

Published in final edited form as:

Dev Neurobiol. 2010 September 15; 70(11): 751–763. doi:10.1002/dneu.20808.

Photoperiodic differences in a forebrain nucleus involved in vocal plasticity: enkephalin immunoreactivity reveals volumetric variation in song nucleus IMAN but not Nif in male European starlings (*Sturnus vulgaris*)

Tyler J Stevenson^{a,*} and Gregory F Ball^a

^a Department of Psychological and Brain Sciences, Johns Hopkins University

Abstract

Seasonal variation in the volume of various song control nuclei in many passerine species remains one of the best examples of naturally occurring adult neuroplasticity among vertebrates. The lateral portion of the magnocellular nucleus of the anterior nidopallium (IMAN) is a song nucleus that is important for song learning and seems to be critical for inducing variability in the song structure that is later pruned via a feedback process to produce adult crystallized song. To date, IMAN has not been shown to exhibit seasonal changes in volume, probably because it is difficult to resolve the boundaries of IMAN when employing histological methods based on Nissl staining. Here, IMAN_{core} volumes were examined in intact photostimulated (i.e. breeding), castrated photostimulated and photorefractory (i.e. non-breeding) male starlings (*Sturnus vulgaris*) to investigate the degree of seasonal variation in brain morphology. We present data demonstrating that the volumes of the total MAN and IMAN_{core} delineated by enkephalin immunoreactivity are greater in photostimulated male starlings as compared to photorefractory males. Moreover, two other regions associated with the song system that have not been investigated previously in the context of seasonal plasticity namely i) the medial portion of MAN (mMAN), and ii) the nucleus interfascialis (Nif) did not display significant volumetric variation. We propose that greater IMAN_{core} volumes are associated with the increase in vocal plasticity which is generally observed prior to production of stereotyped song.

Keywords

songbird; neuropeptide; reproduction; neuroplasticity; learning

INTRODUCTION

Seasonally breeding songbirds in the temperate zone display one of the most dramatic examples of naturally-occurring neuroplasticity and behavior known in extant vertebrate species. In particular, many songbird species exhibit profound seasonal changes in singing behavior. This seasonal variation in singing behavior correlates positively with seasonal changes in testosterone concentrations and the volumes of brain nuclei responsible for song learning and production (Nottebohm, 1981; Brenowitz, 2008; Ball et al., 2008; Tramontin and Brenowitz 2000; Ball, 1999). There is a substantial amount of data consistent with the notion that the vernal increase in photoperiod stimulates gonadal growth and the release of gonadal sex steroid hormones such as testosterone that in turn facilitates the volumetric

*Corresponding Author: Department of Psychological and Brain Sciences, Johns Hopkins University, 3400N Charles St. Baltimore, MD, 21218, tsteve13@jhu.edu.

growth of the song system and singing behavior (Ball, 1999; Tramontin and Brenowitz 2000). However, several studies have demonstrated gonadal or testosterone-independent effects on the volumes of several song nuclei (Bernard et al., 1997; Smith et al., 1997; Gulledge and Deviche, 1998; Tramontin et al., 1999; Dloniak and Deviche, 2001; Sartor and Ball, 2005; Boseret et al., 2006). These findings suggest that factors other than steroids can regulate song nucleus volumes (Ball et al., 2004; Ball et al., 2002).

The song control system that governs singing behavior consists of a series of discrete, interconnected nuclei. One part, the anterior forebrain pathway (AFP) in particular is essential for successful song development and stereotypy in adulthood (Bottjer et al., 1984, Doupe, 1997; Brainard and Doupe, 2002). The AFP circuitry originates in HVC (acronym used as a proper name; which projects to Area X; that in turn projects to the dorsolateral nucleus of the anterior thalamus (DLM); that connects to the lateral nucleus of the magnocellular nidopallium (IMAN) that projects onto the motor output pathway via a connection to the robust nucleus of the archipallium (RA). Recent studies have proposed that IMAN plays a critical role for inducing vocal plasticity in song structure during development and in adulthood (Olviczky et al., 2005; Thompson and Johnson, 2007). In male zebra finches, (*Taeniopygia guttata*) bilateral lesions to IMAN resulted in songs with notes that lacked the normal frequency modulations and produced extremely long bouts of songs deficient in typical phrase types (Bottjer et al., 1984; Scharff and Nottebohm, 1991). In zebra finches, IMAN has been shown to have a core and shell architecture (Johnson and Bottjer, 1992; Johnson et al., 1995). Interestingly, during song development in male zebra finches the volume of IMAN_{shell} undergoes a striking increase in overall volume during early stages of vocal learning followed by an equally substantial decrease by adulthood when birds have acquired stable song patterns (Johnson and Bottjer, 1992; Iyengar and Bottjer, 2002). Moreover, male zebra finches were observed to have increased IMAN cell soma at 50 days compared to female zebra finches (Nixdorf-Bergweiler, 1998). This increase in volume coincides with the sensorimotor phase of song learning when male zebra finches produce highly variable vocal patterns that are gradually modified to match the initially memorized tutor songs (Nixdorf-Bergweiler, 1998). Many seasonally breeding songbirds, in particular European starlings (*Sturnus vulgaris*) exhibit seasonal and age dependent variation in song structure (see Eens, 1997 for a review). It has been proposed that seasonally breeding birds undergo an annual sensorimotor phase resulting in variation in song structure (see Brainard and Doupe, 2002 for a review). It is possible that the annual sensorimotor phase coincides with changes in IMAN_{shell} and/or IMAN_{core} volumes as a result of the yearly induction of variability in song repertoires similar to the sort of structural changes that occur in IMAN during development in association with sensorimotor learning. Traditional Nissl staining methods can define the borders of IMAN_{core}, (Johnson and Bottjer, 1992). However, previous studies attempting to delineate volumetric variation in seasonally breeding birds have produced inconsistent results due to an inability to crisply delineate nucleus boundaries (Airey and DeVoogd, 2000; MacDougall-Shackleton et al., 1998; MacDougall-Shackleton et al., 2005). However, chemical neuroanatomical studies have revealed a variety of neuromodulatory proteins that do clearly delineate the boundaries of IMAN_{core} and in some cases mMAN (Ball et al., 1988; Ball and Balthazart, 2010). By using these alternative approaches to nuclear boundary definition, one can gain insight into the seasonal regulation of IMAN_{core} volume.

