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Anatomically discrete sex differences and enhancement by testosterone of cell proliferation in the telencephalic ventricle zone of the adult canary brain

Jennifer M. Barker [a], Gregory F. Ball [b], Jacques Balthazart [a,*]

[a] GIGA Neurosciences, University of Liege, B-4000 Liege, Belgium
[b] Department of Psychological and Brain Sciences, Johns Hopkins University, Baltimore, MD 21218, United States

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ABSTRACT

Previous work in songbirds has suggested that testosterone increases neuronal recruitment and survival in HVC but does not affect neuronal proliferation in the ventricular zone and that males and females have similar rates of proliferation except at discrete locations. Many of these conclusions are however based on limited data or were inferred indirectly. Here we specifically tested the effects of testosterone on cellular proliferation in the ventricular zone of both male and female adult canaries. We implanted adult birds of both sexes with testosterone or empty implants for 1 week and injected them with BrdU. One day later, we collected their brains and quantified BrdU-positive cells in the ventricular zone (VZ) at different rostro-caudal levels of the brain, ranging from the level where the song nucleus Area *X* occurs through the caudal extent of HVC. Proliferation in the dorsal part of the VZ was low and unaffected by sex or testosterone treatment. In the ventral part of the VZ, females had more proliferating cells than males, but only at rostral levels, near Area *X*. Also in the ventral part of the VZ, testosterone increased proliferation in birds of both sexes, but only in the mid- to caudal-VZ, caudal to the level of Area *X*, around the septum and HVC. We thus demonstrate here that there is both an effect of testosterone and possibly a more subtle effect of sex on cellular proliferation in the adult songbird brain, and that these effects are specific to different levels of the brain.

1. Introduction

Adult vertebrate brains continue to produce new neurons throughout life. This process is particularly prominent in songbirds where seasonal changes in numbers of new neurons in the song system nucleus HVC (used as a proper name; Reiner et al., 2004) during the breeding season are reflected by changes in the volume of the nucleus (Kirn et al., 1994). These new neurons are generated from the ventricular zone (VZ) around the lateral ventricles and migrate throughout the telencephalon (Alvarez-Buylla and Nottebohm, 1988; Scott and Lois, 2007; Balthazart et al., 2008; Vellema et al., 2010). Changes in HVC volume and neuron number take place across seasons based on studies in several species (Nottebohm et al., 1986; Kirn et al., 1994; Smith et al., 1997). Such changes can to some extent be reproduced by administering testosterone to adult female songbirds in species such as the canary (Goldman and Nottebohm, 1983; Rasika et al., 1994; Yamamura et al., 2011) indicating that gonadal hormone fluctuations contribute to the control of some aspects of neurogenesis in the adult canary brain even if the details of how and when these effects of testosterone take place are still partly unclear. Multiple studies have indeed demonstrated seasonal or testosterone-induced changes in the numbers of new neurons migrating to, incorporated or surviving in HVC (Rasika et al., 1994; Yamamura et al., 2011).

Because hormone treatment results in a change in neuron number in HVC, that is usually not observed in the surrounding telencephalic areas, it has been inferred that these changes reflect modifications in neuron recruitment or survival but not in proliferation in the VZ (Rasika et al., 1994; Yamamura et al., 2011). A few studies more directly tested this notion in female canaries and concluded that testosterone increases incorporation into HVC and survival of new neurons but has no effect on proliferation in the ventricle wall (e.g. Brown et al., 1993; Rasika et al., 1994). However in one study of adult male starlings it was found that testosterone increased the number of bromodeoxyuridine-immunoreactive (BrdU-ir) cells near the ventricle wall but since brains were collected weeks after BrdU injection, it is difficult to separate the effects of testosterone on proliferation from reduced cell migration away from the ventricles (Absil et al., 2003).

* Corresponding author at: GIGA Neurosciences, University of Liege, 1 avenue de l'Hopital, B-4000 Liege, Belgium. Tel.: +32 4 366 59 70; fax: +32 4 366 59 71.
E-mail address: jbalthazart@ulg.ac.be (J. Balthazart).

