

## NEUROSYSTEMS

# Estradiol-dependent catecholaminergic innervation of auditory areas in a seasonally breeding songbird

Lisa L. Matragrano,<sup>1</sup> Sara E. Sanford,<sup>1,\*</sup> Katrina G. Salvante,<sup>2,†</sup> Keith W. Sockman<sup>2,3</sup> and Donna L. Maney<sup>1</sup><sup>1</sup>Department of Psychology, Emory University, 26 Eagle Row, Atlanta, GA, USA<sup>2</sup>Department of Biology, University of North Carolina, Chapel Hill, NC, USA<sup>3</sup>Curriculum in Neurobiology, University of North Carolina, Chapel Hill, NC, USA**Keywords:** dopamine, dopamine beta-hydroxylase, norepinephrine, tyrosine hydroxylase, white-throated sparrow

## Abstract

A growing body of evidence suggests that gonadal steroids such as estradiol (E2) alter neural responses not only in brain regions associated with reproductive behavior but also in sensory areas. Because catecholamine systems are involved in sensory processing and selective attention, and because they are sensitive to E2 in many species, they may mediate the neural effects of E2 in sensory areas. Here, we tested the effects of E2 on catecholaminergic innervation, synthesis and activity in the auditory system of white-throated sparrows, a seasonally breeding songbird in which E2 promotes selective auditory responses to song. Non-breeding females with regressed ovaries were held on a winter-like photoperiod and implanted with silastic capsules containing either no hormone or E2. In one hemisphere of the brain, we used immunohistochemistry to quantify fibers immunoreactive for tyrosine hydroxylase or dopamine beta-hydroxylase in the auditory forebrain, thalamus and midbrain. E2 treatment increased catecholaminergic innervation in the same areas of the auditory system in which E2 promotes selectivity for song. In the contralateral hemisphere we quantified dopamine, norepinephrine and their metabolites in tissue punches using HPLC. Norepinephrine increased in the auditory forebrain, but not the midbrain, after E2 treatment. We found that evidence of interhemispheric differences, both in immunoreactivity and catecholamine content that did not depend on E2 treatment. Overall, our results show that increases in plasma E2 typical of the breeding season enhanced catecholaminergic innervation and synthesis in some parts of the auditory system, raising the possibility that catecholamines play a role in E2-dependent auditory plasticity in songbirds.

## Introduction

In seasonally breeding vertebrates, the timing of gonadal steroid release ensures that steroid-dependent reproductive behaviors occur at the appropriate time of year. Behavioral responses to sociosexual signals change dramatically in many species of fish, frogs, birds and rodents when plasma estradiol (E2) rises to breeding levels (reviewed by Maney, 2010; Maney & Pinaud, 2011). To understand how E2 alters behavioral responses to sensory signals, we have studied areas of the auditory forebrain thought to participate in the encoding of behavioral relevance in songbirds. The caudomedial nidopallium (NCM) receives input from the thalamorecipient Field L and is heavily interconnected with the caudomedial mesopallium (CMM). NCM and CMM are thought to be analogous to the supragranular layers of mammalian auditory cortex (Vates *et al.*, 1996) or to

mammalian auditory association cortex (Pinaud & Terleph, 2008; Tremere *et al.*, 2009). These areas have received a great deal of attention, largely because they show selective responses to stimuli that are particularly salient to the listener (Mello & Clayton, 1994; Jarvis *et al.*, 1995; Gentner *et al.*, 2001; Maney *et al.*, 2003; Leitner *et al.*, 2005). We previously reported that in CMM, NCM and the dorsal lateral nucleus of the mesencephalon (MLd, or auditory midbrain) of female white-throated sparrows, expression of the immediate early gene product ZENK (Egr-1) was selective for conspecific song only when plasma E2 was at breeding levels (Maney *et al.*, 2006). In non-breeding females with low plasma E2, the ZENK response to song was indistinguishable from that to frequency-matched tones. E2-dependent changes in the responsiveness of auditory processing centers may therefore promote recognition of and attention to conspecific male song during the breeding season when it is particularly relevant to the female.

Estradiol-induced changes in auditory function, particularly changes involving stimulus salience, could be related to one or more cognitive processes that depend on catecholamines. These compounds are widely understood to shape the response properties of sensory networks involved in selective attention in mammals (see Hurley *et al.*, 2004 and Aston-Jones & Cohen, 2005 for review) and context-dependent

*Correspondence:* Lisa L. Matragrano, as above.  
E-mail: lmatrag@emory.edu

\**Present address:* Division of Infectious Diseases, Emory University School of Medicine, Atlanta, GA, USA.

†*Present address:* Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada.

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modulation of forebrain plasticity (see Castelino & Ball, 2005). In songbirds, it has been hypothesized that catecholaminergic (CA) projections to the forebrain affect the auditory processing of behaviorally relevant social signals such as song (e.g. Appeltants *et al.*, 2002a,b; Cardin & Schmidt, 2004; LeBlanc *et al.*, 2007; Riters & Pawlisch, 2007). Noradrenergic denervation of the forebrain reduces behavioral and neural responses to song as well as behavioral and neural selectivity for sexually stimulating song (Appeltants *et al.*, 2002b; Lynch & Ball, 2008; Vyas *et al.*, 2008). Any modulation of CA systems is therefore likely to affect the auditory processing of conspecific song as well as the resulting behavioral responses. In male zebra finches, catecholamine levels and turnover in the auditory forebrain are regulated by gonadal steroids (Barclay & Harding, 1990). In this study, we investigated the effects of systemic E2 treatment on central CA activity in females of a seasonally breeding species, the white-throated sparrow. We quantified catecholamines and their metabolites using high-pressure liquid chromatography (HPLC) and the density of fibers immunoreactive for the catecholamine synthetic enzymes tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) using immunohistochemistry (IHC).

## Materials and methods

### Animals

All procedures in this study were approved by the Emory University Institute for Animal Care and Use Committee and adhered to NIH standards. We collected sixteen female white-throated sparrows (*Zonotrichia albicollis*) in mist nets in Atlanta, Georgia, USA during the fall of 2007. We determined their sex by polymerase chain reaction analysis of a blood sample (Griffiths *et al.*, 1998) and confirmed sex by necropsy at the end of the study. The birds were housed at the Emory University animal care facility in indoor walk-in flight cages and supplied with food and water *ad libitum*. We held day length constant at 10 : 14 h light : dark, which corresponds to the shortest day the birds would experience during the winter at the capture site. We kept the birds under these conditions for 2 months to ensure that photorefractoriness was broken before the start of the study (Wolfson, 1958; Shank, 1959).

