Estradiol-dependent Modulation of Serotonergic Markers in Auditory Areas of a Seasonally Breeding Songbird

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Abstract

Because no organism lives in an unchanging environment, sensory processes must remain plastic so that in any context, they emphasize the most relevant signals. As the behavioral relevance of sociosexual signals changes along with reproductive state, the perception of those signals is altered by reproductive hormones such as estradiol (E2). We showed previously that in white-throated sparrows, immediate early gene responses in the auditory pathway of females are selective for conspecific male song only when plasma E2 is elevated to breeding-typical levels. In this study, we looked for evidence that E2-dependent modulation of auditory responses is mediated by serotonergic systems. In female nonbreeding white-throated sparrows treated with E2, the density of fibers immunoreactive for serotonin transporter innervating the auditory midbrain and rostral auditory forebrain increased compared with controls. E2 treatment also increased the concentration of the serotonin metabolite 5-HIAA in the caudomedial mesopallium of the auditory forebrain. In a second experiment, females exposed to 30 min of conspecific male song had higher levels of 5-HIAA in the caudomedial nidopallium of the auditory forebrain than birds not exposed to song. Overall, we show that in this seasonal breeder, (1) serotonergic fibers innervate auditory areas; (2) the density of those fibers is higher in females with breeding-typical levels of E2 than in nonbreeding, untreated females; and (3) serotonin is released in the auditory forebrain within minutes in response to conspecific vocalizations. Our results are consistent with the hypothesis that E2 acts *via* serotonin systems to alter auditory processing.

Keywords

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INTRODUCTION

Sensory systems must be adapted to emphasize the stimuli most important for reproduction and survival. No environment is constant, however, and the relative importance of some types of stimuli, particularly social signals, changes over time. As a consequence, the neural substrates that process and encode those stimuli must also change in order to facilitate context-appropriate behavioral responses. What mechanisms govern this plasticity? Recent evidence from fish, frogs, birds, and mammals suggests that one powerful mediator of context-dependent sensory processing is endocrine state; hormones can alter perception of social signals from the "bottom up" (reviewed by Maney, 2010; Maney & Pinaud, 2011). Despite the ubiquity of this phenomenon, the neural processes underlying it are not well understood.

Beach (1948) proposed that hormones act at the sensory periphery to alter how social signals are perceived. It has since become clear that hormones do have profound effects on sensory systems, to the extent that detection thresholds and discrimination can be altered. In both rats and humans, for example, the ability to detect odors is greater during periods of high estradiol (E2) than during other phases of the reproductive cycle, and E2 treatment improves olfactory sensitivity in ovariectomized rats and postmenopausal women (Caruso et al., 2001, 2004; Pietras & Moulton, 1974). Estrogen receptors can be found in sensory organs (Barni et al., 1999; Begay, Valotaire, Ravault, Collin, & Falcon, 1994; Maruska & Fernald, 2010; Noirot et al., 2009; Sisneros, Forlano, Deitcher, & Bass, 2004) and estradiol is likely to impact sensory processing at every level in the brain.

Researchers investigating hormone-modulated sensory function have made tremendous advances in the past five years using songbirds as model systems. For example, we have shown evidence that in seasonally breeding sparrows, systemic E2 treatment can dramatically alter the responsiveness and neurochemistry of auditory areas. In the auditory midbrain, thalamus, and forebrain, immediate early gene (IEG) responses are "selective", meaning the response is greater to conspecific male song than to an irrelevant control sound, only in females with breeding-typical plasma E2 levels (reviewed by Maney & Pinaud, 2011). In untreated, non-breeding females, the genomic response to song is indistinguishable from the response to the control sound. Second, E2 treatment alone, independently of sound, induces IEG expression in auditory regions (Sanford, Lange, & Maney, 2010). E2 thus seems to induce plasticity in the auditory system, making it both more responsive and more selective for the sounds that are important in a breeding context.