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It has also been claimed that there is no sex difference in cell proliferation in the VZ (Alvarez-Buylla and Kirn, 1997) because neuron density or number in multiple telencephalic areas outside the song system do not differ between males and females. However, sex differences in proliferation have been reported in juvenile zebra finches in anatomically discrete brain regions, confined to the ventral and rostral part of the VZ at the level of Area *X* (DeWulf and Bottjer, 2002, 2005). Studies of 15-day-old Bengalese finches also revealed localized sex differences in cell proliferation that the authors related to the development of sex differences in the morphology of the song nuclei HVC and Area *X* (Zeng et al., 2007). Because adult females are often used to investigate cellular effects of testosterone on adult plasticity in songbirds, it is useful to confirm that cells in both sexes respond similarly to the steroid. There is to date no clear confirmation that this is the case in songbirds, whereas studies in mammals indicate that steroids affect proliferation of neural progenitor cells in the adult brain (recent reviews, Galea et al., 2006; Charalampopoulos et al., 2008) and do so in a sex-specific manner (Barker and Galea, 2008). There is evidence that progenitor cells in zebra finches respond differently to dehydroepiandrosterone in vitro, depending on whether they originated from a male or a female brain (Mirzatoni et al., 2010). These uncertainties led us to study in a quantitative manner the effects of testosterone on cell proliferation in the VZ of adult male and female canaries.

2. Materials and methods

2.1. Animals and in vivo treatments

Adult male ($n = 16$) and female ($n = 14$) canaries (*Serinus canaria*) were purchased from a local dealer (Noorderwijk, Belgium) during late summer and housed individually on a 14L:10D light schedule (lights on at 0700 h). All experimental procedures complied with Belgian laws concerning the Protection and Welfare of Animals and the Protection of Experimental Animals, and experimental protocols were approved by the Ethics Committee for the Use of Animals at the University of Liège.

One week after arrival, birds were implanted under the skin between their shoulders with a Silastic™ capsule (12 mm × 0.76 mm ID, 1.65 mm OD; sealed on each end with a 1 mm cap of silicone glue) either packed with 10 mm crystalline testosterone (Sigma–Aldrich) or left empty (control). A recent study from our laboratories indicated that these capsules produce serum concentrations of approximately 1.5 ng/ml in both male and female canaries when measured by enzyme immunoassay 1 and 3 weeks after implantation (Madison F.N., Rouse L.L.Jr., Balthazart J., Ball G.F., submitted for publication). These Silastic capsules of testosterone maintain elevated circulating concentrations of the steroid that are typical of adult sexually mature males for at least 3 weeks (Rasika et al., 1994; Madison F.N., Rouse L.L.Jr., Balthazart J., Ball G.F., submitted for publication), and cause significant growth of HVC in female canaries (Nottebohm, 1980; Sartor et al., 2005; Boseret et al., 2006). Treatments and sex defined 4 experimental groups: control males and females (MC: $n = 9$, FC: $n = 5$) and testosterone-treated males and females (MT: $n = 7$, FT: $n = 9$).

One week later, birds were injected with BrdU (100 mg/kg) five times, every 2 h on a single day starting at 0830 h to ensure extensive labelling of cells replicating their DNA with minimal cellular damage. Previous studies used similar repeated injections of DNA replication markers (e.g., 6 injections of tritiated thymidine performed 8 h apart in Goldman and Nottebohm, 1983) and similar doses were used in many previous studies (e.g., Melleu et al., 2013; Briones and Wood, 2011; Catlow et al., 2009; Jung et al., 2009; see Taupin, 2007 for review). Previous work with BrdU actually showed that lower doses (e.g., 50 mg/kg) do not label all proliferating cells (Cameron and McKay, 2001). We also recently showed that a dose of 100 mg/kg BrdU is cleared from the canary blood within less than 2 h (Barker et al., 2013) so that accumulation of the tracer in the blood that would reach cytotoxic concentrations is very unlikely.

Birds were killed by decapitation 24 h after the third injection. The sex of each bird was confirmed by autopsy. Gonads and female oviduct were weighed as an indication of recent hormonal status. Brains were removed and fixed in 5% acrolein in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 180 min, followed by two 30 min rinses on in PBS and cryoprotected in 30% glucose in PBS for 3 days. Brains were then frozen on dry ice and kept at −80 °C until sectioning in the coronal plane on a cryostat (Leica).