### Hormonal manipulation

Because our goal was to compare the anatomical distribution of the effects of E2 on CA innervation with the previously described effects on the ZENK response (Maney *et al.*, 2006; Sanford *et al.*, 2010), we followed our standard procedures for housing and hormonal manipulation. We transferred the birds in pairs to small rooms where they were housed individually in two adjacent cages (38 × 38 × 42 cm) per room. We held the day length at 10 : 14 h light : dark throughout the experiment to prevent elevation of endogenous plasma E2 (Wolfson, 1958; Shank, 1959). On the day the birds were transferred to individual cages, we implanted each one with a subcutaneous silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96 mm; Dow Corning, Midland, MI, USA) sealed at both ends with A-100S Type A medical adhesive (Factor 2, Lakeside, AZ, USA). In order to balance hormone treatment within each pair, one bird in each pair ( $n = 8$ ) received an empty implant and the other ( $n = 8$ ) received an implant containing 17beta-estradiol (Steraloids, Newport, RI, USA). Capsules were implanted using topical anesthetic (lidocaine; 1%). This dose of E2 increases plasma levels to those typical of the breeding season within 7 days in this species (Maney *et al.*, 2006, 2008) and probably does so within 2 days (Moore, 1983). We have established that 7 days

of hormone treatment results in a dramatic rise in ZENK expression and selectivity in auditory areas (Sanford *et al.*, 2010) and, as our present goal was to reproduce those conditions, we treated for the same amount of time. Seven days after implantation, we decapitated the birds and quickly harvested the brains. After removing the cerebellum in order to clearly visualize the midline, we used a clean razor blade to bisect each brain into hemispheres. We fixed one hemisphere in 5% acrolein as previously described (Maney *et al.*, 2003, 2005) and flash-froze the other in powdered dry ice for HPLC analysis at the University of North Carolina. The hemisphere that was fixed (right or left), the room (one of six identical rooms) and the position of each bird inside the room (to the right or the left of the other bird in that room) were balanced across treatments.

### Immunohistochemistry

We cut the fixed hemispheres into three series of 50- $\mu$ m parasagittal sections using a freezing sliding microtome. We immunolabeled one series for TH and another for DBH using standard IHC protocols (Maney *et al.*, 2001, 2003, 2005; LeBlanc *et al.*, 2007). Briefly, we incubated the first series of sections with an anti-TH antibody (ImmunoStar; Hudson, WI, USA; see section on antisera below) diluted 1 : 2000 (LeBlanc *et al.*, 2007) then labeled the TH with a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA, USA). We visualized the immunolabeling with diaminobenzidine (LeBlanc *et al.*, 2007). For the second series of sections, we pretreated with avidin and biotin, incubated with anti-DBH antibody (ImmunoStar; see section on antisera below) diluted 1 : 16,000, and subsequently labeled the DBH using a biotinylated secondary antibody and the ABC method. We visualized DBH immunolabeling with nickel-enhanced diaminobenzidine (Shu *et al.*, 1988). We processed each series of brain sections in two separate runs of IHC in which the experimental (E2-treated) and blank (empty implants) conditions were balanced across runs. Following IHC, we mounted all of the sections on microscope slides, dehydrated them and coverslipped them in DPX (Sigma, St Louis, MO, USA).

### Antisera

We used a mouse monoclonal antibody generated against denatured TH and purified from rat PC12 cells (ImmunoStar Cat. no. 22941). According to the manufacturer, it recognizes a 62-kDa band corresponding to TH in rat and does not cross-react with dihydropteridine reductase, DBH, phenylethanolamine-*N*-methyltransferase, phenylalanine hydroxylase or tryptophan hydroxylase using Western blot methods. It has a wide species cross-reactivity and has been validated by preadsorption studies in a range of vertebrates (Olsson *et al.*, 2008). This antibody labels a catecholamine-typical pattern of neurons and fibers in a wide variety of birds (e.g., Bailhache & Balthazart, 1993; Moons *et al.*, 1994; Reiner *et al.*, 1994; Soha *et al.*, 1996; Appeltants *et al.*, 2001; Roberts *et al.*, 2001) including white-throated sparrows (Balthazart & Ball, 1996; LeBlanc *et al.*, 2007), and was used by Reiner *et al.* (1994) to perform an exhaustive characterization of the distribution of TH immunoreactivity in birds. Anti-TH antibodies from other sources and anti-dopamine (DA) antibodies produce the same neural distribution in birds (e.g., Bottjer, 1993; Metzger *et al.*, 1996). In our material, the antibody labels all major TH cell groups A1–A15 and fibers in a distribution typical of TH. An antibody against the phosphorylated form of TH (Genetex, Irvine, CA, USA; Cat. no. 16557), which we have validated in preadsorption studies in white-throated sparrows, labels an identical distribution of cells in this species.

To label DBH we used a polyclonal antibody generated in rabbit against DBH purified from bovine adrenal medulla (ImmunoStar Cat. no. 22806). The manufacturer notes that on Western blot the antibody detects a triplet at ~72–74kDa. It labels a norepinephrine (NE)-like distribution of cells and fibers in a variety of birds (e.g., Bailhache & Balthazart, 1993; Karle *et al.*, 1996; Castelino & Ball, 2005; Sockman & Salvante, 2008), including white-throated sparrows (LeBlanc *et al.*, 2007). Sockman & Salvante (2008) reported that preadsorption with antigen supplied by the manufacturer of the antibody eliminates all labeling in brain sections from European starling (*Sturnus vulgaris*).

For each IHC described here, omission of the primary or the secondary antibodies resulted in a complete loss of specific staining in all cases.

### Regions of interest and data collection

Our primary goal in this study was to map the effects of E2 on CA innervation and to determine the extent to which those effects overlap, anatomically, with the effects on the ZENK response we previously described (Sanford *et al.*, 2010). In order to compare the anatomical distributions of the effects of E2 on CA innervation and the effects on the ZENK response, we needed to partition NCM into the same domains as used in our previous study. Sanford *et al.* (2010) divided the region both rostrocaudally and dorsoventrally into four primary domains – rostradorsal (rdNCM), rostroventral (rvNCM), caudodorsal (cdNCM) and caudoventral (cvNCM). Because the dorsal and ventral domains are hodologically and neurochemically similar within both rostral NCM and caudal NCM, and because the effects of E2 on ZENK expression are similar in the dorsal and ventral portions of each domain (Sanford *et al.*, 2010), in the current study we collapsed our regions of interest into rostral and caudal domains (rNCM and cNCM) without the dorsal and ventral divisions (Fig. 1). Like Sanford *et al.* (2010), we also sampled from an apical domain (aNCM) located dorsal to Field L2 (Fig. 1). In the published literature, this region is usually considered part of NCM but may overlap the dorsal portion of Field L (Fortune & Margoliash, 1992).