Several independent groups have shown that in finches and sparrows, E2 alters the physiology of central auditory circuits in both males and females by inducing the expression of genes involved in synaptic plasticity (reviewed by Maney & Pinaud, 2011). Application of exogenous E2 directly into the auditory forebrain enhances the responsiveness and coding efficiency of auditory neurons, demonstrating a possible direct effect. Local application does not, however, increase the selectivity of IEG responses in that region (Tremere, Jeong, & Pinaud, 2009). Furthermore, whereas the auditory forebrain expresses both ER-alpha and ER-beta (Bernard, Bentley, Balthazart, Turek, & Ball, 1999), estrogen receptors have not been described in the subpallial auditory structures of songbirds (Gahr, 2001; Gahr, Guttinger, & Kroodsma, 1993; cf. Martinez-Vargas, Stumpf, & Sar, 1976). In order to fully understand E2-dependent selectivity of genomic responses in the auditory system, we need to look for indirect effects--possibly *via* neuromodulatory systems. In the current study, we have explored a model whereby E2 modulates the processing of conspecific song by acting on the serotonin system (Fig. 1). We have focused on this system because it is widely understood to be estrogen-sensitive (reviewed by McEwen & Alves, 1999) and because it is hypothesized to convey information about both internal state and environmental context to

the auditory system, directly affecting the selectivity of auditory responses (reviewed by Hurley, Devilbiss, & Waterhouse, 2004; Hurley & Hall, 2011). In mammals, serotonergic neurons in the raphe nuclei project throughout the auditory system, for example to the auditory midbrain, thalamus, and cortex (*e.g.,* Hurley & Thompson, 2001; Klepper & Herbert, 1991; Steinbusch, 1981). The system is dynamic and highly plastic in that patterns of activity are modulated by behavioral arousal (Hall, Rebec, & Hurley, 2010) and social context (Hurley & Hall, 2011). Serotonin is released in auditory areas during sound presentation (Hall et al., 2010) and increases auditory selectivity for conspecific vocalizations (Hurley & Pollak, 2005). Clearly, this system is in an ideal position to mediate the effects of internal state, in this case endocrine state, on selectivity for sociosexual signals in seasonal breeders.

The avian serotonergic system has been mapped using a variety of techniques in non-songbirds such as chicken, quail, and pigeons (Challet et al., 1996; Cozzi, Viglietti-Panzica, Aste, & Panzica, 1991; Dube & Parent, 1981; Fuxe & Ljunggren, 1965; Ikeda, Inugai, & Gotoh, 1971; Kaiser & Covey, 1997; Metzger, Toledo, & Braun, 2002; Yamada, Takeuchi, & Sano, 1984). Its anatomical distribution is similar to that of mammals, with cell bodies organized into subdivisions comparable to mammalian cell groups B1-B9 (Dahlström & Fuxe, 1964; Ikeda, Inugai, & Gotoh, 1971; Yamada et al., 1984). The cell bodies are located primarily in the brainstem along the midline, with some groups stretching laterally (Cozzi et al., 1991; Parent, 1981; Yamada et al., 1984). Serotonergic fibers project widely throughout the entire brain, suggesting that like in mammals, this neuromodulator plays important roles in many physiological processes in birds (Cozzi et al., 1991). In the avian species studied so far, serotonergic fibers have been noted in auditory areas (Challet et al., 1996; Kaiser & Covey, 1997; Metzger, Toledo, & Braun, 2002; Zeng et al., 2007). In this study, we aimed to show evidence that in a seasonally breeding songbird, auditory areas are innervated by serotonergic fibers, that the density of this innervation is modulated by plasma E2, and that serotonin is released in the auditory system during the processing of conspecific vocalizations.