All brains were mounted on the cryostat with the rostral tip up and the rostral part of the brain was trimmed until the point where the ventricle adopts a general "Y" shape (distinct lateral movement of the dorsal part of the ventricle) as opposed to the parallel orientation of the left and right ventricles seen in the more rostral sections. This level roughly corresponds to level A4.0 in the Stokes et al. (1974) atlas. In males, this is also the most rostral level where Area *X* appears. From that point on, all 30 μm thick sections were collected in 12 series of 11 sections until the caudal end of the telencephalon (approximately level P0.8-1.0 in Stokes et al., 1974). One of these 12 series of sections was then stained for BrdU so that the periodicity of sampling was one section every 360 μm apart (12 × 30; i.e. there was 330 μm between two sections). The approximate level of these sections in the rostro-caudal axis is schematically indicated in Fig. 1. The position of the Area *X*, septum and HVC is approximate on these sections and is only used as a rough indication of where each section was located in the brain. The absolute reference for locating effects is the number of the section running from 1 to 11 in the rostro-caudal direction.

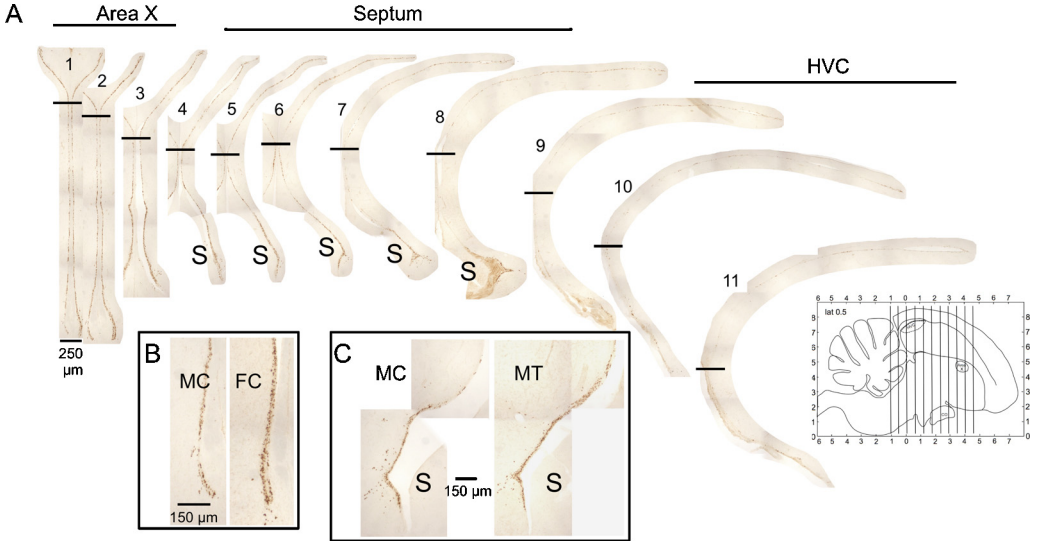

Fig. 1. Representative photomicrographs of stained coronal sections from a control-treated male canary brain illustrating the BrdU labelling of the ventricle wall at successive rostro-caudal levels (from left to right). Levels corresponding to Area *X* (1–3), septum (4–8), and HVC (9–11) are indicated. Horizontal black bars show the separation between 'dorsal' (top) and 'ventral' (bottom) subregions of the lateral ventricles. Note the intensely stained "hot spot" of proliferation in the ventricle wall around the septum (S). Insets illustrate at higher magnification the sex difference in control birds at a rostral level, at the level of Area *X* (B) and the effect of testosterone in males at a caudal level, at the level of the septum (C). The small figure in the lower right corner provides a schematic illustration of the position of the 11 sections that were stained and quantified reported on a sagittal view of the brain as provided in the Stokes et al. (1974) canary brain atlas. Note that this view represents a parasagittal section at 0.5 mm of the mid-line; the full extension of the telencephalon and of nuclei HVC and Area *X* are thus not represented.