Using material from Sanford *et al.* (2010), we recently found that the ZENK response in the nucleus ovoidalis (Ov; auditory thalamus), which is homologous to the mammalian medial geniculate (Karten, 1967), is also sensitive to E2 treatment (T. Tran, unpublished observations; see also Maney & Pinaud, 2011). Maney *et al.* (2006) showed that E2 treatment increases the selectivity of the ZENK response in the auditory midbrain (MLd), which is homologous to the mammalian inferior colliculus (Karten, 1967). In order to test whether E2 treatment alters CA innervation and function in these areas, we estimated the density of immunoreactive fibers in Ov and MLd in addition to the regions of the auditory forebrain described above.

### Image acquisition and regions of interest

We conducted all image analyses while blind to treatment group. We used the 10 × objective on a Zeiss Axioskop microscope attached to a Leica DFC480 camera and Macintosh G5 computer. For all regions we captured rectangular images corresponding to the field of view of the camera (870 × 690 μm). We held the light level constant for all photos, which were ~46 MB in size. We defined the regions of interest within CMM and NCM using the criteria of Sanford *et al.* (2010). We acquired images of CMM and NCM in four consecutive sections between ~350 and ~800 μm from the midline in all birds. The first section chosen was the medial-most in which we could discern CMM and Field L (Sanford *et al.*, 2010). Field L was easily

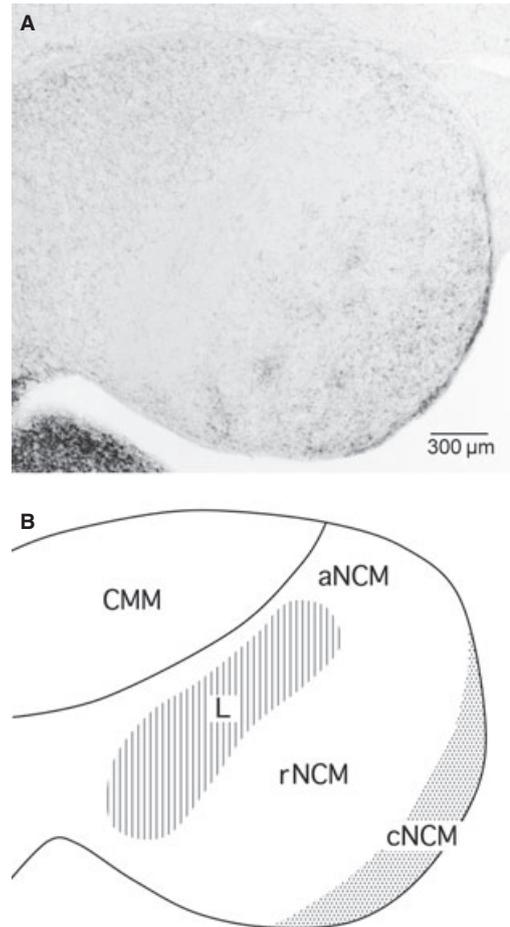


FIG. 1. (A) Parasagittal view of TH immunoreactivity in the auditory forebrain of the white-throated sparrow. (B) We quantified catecholamine innervation, content and metabolites in the following regions of the auditory forebrain (Sanford *et al.*, 2010) – the caudomedial mesopallium (CMM) and three domains of the caudomedial nidopallium (NCM) – rostral NCM (rNCM), apical NCM (aNCM) and caudal NCM (cNCM). Rostral is to the left. Scale bar, 300 μm.

visible in the TH-immunostained series as a region relatively poor in TH immunoreactivity (Reiner *et al.*, 1994); we aligned the DBH immunostained sections with the alternate TH-immunostained series to confirm the location of Field L.

For each series in each bird, five separate images were acquired from the same four sections. From those photos we quantified fiber density in CMM, aNCM, cNCM and two domains of rNCM (dorsal and ventral, which were later combined), as defined by Sanford *et al.* (2010), as follows. To acquire images of CMM, the two upper corners of each photograph were positioned along the dorsal boundary of CMM and one of the lower corners was positioned adjacent to the lamina mesopallium. The remaining four photos, of NCM, were positioned so that they would encompass the domains defined by Sanford *et al.* (2010; see above). When defining our regions of interest for sampling, we opened all four photos at the same time in order to avoid overlap. We first defined cNCM as a strip of tissue ~275 μm from the caudal boundary of NCM. We selected it by tracing the caudal boundary with the IMAGEJ freehand tool and using the straight line tool to define its rostral boundary. The images of cNCM captured the majority of that domain, spanning 870 μm from dorsal to ventral, but did not include the dorsal and ventral tips. The rostral domains

were sampled using the IMAGEJ circle tool. For rNCM we sampled from two areas, one ventral and one more dorsal (Sanford *et al.*, 2010). We sampled the ventral region of interest using a circle  $\sim 550 \mu\text{m}$  in diameter, placed so that its ventral boundary was adjacent to the ventral edge of the telencephalon and its caudal boundary did not overlap with what we defined as cNCM. We defined the dorsal region of interest using a circle  $\sim 550 \mu\text{m}$  in diameter, placed immediately dorsal to the ventral one and not overlapping cNCM. For aNCM we placed a circle with a diameter of  $\sim 350 \mu\text{m}$  dorsal to Field L so that its dorsal boundary lay adjacent to the ventricle. In no case did these circles overlap the region of relatively poor TH immunoreactivity that Reiner *et al.* (1994) identified as Field L. Because we did not conduct tract tracing or any other method that would enable us to discern absolutely the boundary between rNCM and the adjacent subregions of Field L (Vates *et al.*, 1996), it is possible that our samples of aNCM and rNCM may have captured bits of L1 and L3, respectively. We are confident in any case that the rostral regions we sampled correspond exactly to those exhibiting E2-dependent selectivity as described by Sanford *et al.* (2010).

Ovoidalis was visible in two or three sections in each bird. For each series we chose the two sections in which it was the largest, spanning  $150 \mu\text{m}$  from medial to lateral, and traced the outline of the region including its shell and core (Vates *et al.*, 1996; Figs 2A and D). To acquire images of MLd we chose the section in which it was the largest (Maney *et al.*, 2006) and the two immediately medial and lateral to it, so that we sampled from five sections spanning  $600 \mu\text{m}$ . We traced the outline as depicted in Fig. 2B. We also quantified fiber density in a non-auditory region, the apical part of the hyperpallium (HA), to assess the specificity of the effects of E2 on auditory regions. Typical images of HA, which were acquired from the same sections used for Ov, are shown in Figs 2C and F.