The nucleus interface of the nidopallium (Nif) is a song system nucleus that connects to HVC and the auditory system. Nif sends afferent connections to HVC (Nottebohm et al., 1982; Janata and Margoliash, 1996), the main sensorimotor nucleus necessary for song learning and production. Nif is also reciprocally connected with the caudal lateral mesopallium (CLM), a secondary auditory processing area (Vates et al., 1996). Cells located in Nif exhibit strong selectivity to the bird's own song (BOS) and the response is dependent

on behavioral state. Moreover, NIf cell show strong premotor activity that is time-locked to the production of song syllables (Hahnloser and Fee, 2003; Cardin and Schmidt, 2003, 2004). Bilateral lesions of NIf result in significant reductions in spontaneous and auditory activity in HVC, however, song production was not impaired (Cardin et al., 2005). These data suggest then that NIf is a major source of auditory input to HVC, but is not necessary for motor production.

Immunohistochemical studies have demonstrated that NIf is a site for neuromodulation by a number of different transmitter systems. Specifically, neuromodulatory inputs to NIf include catecholaminergic (Soha et al., 1996; Harding et al., 1998; Mello et al., 1998); cholinergic (Ryan and Arnold, 1981a); as well as peptide systems such as vasoactive intestinal polypeptide (VIP) and enkephalin (ENK; Ryan et al., 1981b; Ball et al., 1995). Interestingly, both VIP and ENK clearly delineate the boundaries of the nucleus and reveal a sex difference in the volume of NIf with greater volumes found in male compared to female zebra finches (Ball et al., 1995). Given the importance of auditory feedback for song learning and maintenance of stereotype (Leonardo, 2004, Brainard and Doupe, 2001), and the intimate association of NIf to the song system, this nucleus is a candidate to undergo seasonal variation in volume.

In the present study, we compared the volumes of total MAN, $IMAN_{core}$, mMAN and NIf based on enkephalin immunoreactivity from male starlings that were either photostimulated intact, photostimulated castrate or photorefractory. We found that $IMAN_{core}$ volumes were significantly greater in photostimulated birds independent of gonadal state and suggest that the variation in $IMAN_{core}$ volume is associated with changes in vocal plasticity in a seasonally breeding songbird.

METHODS

Twenty four wild caught adult male European starlings were caught using a drop down V-trap in early March 2008 in Conneautville, PA (41° 45' N lat., 80° 22' W long). Birds were transported to the Johns Hopkins University and group housed (4 per cage). All birds were placed on the natural photoperiod experienced on the date of capture (11L:13D) in order to maintain photosensitivity. Birds were treated and handled in accordance with all appropriate animal care guidelines and permits.

Treatment Groups

Seven days after acclimation to laboratory conditions, all starlings were laparotomized under isoflurane (3–4% induction, then 1–2% maintenance) and the dimensions of the left testis were measured with calipers. Testis volume was determined using the equation $V = 4/3\pi a^2 b$, where a is half the width and b is half the length. All birds were observed to have undeveloped testis indicative of a photosensitive state and suggest that the birds were not currently exhibiting gonadal recrudescence (Mean \pm SEM = 58 ± 4 mm³). These data indicate that starlings had not experienced elevated levels of gonadal steroids prior to the present experiment. Eight adult males were selected for castration, starlings were anesthetized and a small incision was made in the lower abdomen and the testes removed with forceps. For the sham surgeries eight adult males were anesthetized and a small incision was made in the lower abdomen and the testes were touched with forceps but not removed. After sacrifice the body cavity was inspected for testicular fragments and none were found in castrates. The eight castrated and intact males were housed individually on short daylengths to maintain sensitivity. Two weeks prior to the termination of the experiment, all males were transferred to long daylengths (16L:8D) to induce gonadal growth and a photostimulated state. Another group of eight males were placed on the same long photoperiod for nine weeks to induce photorefractoriness. The photoperiods utilized in

this experiment have previously shown to be effective to induce photostimulation and subsequent photorefractory states (Dawson et al., 2001). The photoperiod treatment conditions were arranged such that all birds were in the required photoperiodic condition so that their brains were collected on the same calendar day. To determine male reproductive condition, beak coloration was assessed throughout the experiment; and blood was collected for testosterone determination and testis volume was measured at the time of brain collection. The color of the beak is a reliable indicator of whether testosterone is present or absent in the circulation in European starlings. Yellow means that testosterone is detectable by standard assay methods such as radioimmunoassay while black means that testosterone is undetectable (Ball and Wingfield, 1987; Gwinner, 1975; Dawson, 1983). Because a yellow beak is a reliable indicator of the presence of testosterone, measuring beak color provides a reliable indicator of the transition across reproductive states (Ball and Wingfield, 1987; Gwinner, 1975; Bernard and Ball, 1995; Falk and Gwinner, 1988). Beak scores were measured on a four point scale with a score of 0 equaling completely black; 1 equals black base with a little yellow tip; 2 equals a yellow beak with a slightly black coloration; and 3 is a yellow beak with blue base. At the termination of the experiment, eight intact males had bright yellow beaks, whereas the castrates and photorefractory males had black beaks indicative of no detectable testosterone being present in the circulation.