The anatomical and functional organization of central auditory pathways in songbirds largely resembles those found in other vertebrates, including mammals (Fig. 2; reviewed by Maney & Pinaud, 2011). Auditory input is transduced in the cochlea, ascends through brainstem areas analogous to mammalian cochlear nuclei, and arrives at the dorsal lateral mesencephalic nucleus (MLd), the avian homolog of the mammalian inferior colliculus (Karten, 1967). MLd neurons send direct projections to the thalamic nucleus Ovoidalis (Ov), the avian homolog of the ventral medial geniculate (Karten, 1967). Both structures participate in auditory discrimination and are tuned to behaviorally relevant signals (Amin, Gill, & Theunissen, 2010; Woolley, Fremouw, Hsu, & Theunissen, 2005). Ov projects to a pronounced lobe in the forebrain that contains auditory areas (Fig. 2). Inside this lobe, the caudomedial nidopallium (NCM) receives input from the thalamo-recipient Field L and is heavily interconnected with the caudomedial mesopallium (CMM). NCM and CMM are analogous to the supragranular layers of mammalian auditory cortex (Vates, Broome, Mello, & Nottebohm, 1996) or to mammalian auditory association cortex (Pinaud & Terleph, 2008; Tremere et al., 2009) and respond selectively to behaviorally relevant stimuli. For example, the factors that affect behavioral responses to song, such as attractiveness or familiarity, also affect the expression of the IEG ZENK (*e.g.,* Gentner, Hulse, Duffy, & Ball, 2001; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003; Terpstra, Bolhuis, Riebel, Van Der Burg, & Den Boer-Visser, 2006). Changes in the behavioral relevance of a signal are thus expected to be accompanied by changes in the ZENK response to that signal (Maney, Cho, & Goode, 2006).

We have shown such changes in seasonally breeding sparrows. When plasma E2 reaches breeding levels, which presumably happens when conspecific male song peaks in behavioral relevance (see Maney, 2010), ZENK responses in the auditory forebrain, thalamus, and midbrain become selective for that stimulus over a non-relevant sound (reviewed by Maney & Pinaud, 2011). In the current study, we tested whether this increase in selectivity is accompanied by an E2-mediated change in the serotonergic innervation of auditory areas (Fig. 3). We manipulated plasma E2 in female white-throated sparrows (*Zonotrichia albicollis*) and measured the subsequent effects on the density of serotonergic fibers and levels of the serotonin metabolite 5-hydroxyindoleacetic acid, 5-HIAA, in auditory areas. If E2 mediates seasonal changes in sensory tuning, then we expected these markers to be higher in females with breeding-typical E2 levels than in untreated, non-breeding females.

In a second experiment, we addressed the function of E2-induced increases in serotonergic innervation of auditory areas. We reasoned that serotonergic input may regulate auditory responses *via* two independent mechanisms. First, E2 may increase the density of terminals that release serotonin in a nonsynaptic, or tonic fashion, altering the responsiveness or spontaneous firing activity in auditory areas (Beaudet & Descarries, 1978). Such changes, which may be sustained over prolonged periods, may enable auditory areas to respond differently to the same input depending on the season or social context. In this scenario, serotonergic neurons projecting to auditory regions need not themselves respond to song. Second, the serotonergic neurons may themselves fire and release transmitter in a phasic fashion during auditory stimulation (Cransac, Cottet-Emard, Hellstrom, & Peyrin, 1998; Hall et al., 2010), rapidly altering the responsiveness and selectivity of target cells. In order to test whether serotonin plays a priming role only or whether it is released rapidly during auditory stimulation, we performed a song playback experiment. We predicted that if serotonergic cells actively fire in response to conspecific song, exposure to song playback would increase local levels of serotonin metabolite rapidly in auditory areas.

METHODS

Animals

All procedures in this study adhered to NIH standards and were approved by the Emory University Institute for Animal Care and Use Committee. We collected female white-throated sparrows in mist nets in Atlanta, Georgia during fall migration. We determined their sex by polymerase chain reaction (PCR) analysis of DNA extracted from a blood sample (Griffiths, Double, Orr, & Dawson, 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 while wintering at the capture site. We kept the birds under these conditions for two months to ensure that they were not photorefractory prior to the start of the study (Shank, 1959; Wolfson, 1958).

Experiment 1: Effects of E2 treatment on serotonergic fiber density and 5-HIAA

Hormonal Manipulation—We transferred the birds in pairs to small, acoustically isolated rooms where they were housed one per cage ($38 \times 38 \times 42$ cm) in two adjacent cages. On the day of transfer, we implanted each bird with a subcutaneous silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI) sealed at both ends with A-100S Type A medical adhesive (Factor 2, Lakeside, AZ). One bird in each pair (n = 8) received an empty implant and the other (n = 8) received an implant containing 17β-estradiol (Steraloids, Newport, RI). This dose of E2 elevates plasma levels to those typical of the breeding season within seven days in this species (Maney et al., 2006, 2008) and

likely does so within two days (Moore, 1983). After seven days of E2 treatment, which induces selectivity of sound-induced ZENK responses (Sanford et al., 2010) as well as enhanced catecholaminergic innervation of auditory areas (Matragrano, Sanford, Salvante, Sockman, & Maney, 2011), we rapidly decapitated the birds, quickly harvested the brains, and bisected each brain into two 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 high performance liquid chromatography (HPLC) analysis. The hemisphere that was fixed (right or left) was balanced across treatments.