#### Estimation of TH- and DBH-immunoreactive fiber density

Our hypothesis was that E2 treatment would increase the density of the CA innervation, in other words the total volume of CA fibers innervating our regions of interest (Kritzer & Kohama, 1998; LeBlanc *et al.*, 2007). We therefore estimated immunoreactive fiber density from our acquired images in units of area (e.g., Kritzer & Kohama,

1998; Gerhardt *et al.*, 2002; Poeggel *et al.*, 2003; Maney *et al.*, 2005; LeBlanc *et al.*, 2007; de Vries *et al.*, 2008). Using IMAGEJ (version 1.41o; National Institutes of Health, Bethesda, MD, USA), we converted each image to 8-bit scale and selected TH- or DBH-immunoreactive fibers using the thresholding feature (Maney *et al.*, 2005). The level of background staining varied somewhat from section to section so, in order to best distinguish labeled fibers from the background, we set the threshold manually for each image. In each case, we adjusted the threshold such that the pixels selected by IMAGEJ agreed with what the observer considered, by eye, to be labeled fibers. The same observer (L.L.M.) set the threshold for all images. We performed this procedure in two to five images per region (see above). We then calculated the area covered by the fibers, in square microns per square mm, for each region by summing the total area covered by fibers and dividing by the total area measured. Because Sanford *et al.* (2010) showed that the ZENK response is modulated similarly in the dorsal and ventral areas of rNCM, we combined our dorsal and ventral rNCM samples.

#### Test of inter-rater reliability

Like most published methods for estimating fiber density, the manual adjustment of threshold is based on the observer's subjective opinion of which parts of the image represent immunoreactive fibers. We therefore performed a test of inter-rater reliability. For this analysis we used 60 images of DBH staining in CMM, which contained the lightest and least obvious fibers of all the labeling in our study (see Fig. 3B). Fifteen different animals were represented in this sample. Two different observers chose thresholds independently for these 60 images. Both observers used the same computer monitor located in the same room, and the lighting in the room was held constant.

#### Quantification of catecholamines and metabolites

The methods for quantification of catecholamines and metabolites are published elsewhere (Sockman & Salvante, 2008), and we reiterate the relevant portions here. We determined the concentration of catecholamines and metabolites by reversed-phase HPLC with

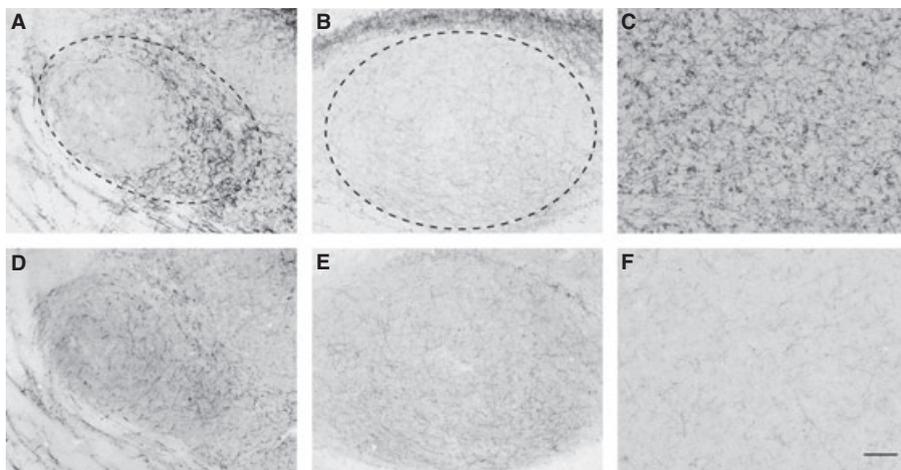


FIG. 2. Fibers immunoreactive for (A–C) TH or (D–F) DBH in the auditory thalamus (Ov; A and D), the auditory midbrain (MLd; B and E) and the apical part of the hyperpallium (HA; C and F), a visual area, in an estradiol-treated female white-throated sparrow. The regions of interest in which we quantified fiber density are delineated by dashed lines in A and B. The apparent cell bodies in HA are actually immunoreactive fibers forming basket-like structures (Metzger *et al.*, 1996). Rostral is to the left. Scale bar,  $100 \mu\text{m}$ .

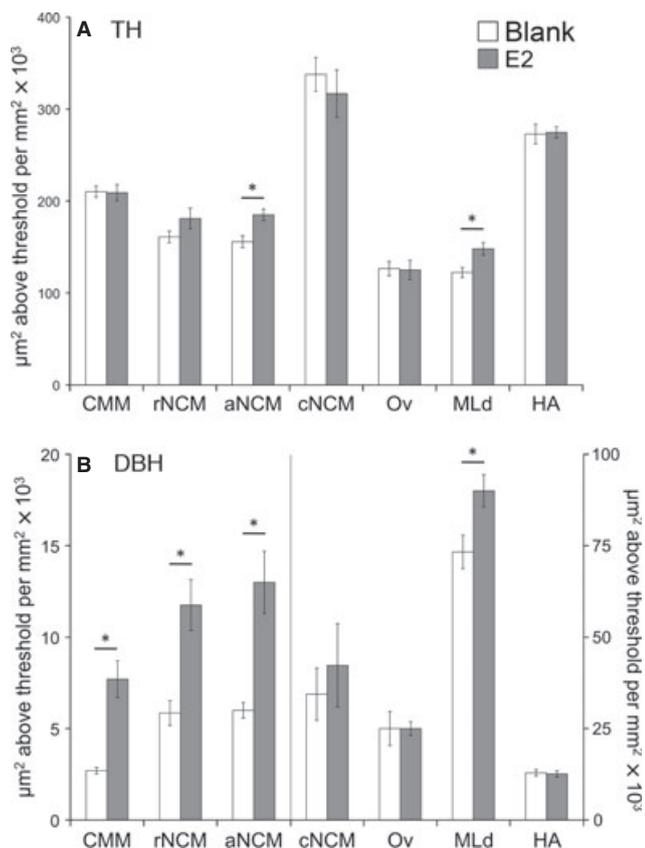


FIG. 3. Estradiol (E2) treatment increased the estimated density of fibers immunopositive for (A) TH in aNCM and MLd; and (B) DBH in CMM, rNCM, aNCM and MLd. MLd, auditory midbrain; HA, apical part of the hyperpallium; Ov, n. Ovoidalis. Other abbreviations, see Fig. 1. \* $P < 0.02$  for E2 vs. condition. See text for actual  $P$ -values.