Hormone Assay

Samples for hormone analysis were taken via puncturing the alar (wing) vein with a 25-gauge needle and 300–500 μ l of blood was collected into heparinized tubes. The blood samples were transferred into microfuge tubes and centrifuged at 8900 rpm for 10 minutes. The supernatant was removed with a Pasteur pipette and stored in Eppendorf vials at -20° until assayed for testosterone. The serum was analyzed in a single run of duplicates (50 μ l) using a commercially available 125 I Coat-A-Count kit for total testosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA). This kit consistently provides reliable hormone concentrations and has been previously used in a number of avian species including starlings (Stevenson et al., 2008; Stevenson and Ball, 2009). The antiserum is highly sensitive for testosterone (i.e. 100 pg/ml) and shows negligible cross reactivity with other steroids including dihydrotestosterone (<3.5%); 17β -estradiol (< 0.01%); corticosterone (< 0.01%). The intra-assay coefficient of variation averaged 11%.

Perfusion

At the end of the experiment, birds were sacrificed and brains removed. Birds were deeply anaesthetized with secobarbital (50mg/ml im, Sigma). Then, the birds were transcardially perfused with 0.1M phosphate buffered saline (PBS) pH 7.5, followed by 4% paraformaldehyde. Then the brains were dissected out and placed in 4% paraformaldehyde and left overnight at 4° C. The following morning, the brains were transferred to a sucrose solution (30% sucrose in 0.1 M PBS) and left overnight at 4° C. The brains were then frozen with powdered dry ice for five min., and left in the freezer (-70° C) until sectioning. Brains were sectioned coronally (40 μ m) using a cryostat, every fourth section was collected and placed in tissue wells containing 0.1M PBS.

Antibody Specificity

Immunocytochemistry was carried out on all brains in random order such that the time from tissue collection to processing was equivalent for all groups. Control tests were conducted to validate the primary antibody used in this protocol. Tissue sections were either incubated in the absence of primary/secondary antibody or presaturated with enkephalin antigen (Bachem). The absence of the primary and secondary antibody eliminated enkephalin immunoreactivity. Furthermore, preabsorption of the enkephalin antibody (1:2000

methionine enkephalin; ENK Immunostar Inc.) with 100 µg of enkephalin (Bachem) significantly reduced enkephalin immunoreactivity.

Immunocytochemistry

The immunocytochemistry protocol commenced with the sections washed in 0.1 M phosphate buffer saline (PBS) three times, once in 0.5% H₂O₂ for 15 min., then washed three times in 0.1 M PBS and left overnight in tyramide blocking reagent solution (TNB; Perkin Elmer, TSA Biotin System) at 4°C. The following day sections were incubated in TNB and primary antibody (1:2000 methionine enkephalin; ENK Immunostar Inc.) for 1 hr at room temperature and then placed at 4°C overnight with agitation. The sections were then washed three times in 0.1M PBS with 1% Triton X (PBS/T; Fisher Scientific Laboratories), then incubated in biotinylated secondary antibody (goat anti rabbit IgG, 1:250) for 1 h, washed three times in 0.1% PBS/T, incubated in avidin biotin horseradish-peroxidase complex (Vectastain ABC, Elite Kit 1:200) for 1 h and then washed again three times in 0.1% PBS/T. Sections were then incubated in biotinylated tyramine (1:150; Perkin Elmer) and then washed three times in 0.1% PBS/T. Followed by one hour incubation in horseradish peroxidase (Perkin Elmer, TSA Biotin System, 1:200) and subsequently washed three times in 0.1% PBS/T. Antibodies were visualized by incubating the sections with diaminobenzidine (Sigma Fast DAB) for 4 minutes. Finally, sections were washed three times with 0.1 M PBS and mounted onto gelatin coated microscope slides. Sections were then serially dehydrated in ethanol and then placed in xylene for ten minutes. The slides were coverslipped using Permount (Fisher).

Song Nucleus Volume Reconstruction and Statistical Analysis

We measured the volume of HVC, Area X, and RA using thionin, a dye that stains for Nissl bodies. We collected 40 µm sections from regions of interest and placed in .1 M PBS solution, then mounted onto gelatin-coated microscope slides. The slides were dried, stained with thionin for two minutes, serially dehydrated in ethanol at 50%, 75%, 95%, 100% for one minute and a final step in 100% ethanol for five minutes. The slides were then cleared in xylene (Fisher Scientific) then coverslipped with Permount (Fisher Scientific). The boundaries of IMAN_{core}, mMAN, and NIF were defined by enkephalin immunoreactivity. In brief, brain regions of interest were digitized using a bright field light microscope (Zeiss Axioskop, Carl Zeiss, Thornwood NY) with a CCD camera connected to a MacIntosh computer. For each image, the area of the brain region was measured using Openlab 5.0.2 (Improvision, Lexington, MA). The volume of each region was then reconstructed combining the areas of subsequent sections with the sampling interval (160 µm) using the formula for a truncated cone (developed by Smith et al., 1995) as used previously in starlings (Bernard and Ball, 1995; Bentley et al., 1999; Bernard and Ball, 1997). For each bird we used the average volume of the left and right hemispheres, summed the values across sections and then multiplied by the width of the interval. The volumes of all nuclei examined could be successfully delineated in all males.

