F, medium: 5M/5F and high: 5M/5F; week 3, control: 6M/5F, low: 5M/5F, medium: 4M /6F and high: 6M/6F.

Starting immediately after these subcutaneous implantations, all male and female canaries were individually housed in sound attenuated chambers (41 cm × 48 cm × 51 cm). Song behavior was recorded for thirty minutes, three times per day for either seven or twenty-one days, depending on treatment group. Isolation chambers were outfitted with a microphone (BT-MP8087 Mini microphone; B&H Foto and Electronics Corp, New York, NY) and camera (KPC-600 Pinhole Camera 3.6 mm; B&H Foto and Electronics Corp, New York, NY) connected to computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time video and audio surveillance recording. Behavioral recordings (audio and video) began immediately after lights on (0900 h), four hours after lights on (1300 h), and thirty minutes before lights off (1630 h). All canaries remained on an 8L:16D photoperiod for the duration of the study as it was necessary to examine the effects of the steroid hormone treatment in the absence of any other cues characteristic of breeding condition.

2.2. Behavioral quantification

Sound files (converted from audio/video file format .mp4 to audio only .wav files; the video information was not analyzed in