Immunohistochemistry—We cut the fixed hemispheres into 50 μm parasagittal sections using a freezing sliding microtome. We then used a standard immunohistochemistry (IHC) protocol (LeBlanc, Goode, MacDougall-Shackleton, & Maney, 2007; Maney et al., 2006; Matragrano et al., 2011) to immunolabel serotonin transporter (SERT). SERT is less liable to metabolism than serotonin; therefore immunolabeling with an anti-SERT antibody is a more stable marker of serotonergic fibers and has been shown to be a better indicator of serotonin axons than an anti-serotonin antibody (Nielsen, Brask, Knudsen, & Aznar, 2006). For this study, we used every third section; the first two series of sections were used for a separate study (Matragrano et al., 2011; see Discussion). Sections were incubated with an anti-SERT antibody (ImmunoStar; Hudson, WI; see below) diluted 1:5000 (Meyer, Grande, Johnson, & Ali, 2004), followed by a biotinylated antibody against anti-rabbit IgG (Vector, Burlingame, CA) diluted 1:250. We visualized the label *via* the ABC method (Vector) followed by diaminobenzidine. We processed all of the brain sections in a single run of IHC. Following IHC, we mounted all of the sections onto microscope slides, dehydrated them, and coverslipped in DPX (Sigma, St. Louis, MO). In order to better our understanding of the distribution of SERT-immunoreactive (ir) fibers, which has not been described in birds, we also processed coronal brain sections from several additional birds not otherwise used in this study.

The anti-SERT antibody was a rabbit polyclonal antibody generated against a synthetic peptide sequence corresponding to amino acids (602–622) of rat SERT coupled to keyhole limpet hemocyanin (ImmunoStar Cat#24330). We validated the specificity of the SERT antibody in brain sections of a white-throated sparrow not in the current study, following the procedure described by Saper and Sawchenko (2003). All labeling at antibody concentrations of 1:25,000 was eliminated by preadsorption of the antibody with 50 μg/ml of the SERT peptide (ImmunoStar Cat#24332). Omission of the primary or the secondary antibodies resulted in a complete loss of specific staining.

Regions of Interest—Our primary goals in this study were to test the effects of E2 on SERT-ir innervation of auditory areas and to determine the extent to which those effects overlap anatomically with previously described effects on the ZENK response (Sanford et al., 2010) and catecholaminergic innervation (Matragrano et al., 2011). In order to compare the anatomical distributions of the effects of E2 on SERT-ir innervation and the previously described effects, we needed to partition NCM into the same domains as in our previous studies. In the past, we have divided the region both rostro-caudally and dorso-ventrally into four primary domains (Sanford et al., 2010): rostro-dorsal (rdNCM), rostro-ventral (rvNCM), caudo-dorsal (cdNCM) and caudo-ventral (cvNCM). Because the dorsal and ventral domains of cNCM are similar hodologically and neurochemically, and because E2 does not affect ZENK expression in either domain (Sanford et al., 2010), we combined them into one caudal domain. As in our previous work, we also sampled from an apical domain (aNCM) located dorsal to Field L2 (Fig. 3). In the published literature, this region is usually considered part of NCM but may overlap the dorsal portion of Field L (Fortune & Margoliash, 1992).