One-way ANOVAs were conducted for beak scores, plasma testosterone concentrations, and brain nucleus volume. For testis volume, the castrates were removed from the statistical analysis and a t-test was conducted between intact photostimulated and photorefractory starlings. Tukey's post hoc tests were conducted to evaluate pair-wise comparisons. Pearson's correlation tests were conducted to evaluate inter-hemispheric differences in song nucleus volumes IMAN_{core}, mMAN and NIF that were obtained using enkephalin immunoreactivity.

RESULTS

Assessment of Reproductive State

A one-way ANOVA revealed a significant difference in beak scores across treatment groups ($F(2,23) = 72.21, P < 0.001$; Fig. 1A). Tukey's post-hoc analysis demonstrated that intact males had significantly higher beak scores compared to castrate ($p < 0.001$) and photorefractory males ($p < 0.001$). Furthermore, castrated males had higher beak scores compared to photorefractory males ($p < 0.01$). A t-test revealed a significant difference in testis volume between photostimulated and photorefractory starlings ($t(14) = 12.18, p < 0.001$). A one-way ANOVA for plasma testosterone concentrations showed a significant difference across treatment groups ($F(2,23) = 9.97, P < 0.001$; Fig 1C). Tukey's post-hoc analysis revealed a significant difference with intact males having greater amounts of testosterone compared to castrates ($p < 0.005$) and photorefractory birds ($p < 0.005$). However, there was no significant difference between castrated and photorefractory males ($p = 0.96$ ns).

Song Nucleus Volumes

A one-way ANOVA revealed a significant difference in HVC volume across treatment groups ($F(2,23) = 5.38, P < 0.05$; Fig. 2A). Tukey's post-hoc analysis demonstrated that intact males had significantly larger HVC volumes compared to photorefractory birds ($p < 0.01$). However, there were no significant differences between intact and castrate ($p = 0.29$), and castrate and photorefractory males ($p = 0.21$). There was a significant difference in the volumes of Area X across treatment groups $F(2,23) = 11.53, P < 0.001$; Fig. 2B). Intact starlings had significantly greater Area X volumes compared to castrates ($p < 0.005$) and photorefractory starlings ($p < 0.001$). There was no significant difference between castrated and photorefractory starlings ($p = 0.61$). There was a significant difference in RA volume across treatment groups ($F(2,23) = 8.86, P < 0.005$; Fig. 2C). Intact starlings had significantly greater RA volumes compared to castrates ($p < 0.05$) and photorefractory starlings ($p < 0.001$). There was no significant difference between castrated and photorefractory starlings ($p = 0.35$).

A one-way ANOVA revealed a significant difference in total MAN volumes across the treatment groups ($F(2,23) = 5.29, P < 0.05$; Fig. 3A, Fig. 4). Tukey's post-hoc test showed that intact males were significantly greater MAN volumes compared to photorefractory males ($p < 0.05$). However, there was no significant difference between intact versus castrates ($p = 0.86$, ns) and castrates versus photorefractory males ($p = 0.06$). A one-way ANOVA demonstrated a significant difference in $IMAN_{core}$ volumes across treatment groups ($F(2,23) = 8.66, P < 0.005$; Fig. 3B). Intact males had significantly greater $IMAN_{core}$ volumes compared to photorefractory males ($p < 0.005$). Furthermore, castrated males had significantly greater volumes compared to photorefractory males ($p < 0.01$). However, there was no significant difference between intact and castrated males ($p = 0.97$, ns). There was no significant difference across treatment groups on measures of mMAN volumes ($F(2,23) = 2.16, P = 0.14$, ns; Fig. 3C). There was no significant difference in Nif volumes across treatment groups ($F(2,23) = 0.97, P = 0.39$, ns; Fig. 3D). Pearson's correlations were conducted for $IMAN_{core}$, mMAN and Nif volumes to evaluate the relationship between the measurements collected from the left and right hemispheres. All song nucleus volumes had significantly positive inter-hemisphere correlations ($IMAN_{core}$: $r = 0.77; P < 0.0001$; mMAN: $r = 0.80; P < 0.0001$; Nif: $r = 0.53; P < 0.0001$).

DISCUSSION

This study provides evidence for the occurrence of photoperiodic changes in the volume of $IMAN_{core}$, but not the closely associated mMAN or Nif in male European starlings. The

song nucleus volumes of both intact and castrated photostimulated males had significantly greater IMAN_{core} volumes compared to photorefractory males. These data suggest that the increase in photoperiod can regulate IMAN_{core} volume in male European starlings independently of gonadal state. We also found that the volume of mMAN and Nif were relatively constant across the treatment conditions. These findings provide novel insight into the photoperiodic regulation of brain regions that are necessary for song learning and maintenance. We also replicated previous studies in starlings indicating that photoperiod and gonadal testosterone can regulate the volume of song nuclei such as HVC, RA and Area X (e.g., Bernard and Ball, 1995; Riters et al. 2002) as has been shown in a variety of other songbird species (Tramontin and Brenowitz, 2000).