electrochemical detection (Kilts *et al.*, 1981). Because the number of brain regions we could sample via HPLC was limited (see below), we decided to sample regions in which we had previously shown E2-dependent selectivity of the ZENK response – NCM, CMM and MLd (Maney *et al.*, 2006). The chromatographic system consisted of an SM-909 isocratic HPLC pump (ANSPEC, Ann Arbor, MI, USA), Basic+ Marathon type 816 Autosampler (Spark Holland, Netherlands), Model 400 potentiostat (EG&G Princeton Applied Research, Oak Ridge, TN, USA) and TURBOCHROM software Version 4.1 (Perkin Elmer, Waltham, MA, USA) running on a PC. We separated the compounds using a Monochrom C18 3- $\mu$ m column (100  $\times$  4.6 mm; MetaChem, Torrance, CA, USA) with a mobile phase consisting of sodium phosphate (7.1 g), citric acid (5.76 g), disodium EDTA (50 mg), sodium octyl sulfonate (350 mg) and methanol (130 mL) topped-up to one liter total volume with double-distilled deionized water, with pH lowered to 3.9 with hydrochloric acid. We filtered the mobile phase through a 20- $\mu$ m filter (Kontes Scientific Glassware and Instruments, Vineland, NJ, USA) before use, and flow rate was 0.8 mL/min. We maintained the electrode potential at 650 mV with respect to an Ag/AgCl reference electrode. We prepared standard solutions containing a fixed amount (30 ng) of the internal standard (isoproterenol; Sigma) and variable amounts of each of the four external standards (Sigma) – DA, NE, dihydroxyphenylacetic acid (DOPAC; the principal metabolite of dopamine) and 3-methoxy-4-hydroxyphenylglycol (MHPG; the principal metabolite of norepinephrine). We included a five-point standard curve in each assay

(5  $\times$  50  $\mu$ L injections), and used linear regression to fit a line through the standard curve points ( $r^2 > 0.99$  for all four components in each assay).

We sectioned the frozen, non-fixed hemispheres at 10  $^{\circ}$ C in the sagittal plane at 300  $\mu$ m on a cryostat, thaw-mounted the sections onto glass slides, and rapidly re-froze them on pulverized dry ice. Using a chilled custom-made, thin-walled stainless steel spring-loaded punch tool, we micropunched a region of CMM (1 mm i.d.) and of NCM (1.5 mm i.d.), for each of which the anatomical boundaries have been described (Sockman *et al.*, 2002). Because the diameter of the punch tool was greater than the diameters of any of the individual domains within NCM, we did not further divide NCM into domains for the HPLC analysis. We took a micropunch (1 mm i.d.) from a region of MLd from two consecutive sections. We expelled the tissue punches into 1.9-mL polypropylene microcentrifuge tubes (one for each brain region), froze them on dry ice and stored them at  $-80$   $^{\circ}$ C until assay. Immediately before assay, we added mobile phase (225  $\mu$ L) containing 30 ng isoproterenol to each tube. We sonicated the samples and then centrifuged them at 16 000 g for 15 min at 4  $^{\circ}$ C. We aspirated the supernatant and injected 50  $\mu$ L from each sample into the HPLC system. We calculated catecholamine and metabolite concentrations (pg) by first correcting the peak heights for percentage recovery of the internal standard (i.e., the height of the isoproterenol peak) for each sample and then comparing the values to those obtained for the corresponding catecholamines or metabolites in the standard curve.

#### Statistical analysis

All of our dependent variables were measured in the same set of animals and are linked to catecholamine synthesis. We therefore could not assume that they were independent of each other. For this reason we began our analysis with a single repeated-measures MANOVA that included data acquired by IHC and HPLC from all birds. Because the HPLC data set included only three regions of interest (CMM, NCM as a single region, and MLd), we could not include all of the IHC data on enzymes, which were measured in seven separate regions, in this initial analysis. The IHC data were therefore analyzed separately, with all regions of interest included, in a second analysis (see below). For the initial omnibus analysis of CMM, NCM and MLd only, because CA innervation was measured in individual domains whereas our HPLC data were acquired from a single larger sample, we needed to collapse the NCM IHC data for individual domains into a single value for NCM for each bird. To do so, we calculated an average estimated percentage area covered by fibers for each enzyme by summing the total area covered by immunoreactivity in rNCM and cNCM and dividing by the total area measured for all regions summed. aNCM was not included in this calculation because it lies dorsal to Field L, outside the area of the micropunch. This calculation produced an estimate of the average area covered by fibers immunoreactive for TH or DBH throughout the part of NCM that was sampled for HPLC analysis. We then conducted a repeated-measures MANOVA with region of interest (CMM, NCM, or MLd) and catecholamine measure (TH, DBH, DA, NE, DOPAC or MHPG) as the within-subject variables, and hormone treatment (blank or E2) as the between-subjects variable. In this procedure, therefore, we tested for an effect of E2 treatment on most of the related variables together as a whole before proceeding to analyze the data for each measure or individual region separately.

In order to determine the effect of E2 treatment on estimated TH and DBH fiber innervation in our regions of interest, including Ov, and the domains of NCM, we performed a separate repeated-measures

MANOVA with enzyme (TH or DBH) and region of interest (CMM, rNCM, aNCM, cNCM, Ov and MLd) as the within-subject factors, and hormone treatment and hemisphere as the between-subjects factors, followed by multivariate *F*-tests for each region of interest. To analyze the HPLC data in the other hemisphere, we did a repeated-measures MANOVA with region of interest (CMM, NCM or MLd) and compound (DA, NE, DOPAC or MHPG) as the within-subject variables, and hormone treatment (blank or E2) and hemisphere as the between-subjects variables, followed by multivariate *F*-tests for each region of interest. Effect sizes were calculated using Cohen's *d*. Because we hypothesized that immunoreactivity for TH and DBH in HA would be unaffected by E2 treatment and thus wanted to minimize Type II rather than Type I error, we analyzed the data from that region in a separate multivariate *F*-test.

In order to better compare the effect of E2 treatment on the estimated density of CA fiber innervation among the regions, we normalized the values from the E2-treated birds to evaluate the percentage change relative to controls. We then performed a repeated-measures ANOVA with normalized estimated fiber density (TH or DBH) and region of interest as within-subject variables. We followed that analysis with *post hoc* pairwise Student's *t*-tests to compare the effects of E2 in each region to that in the other regions.