Histochemical delineation of song nucleus volume

Nissl staining methods have traditionally been employed to delineate song nucleus boundaries for volumetric analyses. However, Nissl stains are limited in the information they provide. Nissl stains label more prominently components of a cell that are highly basophilic (i.e. ribosomes, Raine, 1989). Since ribosomes are essential organelles involved in protein synthesis; this method provides an indication of relative cellular activity with darker stains associated with more active cells (Raine, 1989). Nissl staining in general works extremely well for delineating the boundaries of many song nuclei (i.e. HVC, RA and Area X), however, other methods work better in the case of other nuclei (e.g., IMAN, Nif). Other histological markers have been demonstrated to provide an alternative method for measuring the volumes of song control nucleus by clearly delineating the boundaries of individual nuclei (Bernard and Ball, 1995; Gahr, 1990). Specifically, the Nissl stain is based on a grade of intensity and in some brain regions the borders are challenging to identify whereas the pattern of enkephalin immunoreactivity in the case of IMAN, for example, is more of a binary decision (i.e. present versus absent). It has been argued that a comprehensive analysis of song nucleus neuroplasticity requires the combination of cytoarchitectural, cytochemical and hodological experiments (Ball et al., 1994; Gahr, 1997; also see Vogels, 1997; Ball and Balthazart, 1997; Bottjer and Johnson, 1997; Brenowitz and Smith, 1997). The advantage of using enkephalin as a histological marker is that it provides a clear distinction of IMAN_{core} from other surrounding brain areas (see Fig. 4; Fig. 5). Taken together, the use of ENK immunoreactivity in the current experiment provides a direct means to determine volumetric changes in a telencephalic song nucleus, IMAN_{core}.

Functional Significance of Seasonal Changes in IMAN Volume

The seasonal variation in the song control system was initially observed for three song nucleus, HVC, RA and Area X based on studies in canaries (Nottebohm, 1981). The initial hypothesis for the functional significance of seasonal variation in song control nucleus proposed that the greater volumes of HVC and RA nuclei were necessary for song learning (Nottebohm, 1981; Nottebohm et al., 1981) and was consequently associated with an increase in song repertoire sizes (DeVoogd et al., 1993). However, the validity of this claim has been questioned by the fact that species that exhibit no seasonal change in song repertoire do exhibit marked seasonal changes in volume (e.g., Brenowitz et al., 1991) and by a study that demonstrated that population differences in repertoire size is positively correlated with large volumes of HVC and RA observed in marsh wrens (*Cistothorus palustris*) developed independently of song learning (Brenowitz et al., 1995). Another set of hypotheses proposed to explain seasonal variation in song nucleus volume attributes this variation to seasonal variation in song performance (e.g., Smith et al., 1995; 1997). According to these hypotheses the quality of song performance (e.g., degree of song stereotypy) (Smith et al., 1995; 1997) and/or the rate at which birds engage in singing behavior (Sartor and Ball, 2005) are associated with greater HVC volumes. There is

substantial support from a variety of species for these types of explanations of the function of song system plasticity (Brenowitz, 2008).

In starlings, HVC and RA do exhibit variation in song nucleus volume as a function of photoperiodic state (Bernard and Ball, 1995; Bernard et al., 1996; Bentley et al., 1999) or season (Riters et al., 2000). The variation in song nucleus volumes are positively correlated with song bout length (Bernard et al., 1996) and singing rate (Sartor and Ball, 2005). Unlike HVC, IMAN is not required for song production in adult birds, but it is necessary for normal song learning in juveniles (based on studies in zebra finches; Bottjer et al., 1984) and plays a role in producing song variability in adult and juvenile zebra finches (Olveczky et al., 2005, Thompson et al., 2007; Aronov et al., 2008). We propose that the photoperiodic variation in IMAN_{core} volumes is associated with increased singing performance as a result of the song remodeling that is associated with the yearly plasticity in stereotypy and other measures of song performance. Support for this hypothesis requires comparing the amount of variability in song structure with IMAN_{core} volumes during the annual sensorimotor phase of song learning that occurs in male starlings (Eens, 1997). In male zebra finches, IMAN_{shell} exhibits a marked increase in overall volume during the early stages of vocal development followed by a dramatic retraction by adulthood when birds have acquired stable song patterns (Johnson and Bottjer, 1992). The decrease in IMAN volume is paralleled by a substantial decline in the numbers of IMAN neurons (Bottjer and Sengelaub, 1989). Moreover, the total number of DLM neurons remains stable throughout this period and the extensive changes in DLM to IMAN circuit are presumably attributable to the dynamic rearrangements at the level of individual DLM axon arbors over the period of song development (Iyengar and Bottjer, 2002). These findings indeed suggest that the volumetric changes in IMAN during song development in juvenile finches are associated with changes in vocal plasticity.

European starlings exhibit seasonal variation in song bout length with longer bouts associated with the breeding period (Riters et al., 2000; Eens et al., 1994; Eens, 1997). The annual change in song behavior is associated with the seasonal variation in the volumes of HVC, Area X and RA with larger volumes occurring in association with breeding conditions (Bernard et al., 1996; Riters et al., 2000). Here, we also observed photoperiodic-induced volumetric changes in HVC, RA and Area X. Moreover, HVC volumes in photostimulated castrates were intermediate to intact and photorefractory starlings. Whereas nucleus RA and Area X were both significantly smaller in photostimulated castrates compared to intact. These findings are reminiscent of those previously reported in white-crowned sparrows in which the growth of HVC precedes RA and Area X (Tramontin et al., 2000). Specifically, we observed a slight increase in HVC volume in castrates without an increase in Area X and RA. Since photostimulated castrates did not have gonadal steroids, HVC may have been unable to cause the full trans-synaptic effects on RA and Area X. Thus, these findings appear to provide further support for the hypothesis that there is sequential growth of the song system (i.e. RA and Area X after HVC) and that these volume increases in RA and Area X are dependent on the initial increase in HVC volume. Interestingly, the significant increase in IMAN_{core} volume observed in photostimulated castrates suggests that gonadal steroids are not necessary for regulating the seasonal change in IMAN_{core} volume. It is tempting to speculate that IMAN_{core} growth may actually precede the volumetric changes in HVC, RA, and Area X; in which the former is essential for inducing vocal plasticity while the later is critical for the development of stereotyped song.