In order to determine whether TH- or DBH-immunoreactivity predicted the levels of catecholamines or metabolites in each region, we ran Pearson correlation tests relating the percentage area covered by TH immunoreactivity to DA and its metabolite DOPAC, and the estimated percentage area covered by DBH immunoreactivity to NE and its metabolite MHPG in each region of interest (total of 12 Pearson tests). For this analysis, NCM was treated as a single region and we used the averaged IHC data for individual domains (see above). We evaluated the correlations with the sequential Bonferroni correction for multiple comparisons (Rice, 1989).

## Results

When the data were analyzed together in a single repeated-measures MANOVA (see Materials and methods), we found no reliable effect of treatment ( $F_{1,14} = 0.758$ ;  $P = 0.399$ ). There was, however, a significant interaction between hormone treatment and region (Wilks' lambda,  $F_{2,13} = 7.508$ ;  $P = 0.007$ ), meaning that there was a significant effect of E2 treatment that depended on the region of interest. There was also a significant interaction between hormone treatment and compound measured (Wilks' lambda,  $F_{5,10} = 3.713$ ;  $P = 0.037$ ), which indicated that E2 treatment may have affected some of our measures but not others. We then proceeded to the analyses of data collected by each method (IHC or HPLC).

### *Effects of E2 treatment on catecholaminergic innervation of auditory areas*

The effects of E2 treatment on estimated TH and DBH fiber density are plotted in Fig. 3. A repeated-measures MANOVA that considered all regions of interest, including the domains of NCM, revealed a highly significant effect of E2 treatment on immunoreactivity for CA enzymes ( $F_{1,12} = 15.541$ ;  $P = 0.002$ ). *Post hoc* pairwise comparisons showed that E2 significantly increased the estimated density of TH innervation in aNCM ( $F_{1,15} = 9.615$ ,  $P = 0.009$ ,  $d = 1.614$ ) and MLd ( $F_{1,15} = 7.515$ ,  $P = 0.018$ ,  $d = 1.455$ ). Estimated DBH fiber density was increased in CMM ( $F_{1,15} = 27.809$ ,  $P < 0.001$ ,  $d = 2.427$ ), rNCM ( $F_{1,15} = 15.372$ ,  $P = 0.002$ ,  $d = 1.899$ ), aNCM ( $F_{1,15} = 15.133$ ,  $P = 0.002$ ,  $d = 2.006$ ) and MLd ( $F_{1,15} = 8.485$ ,  $P = 0.013$ ,

$d = 1.327$ ). We did not detect an effect of E2 on the estimated density of TH or DBH innervation of cNCM (TH –  $F_{1,15} = 0.447$ ,  $P = 0.516$ ,  $d = 0.329$ ; DBH –  $F_{1,15} = 0.288$ ,  $P = 0.601$ ,  $d = 0.293$ ).

### *Relative effects of E2 treatment on CA fiber density in regions of interest*

A repeated-measures ANOVA on normalized values from the E2-treated birds showed significant effects of enzyme ( $F_{1,7} = 32.76$ ;  $P = 0.001$ ) and region ( $F_{6,42} = 10.05$ ;  $P = 0.001$ ) and a significant interaction between the two ( $F_{6,42} = 8.39$ ;  $P < 0.001$ ). *Post hoc* pairwise comparisons between the areas revealed that, in general, the rostral areas of NCM responded more to E2 treatment than did the caudal area.

### *Effects of E2 treatment on levels of catecholamines and their metabolites*

A repeated-measures MANOVA with compound and region as within-subjects measures showed no main effect of hormone treatment ( $F_{1,12} = 1.472$ ;  $P = 0.248$ ); however, there was an interaction between treatment and region ( $F_{2,11} = 8.005$ ;  $P = 0.007$ ). *Post hoc* pairwise comparisons revealed that the effects of E2 treatment were limited to CMM, in which NE increased ( $F_{3,15} = 6.876$ ,  $P = 0.022$ ,  $d = 1.386$ ; Fig. 4). We detected no other effects of hormone treatment on the concentrations of catecholamines or their metabolites in CMM, NCM or MLd (Fig. 4).

### *Interhemispheric differences*

The repeated-measures MANOVAs conducted on the IHC data showed an overall effect of hemisphere ( $F_{1,12} = 5.059$ ;  $P = 0.044$ ), and the parallel analysis of the HPLC data showed a significant compound  $\times$  hemisphere interaction ( $F_{3,10} = 4.564$ ;  $P = 0.029$ ). In neither analysis was there an interaction between hemisphere and treatment or between hemisphere and other variables that included treatment. Therefore, E2 treatment affected our variables of interest equally in both hemispheres in all cases. The two interhemispheric differences we found for enzyme immunoreactivity were in the same direction; our estimation of TH immunoreactivity was higher in left than right rNCM ( $F_{1,15} = 4.850$ ,  $P = 0.048$ ,  $d = 0.95$ ), and the estimated DBH immunoreactivity was higher in the left than the right cNCM ( $F_{1,15} = 4.977$ ,  $P = 0.046$ ,  $d = 1.031$ ). NE and its metabolite MHPG were both higher in the right MLd than the left (NE –  $F_{1,15} = 5.481$ ,  $P = 0.037$ ,  $d = 1.725$ ; MHPG –  $F_{1,15} = 13.298$ ,  $P = 0.003$ ,  $d = 1.089$ ).

### *Correlations between enzyme immunoreactivity and catecholamine levels*

In order to test whether enzyme immunoreactivity predicted the levels of catecholamines and their metabolites, we looked for correlations between estimated TH fiber density and DA or DOPAC levels and between estimated DBH fiber density and NE or MHPG levels in CMM, MLd and NCM (rNCM and cNCM combined; see Materials and Methods). Pearson correlation tests revealed that estimated TH fiber density did not predict levels of DA or DOPAC as measured by HPLC. We detected no significant correlations between the percentage area stained and DA or DOPAC levels in any of the regions of interest. Similarly, in most regions estimated DBH fiber density did not predict

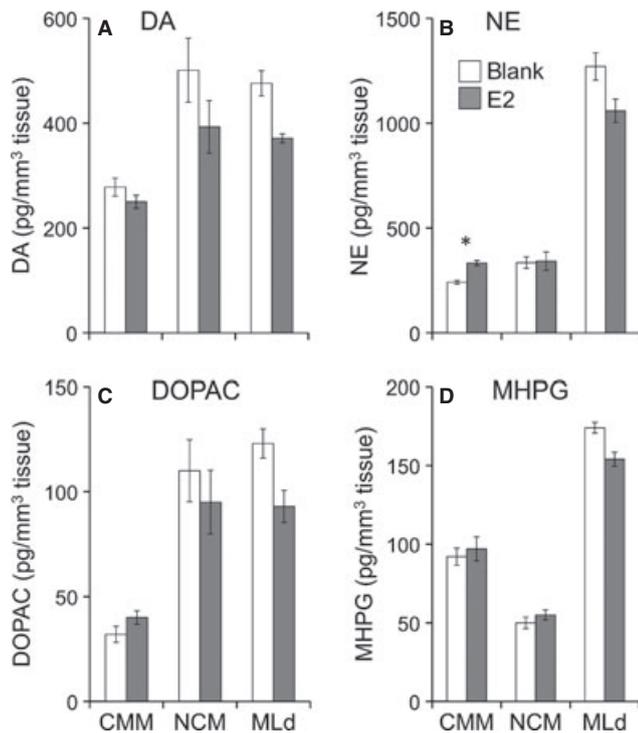


FIG. 4. The effects of estradiol (E2) treatment on (A) DA and (B) NE and their metabolites (C) DOPAC and MHPG in the caudomedial mesopallium (CMM), the caudomedial nidopallium (NCM) and the auditory midbrain (MLd) in female white-throated sparrows. Average protein content was  $0.1 \text{ mg/mm}^3$ . \* $P = 0.022$ .

levels of NE or MHPG as measured by HPLC. The only notable correlation, between DBH immunoreactivity and the concentration of NE in CMM ( $R^2 = 0.334$ ,  $P = 0.019$ ) was clearly driven by the significant effect of E2 on both measures (Fig. 5). We also tried correlating the estimated fiber density of each enzyme with the summed concentrations of all of its downstream products, in other words DBH immunoreactivity with NE and MHPG levels summed, and TH immunoreactivity with all four products summed, but detected no significant correlations.

#### Reliability of manual thresholding

Our analysis showed manual thresholding to be highly reliable. The mean thresholds and SDs for observers 1 and 2 were  $208.36 \pm 21.11$  and  $208.34 \pm 21.31$ , respectively, and the correlation between them was  $R = 0.98$ . There was no difference between the thresholds set by observers 1 and 2 when compared using a paired  $t$ -test ( $P = 0.976$ ) and the effect size was very low ( $d = 0.0009$ ). Thresholds set by observers 1 and 2 were, on average, 1.71% different (SD = 1.28%) and never differed by  $> 5\%$ . They were within one unit of each other in 30% of cases. When the direction of the difference was taken into account, the thresholds selected by the two observers were, on average, 0.01% different.

#### Discussion

In this study, we provide evidence that the CA innervation of the auditory system in songbirds is sensitive to E2. We found that in the auditory forebrain (CMM and NCM) and midbrain (MLd), a rise in plasma E2 from non-breeding to breeding levels causes significant

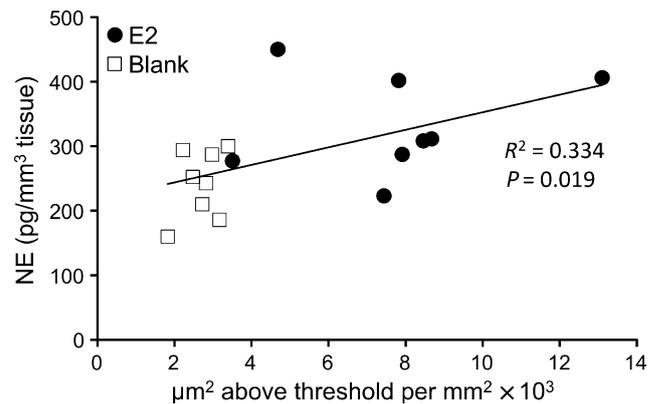


FIG. 5. Correlation between the density of DBH-immunoreactive fibers and the level of NE in the caudomedial mesopallium (CMM). The relationship between DBH immunoreactivity and NE was probably driven by the significant effect of estradiol (E2) on both variables (see Figs 3B and 4B).

increases in the estimated density of the innervating CA fibers. This finding is consistent with our previous report that E2 treatment increases the number of TH-immunoreactive cells in the ventral tegmental area, a major source of DA in the forebrain, and the locus coeruleus, the primary source of NE (LeBlanc *et al.*, 2007). Both of these brainstem CA cell groups, which probably project to the auditory forebrain (Mello *et al.*, 1998; Ribeiro & Mello, 2000; Appeltants *et al.*, 2001, 2004), contain estrogen receptors (Maney *et al.*, 2001) and therefore could be directly affected by E2 treatment.

As we have reported previously for the ZENK response, CA innervation in some areas of the auditory forebrain was not sensitive to the effects of E2. Unlike the rostral areas of NCM, the caudomedial edge (cNCM) does not respond to E2 treatment with increased ZENK expression, nor does it respond with increased selectivity for song over tones (Sanford *et al.*, 2010). Here, we showed that the CA innervation of this region is similarly insensitive to E2 treatment; whereas estimated CA fiber density increased in the rostral areas of NCM after E2 treatment we detected no such change in cNCM (Fig. 3). The excellent anatomical match between E2-induced ZENK expression and effects on CA fiber density suggest strongly that the two may be related. This hypothesis is further supported by our observation of CA basket-like structures surrounding ZENK-immunoreactive cells in NCM (L.L. Matragrano, unpublished observations). Our results add to existing evidence on connectivity, electrophysiological responses and neurochemical markers (Pinaud *et al.*, 2006) that the rostral and caudal domains of NCM are perhaps functionally distinct. Future work should focus on the distinction between the rostral and caudal domains and the delineation of the rostral domains from regions of Field L proper.

How might CA input affect auditory selectivity? If the CA cells that project to auditory areas themselves fire in response to song, the ensuing catecholamine release may cause or modulate subsequent activity in a way that produces selective responses. Gale & Perkel (2010) recently demonstrated that, in male zebra finches, TH-immunoreactive cells in the ventral tegmental area fire in response to sound and in fact are highly selective for conspecific song. LeBlanc *et al.* (2007) reported that in E2-treated female white-throated sparrows, hearing song induces ZENK expression not only in the ventral tegmental area but also in the locus coeruleus, substantia nigra and periaqueductal gray, and that these responses are selective for song only in birds with physiological breeding levels of plasma E2. This effect was particularly striking in the locus coeruleus; in

E2-treated birds, the ZENK response to song was three-fold higher than the response to tones, but in blank-implanted birds the responses to song and tones were identical. Although LeBlanc *et al.* reported that the ZENK response in CA cell groups of the brainstem was not localized in TH-positive cells, other authors have reported colocalization of immediate early gene expression and TH in these cell groups after presentation of song or other social stimuli (Bharati & Goodson, 2006; Goodson *et al.*, 2009; Nordeen *et al.*, 2009).