Functional Significance of mMAN and Nif Volume

The functional significance of mMAN for song learning and production has been studied to a lesser extent compared to IMAN in all songbird species. mMAN sends projections to HVC (Nottebohm et al., 1982; Bottjer et al., 1989) and receives input from the DLM and RA

(Foster et al., 1997; Vates et al., 1997). The transient inactivation of mMAN using TTX does not significantly affect song structure in zebra finches, suggesting that an intact mMAN is not necessary for either song production or vocal plasticity (Olveczky et al., 2005). The lack of seasonal variation in nucleus volume provides further evidence suggesting that mMAN is not necessarily involved with changes in song production and/or song quality. It remains to be determined what the functional significance of mMAN is in relation to song. Given its connections with other brain nucleus involved in the regulation of song development and production it is certainly reasonable to postulate that it does play a role.

Nif receives afferent connections from the thalamic nucleus Uva and sends efferent connections directly into HVC (Nottebohm et al., 1982). It has been postulated that Nif provides key auditory input to the song system (Janata and Margoliash, 1999) for the sort of sensory-motor integration that is essential for song development. However, lesions of Nif do not prevent successful song learning in male zebra finch (Gardner and Fee, 2007), suggesting that auditory input to HVC also originates from auditory processing areas other than Nif. Uva also receives auditory input and can gate auditory responses to song in HVC and Nif (Coleman et al., 2007) so it may play an important role in sensory-motor integration needed for song learning and maintenance. Here we present evidence that Nif does not undergo photoperiodic variation in nucleus volume when the boundaries of the nucleus are clearly defined by enkephalin immunoreactivity. However, the delineation of Nif by enkephalin immunoreactivity does reveal a sex difference in volume based on studies in zebra finches (Ball et al., 1995). Thus this delineation of Nif has functional significance.

Neuromodulatory role of Enkephalin in IMAN, mMAN and Nif

Many neuromodulators have been identified in IMAN_{core}; mMAN and Nif (Soha et al., 1996; Harding et al., 1998; Mello et al., 1998; Ryan and Arnold, 1981a; Ryan et al., 1981b; Ball et al., 1995). In HVC, RA, and Nif catecholamines and cholinergic activity modulate auditory processing and/or motor production (Shea and Margoliash, 2003; Cardin and Schmidt, 2003, 2004). Few studies have investigated the functional significance of enkephalin in songbird species. Pharmacological studies have suggested that peripheral enkephalin administration can influence the frequency of song behavior (Riters, 2010). Enkephalin antagonist increased singing behavior (Riters et al., 2005) whereas agonist decreased singing behavior in male starlings (Schroeder et al., 2006). These findings suggest that opioids may regulate the motivation to engage in song production and not necessarily vocal plasticity or song quality. Further experiments determining how the opioid system modulates the electrochemical properties during song perception or song production are needed to formulate hypotheses regarding neuromodulatory control during song learning and maintenance.

Testosterone Independent Regulation of Song Control System

A number of studies have demonstrated that a change in photoperiod can have profound effects on the volume of song control nuclei independent of testosterone and/or gonad state (Bernard et al., 1997; Smith et al., 1997; Gullledge and Deviche, 1998; Tramontin et al., 1999; Bentley et al. 1999; Dlaniak and Deviche, 2001; Boseret et al., 2006). Here, we provide further support for testis independent regulation of a nucleus within the song system. The neural changes associated with the reduction in IMAN during development include a decline in neuron number (Bottjer and Sengelaub, 1989). However, the neural attributes associated with the photoperiodic increase in IMAN_{core} volume are not currently known in part because seasonal changes in IMAN_{core} volume have not been previously reported. Several neural attributes have been observed to account for the neuroplasticity in the song system (see Tramontin and Brenowitz, 2000; Brenowitz, 2004 for a review). One plausible explanation for the photoperiodic regulation of IMAN_{core} observed in this study

stems from evidence for an increase in axonal arboration from DLM efferent projections during development (Johnson and Bottjer, 1992). This hypothesis would predict that DLM efferent connections would vary considerably across reproductive states. A second hypothesis for the observed increase in IMAN_{core} volume is the result of greater neuronal soma sizes (Nixdorf-Bergweiler, 1998). Male zebra finches exhibit larger soma sizes during the sensorimotor phase of song learning and this may contribute to the increase in IMAN_{core} reported in the present study. How the change in photoperiod results in the recruitment of genes involved in these types of neuroplasticity requires further exploration.

However, even though the gonads of the starlings at capture had not undergone recrudescence, it remains possible that the IMAN_{core} volume had already started to increase. Previous studies have shown that several song nuclei are close to fully recrudescenced prior to the vernal increase in plasma testosterone concentrations (Tramontin et al., 2001; Ritters et al., 2002; Caro et al., 2005; Caro et al., 2006). An alternative explanation for the observed data is therefore that there was substantial growth in the IMAN_{core} by the time of capture even though the birds had relatively small gonads. As a result, IMAN_{core} volumes could have increased in intact and castrates prior to photostimulation and the effect of gonadectomy may not have resulted in the regression of the nucleus volume before the end of the experiment. Nevertheless, the data presented here provide support for marked changes in IMAN_{core} volume that is dependent on the reproductive state.