The CA cells in the brainstem need not be actively firing during song in order to play a neuromodulatory role in E2-dependent auditory selectivity. Some CA terminals may release transmitter in a nonsynaptic or paracrine fashion, which may alter the responsivity or spontaneous firing activity in forebrain neurons (reviewed by Beaudet & Descarries, 1978). Such changes, which may be sustained over prolonged periods (Reader *et al.*, 1979) may enable forebrain areas to respond differently to the same input depending on the behavioral state of the animal (e.g., season or social context), without changes in the selectivity of CA neurons themselves. Seasonal changes in catecholamine-based neuromodulation may enable auditory systems of female songbirds to respond selectively to male song during the breeding season and to respond less selectively outside it.

In addition to mediating changes associated with the transition from a non-breeding to a breeding context, catecholamines may play a role in encoding more detailed information about social context within the breeding season. For example, in male zebra finches singing in different social contexts, NE depletion eliminates context-dependent suppression of ZENK expression in the song system (Castelino & Ball, 2005). Social context has also been associated with catecholamines in auditory structures; in female canaries, NE depletion reduces behavioral preferences for attractive male song while eliminating the selectivity of ZENK responses for those songs (Appeltants *et al.*, 2002b; Lynch & Ball, 2008; Vyas *et al.*, 2008). Female European starlings exposed for sustained periods to high-quality male song have higher levels of DBH and catecholamine metabolites in NCM than those exposed to sustained periods of low-quality song (Sockman & Salvante, 2008). CA innervation and release in the auditory forebrain is therefore sensitive to stimulus quality and thus the quality of potential mates, and may play a role in context-dependent mate choice. Because hearing song can stimulate the secretion of reproductive hormones (Hinde & Steel, 1978; Morton *et al.*, 1985; Bentley *et al.*, 2000; Maney *et al.*, 2007), it is possible that the effects of hearing song on CA systems are mediated by plasma E2. Our present results strongly support this hypothesis. Whereas Sockman & Salvante (2008) found that song quality modulated catecholamine metabolites and presumably catecholamine release, we found no evidence that release was affected by E2 treatment. This discrepancy could be due to the auditory environment; in the previous study, females heard song whereas the birds in the present study were housed with no auditory stimuli aside from a female companion. Experiments are underway to explore the effects of hearing song on CA synthesis and metabolism and the modulation of those effects by E2.

Although lateralization of CA function was not our primary focus in this study, we included hemisphere as a factor in our analysis in order to control for possible effects. We found that in some domains of NCM, immunoreactivity for TH or DBH was higher in the left than in the right hemisphere. In MLd, the auditory midbrain, levels of NE and its metabolite MHPG were higher on the right. These findings add to a small but growing literature showing lateralization of function in the songbird auditory forebrain and midbrain as demonstrated by ZENK expression (Avey *et al.*, 2005), electrophysiological recording (George

*et al.*, 2004; Phan & Vicario, 2010), blood oxygen level-dependent (BOLD) responses (Poirier *et al.*, 2009) and behavioral assay after retrodialysis (Remage-Healey *et al.*, 2010). This literature shows evidence of left hemisphere dominance in a variety of species and demonstrates that specific functions, such as auditory selectivity for conspecific song, can even be lateralized at the level of the midbrain (Poirier *et al.*, 2009). In our study, interhemispheric differences did not depend on E2 treatment, meaning that both hemispheres responded equally to the hormone and that CA activity may differ between hemispheres at all times of the year.

We predicted that CA fiber density in each region would be related to catecholamine or metabolite concentrations. We did not, however, find correlations between the estimated density of enzyme-immunoreactive fibers and the concentrations of catecholamines or their metabolites. In other words, the results from our IHC measurements did not predict the results of the HPLC measurements. The most notable correlation, between estimated DBH immunoreactivity and the concentration of NE in CMM, was driven by the effects of E2 on both variables independently. The lack of a relationship between the IHC and HPLC data raises important questions about what is actually being measured by each method and how we should expect them to relate. Because the synthetic enzymes may require posttranslational modifications such as phosphorylation, and because there are multiple downstream products of these enzymes, the relationships between the enzymes and any single product may not be linear. Even if the levels of the enzyme can be used to predict the level of monoamine and *vice versa*, because we have shown evidence for interhemispheric differences in both catecholamines and their synthetic enzymes, we cannot expect levels of these enzymes to relate absolutely to catecholamine levels in the contralateral hemisphere in this study. In order to determine how CA activity as measured by IHC relates to that measured by HPLC, both methods would need to be used in the same sample, for example in alternate series of sections. The fact that we detected more effects of E2 manipulation using IHC than with HPLC suggests either that the former may be a more sensitive measure than the latter or that E2 may affect the capacity to synthesize catecholamines without stimulating their synthesis directly.

In this study, we have shown that changes in plasma gonadal steroids, such as during the transition from a non-breeding to a breeding state, can significantly alter the neurochemical makeup of sensory regions involved in the processing of sociosexual signals. Breeding levels of plasma E2 increased the estimated CA innervation of the auditory forebrain (NCM and CMM) and midbrain (MLd) in female sparrows that use auditory signals to choose mates. Estrogen-dependent CA innervation of sensory areas may be a conserved mechanism by which sensory systems are tuned and sensory responses modulated in order to ensure a good match between the social context of a signal and the subsequent behavioral response (reviewed by Maney & Pinaud, 2011). Future work should explore the role of CA input in auditory selectivity and its modulation by gonadal steroids, using electrophysiological or real-time optical techniques in seasonally breeding species.

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## Abbreviations

aNCM, apical NCM; CA, catecholaminergic; CMM, caudomedial mesopallium; cNCM, caudal NCM; DA, dopamine; DBH, dopamine beta-hydroxylase; DOPAC, dihydroxyphenylacetic acid; E2, estradiol; HA, apical part of the hyperpallium; HPLC, high-pressure liquid chromatography; IHC, immunohistochemistry; MHPG, 3-methoxy-4-hydroxyphenylglycol; MLd, dorsal lateral nucleus of the mesencephalon (auditory midbrain); NCM, caudomedial nidopallium; NE, norepinephrine; Ov, nucleus ovoidalis (auditory thalamus); rNCM, rostral NCM; TH, tyrosine hydroxylase.

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