Summary and Conclusions

The prevailing hypothesis in the study of neuroplasticity in songbirds is that the vernal increase in testosterone concentrations drives the seasonal change in song nucleus volume (Brenowitz, 2008; Ball et al., 2008; Tramontin and Brenowitz 2000; Ball, 1999). Here, we provide the first report of a photoperiodic increase in IMAN_{core} volume, a nucleus that is critical for inducing vocal plasticity in song structure (Olviczky et al., 2005; Thompson and Johnson, 2007). It remains to be clarified how seasonal changes in the volume of song nuclei and song performance can be enhanced by environmental factors such as long photoperiods prior to the bird experiencing high testosterone concentrations or even in the absence of significant testosterone as was shown in this study. One hypothesis that emerges from the current findings is that variation in the neural attributes of IMAN_{core} may underlie the induction of vocal plasticity during song development and the annual sensorimotor integration of new song types in adulthood.

Acknowledgments

We would like to thank Adam Troyer for his assistance trapping starlings and Marc Calabrese for his technical assistance. This research was supported by NIH/NINDS RO1 35467 to GFB; TJS was supported by an NSERC PGS-D 334570.

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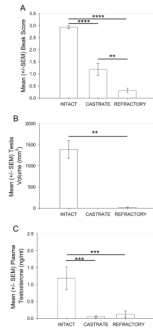


Figure 1.

The reproductive state in male European starlings. A) Mean (+/- SEM) beak score; B) Mean (+/- SEM) testis volume; C) Mean (+/- SEM) plasma testosterone concentrations at sacrifice. Significance was determined at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$ and asterisks indicate significant differences.

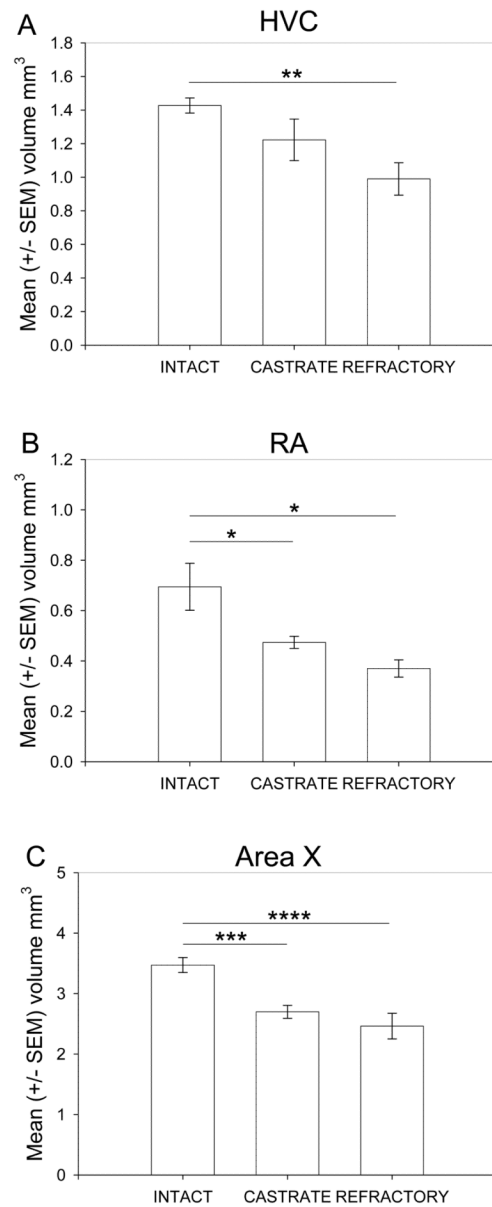


Figure 2. Nissl defined boundaries for HVC, RA and Area X volumes. A) Mean (+/- SEM) HVC volumes; B) Mean (+/- SEM) RA; and C) Mean (+/- SEM) Area X volumes. Significance was determined at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$ and asterisks indicate significant differences.

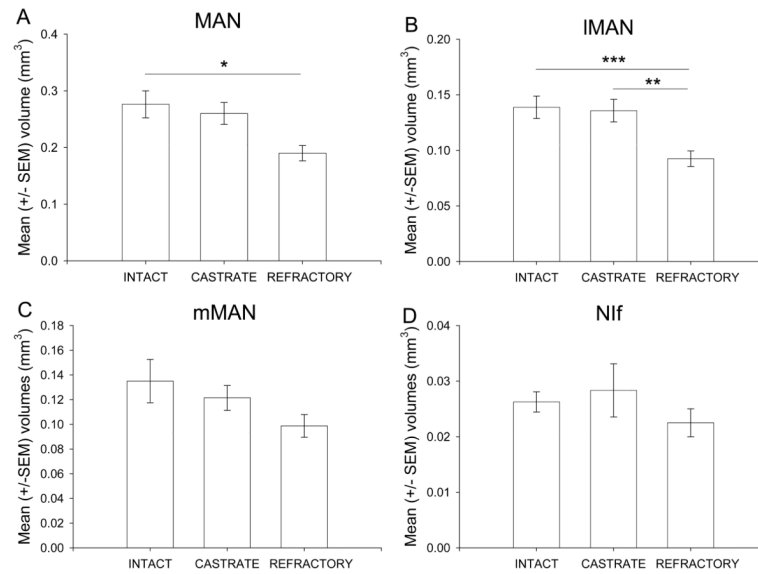


Figure 3. Enkephalin immunoreactivity defined variation in song nuclei IMAN and auditory nuclei Nif volumes. A) Mean (+/- SEM) total MAN volumes; B) Mean (+/- SEM) IMAN volume; C) Mean (+/- SEM) mMAN volumes; D) Mean (+/- SEM) Nif volume. Significance was determined at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$ and asterisks indicate significant differences.

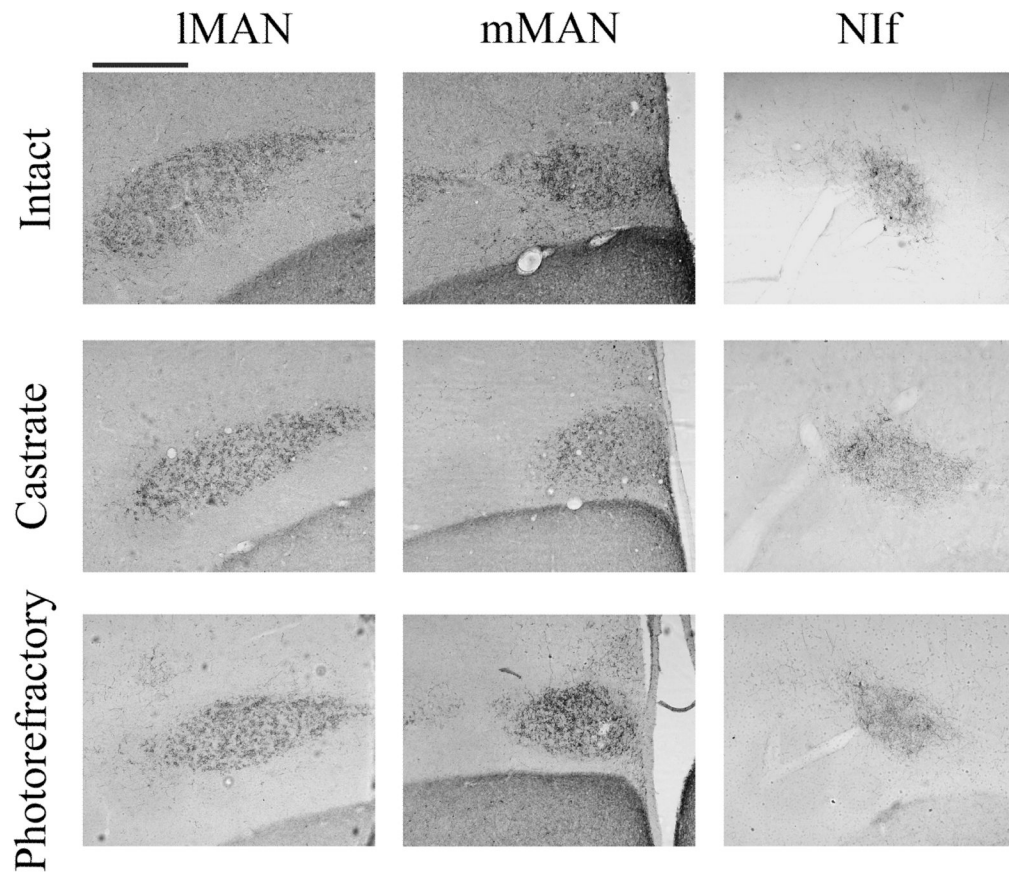


Figure 4. Photomicrographs of representative sections for IMAN, mMAN and NIf across treatment conditions. The columns represent the song nuclei IMAN, mMAN and NIf. The rows represent the treatment groups: photostimulated intact, photostimulated castrate and photorefractory male European starlings. Scale bar represents 130 μ m.

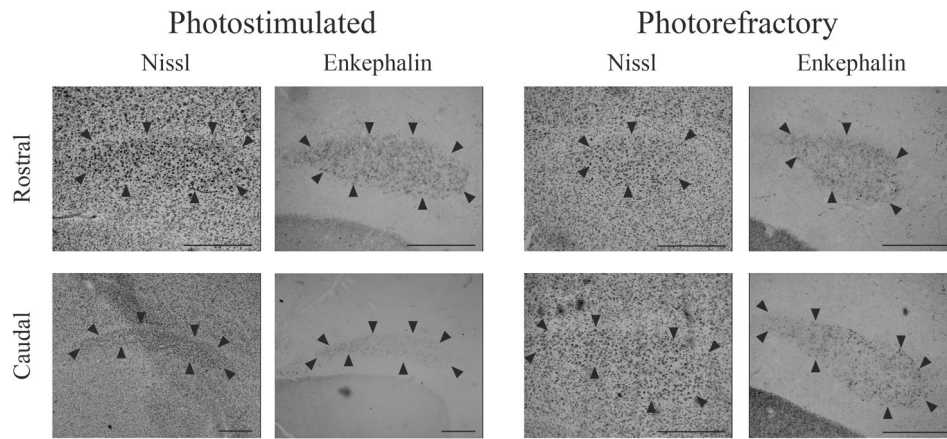


Figure 5. Photomicrographs of $IMAN_{core}$ from intact photostimulated and photorefractory starlings defined by Nissl stain and enkephalin immunoreactivity. The columns are sequential sections that compare enkephalin immunoreactivity and Nissl stain for the two treatment groups. The rows indicate sections taken from the rostral and caudal regions of $IMAN_{core}$. Arrows indicate boundaries used to delineate the volume using enkephalin immunoreactivity and approximated boundaries in Nissl stained sections. Scale bar represents 130 μm .