We conducted all image acquisition and analyses while blind to treatment group. Regions of interest were identified with reference to Stokes et al. (1974) and Vates et al. (1996). To acquire the images we used the 10x objective on a Zeiss Axioskop microscope attached to a Leica DFC480 camera and Macintosh G5 computer. We captured rectangular images (approximately 46 MB in size) corresponding to the field of view of the camera (870×690 µm), holding the light level and exposure time constant for all photos. For each bird, we acquired images of NCM and CMM in four consecutive sections between ~350 and ~800 µm from the midline. Five separate images, each containing either CMM, aNCM, rdNCM, rvNCM, or cNCM, were acquired from each of the four sections. With the exception of CMM, for which the entire photo was used, all regions of interest were selected in the photos using ImageJ (version 1.41o, National Institutes of Health, Bethesda, MD) as previously described (Matragrano et al., 2011; Sanford et al., 2010; see Fig. 3).

To acquire images of CMM, the upper corners of the field of view of the camera were positioned along the dorsal boundary of CMM and one of the lower corners was positioned adjacent to the lamina mesopallium (Fig. 3). We defined cNCM as a strip of tissue approximately 275 µm from the caudal boundary of NCM (Sanford et al., 2010; Matragrano et al., 2011). The acquired images of cNCM captured the majority of that domain, spanning 870 µm from dorsal to ventral (Fig. 3). For aNCM, we placed a circle approximately 350 µm in diameter dorsal to Field L and just ventral to the ventricle. For rdNCM and rvNCM, we placed two circles, each approximately 550 µm in diameter, into the dorsal and ventral portions of this region (Matragrano et al., 2011; Sanford et al, 2010) so that they did not overlap with Field L or with the region we defined as cNCM. Because we did not conduct tract tracing or another method that would enable us to discern absolutely the boundary between the rostral domains of NCM and the adjacent subregions of Field L (Vates et al., 1996), it is possible that our samples of aNCM, rdNCM and rvNCM may have captured a bit 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). All five photos of each section of auditory forebrain were viewed at the same time to ensure that the regions of interest we selected in each section did not overlap.

In addition to the auditory forebrain, we estimated the density of SERT-ir fibers in the auditory midbrain (MLd) and thalamus (Ov). Five sections of MLd, spanning 600 µm, were chosen that encompassed the largest portion of this nucleus (Maney et al., 2006; Matragrano et al., 2011). We traced the outline of MLd in each acquired photograph using the ImageJ freehand tool. Ov was visible in at least three sections of each brain. We photographed the three consecutive sections in which Ov was the largest. Because the core region of Ov did not contain SERT-ir fibers (see also Belekhova et al., 2002; Kaiser & Covey, 1997); we included only the shell region in our analysis (Zeng, Li, Zhang, & Zuo, 2007). The Ov shell was traced with the ImageJ freehand tool. Finally, we estimated the density of fibers in a non-auditory region, the apical part of the hyperpallium (HA; see Stokes et al., 1974; Reiner et al., 2004), to test the specificity of the effects of E2 on auditory regions. We photographed HA in the same three brain sections we used for the Ov shell. Like CMM, the entire photo of HA was analyzed for fiber density.

Image acquisition and estimation of SERT-immunoreactive fiber density—We converted all the photos to 8-bit scale and selected SERT-ir fibers using the thresholding feature in ImageJ (Maney et al., 2005; Matragrano et al., 2011). Our method of selecting immunolabeled fibers has been fully validated and has high interrater reliability and low variability. Briefly, the thresholds selected by two independent observers were highly correlated ($R = 0.98$), not different from each other ($P = 0.976$; d = 0.0009), and were within one unit of each other in 30% of cases (Matragrano et al., 2011). In the present study, the same observer set the threshold for all images with the same lighting and computer monitor

(LLM). We performed this procedure in 3–5 images per region (see above). For each region, we summed the total area covered by fibers and divided this sum by the total area measured to yield the area covered by the fibers in square microns per square mm of area measured.

Quantification of the serotonin metabolite and total protein—We measured the concentration of serotonin metabolite in the auditory pathway *via* HPLC. Because the number of brain regions we could sample was limited, 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). We collected NCM, CMM and MLd with micropunch tools (i.d.: 1.5, 1.0 and 1.0 mm, respectively) in sagittal sections (300 μm) from the frozen, non-fixed hemispheres. All of the sectioning and sampling was done in one day by the same person using the same punch tool for each region. The regions of interest were located by referring to Sockman, Gentner & Ball (2002) as well as two sets of Nissl-stained brain sections from white-throated sparrow. For NCM and CMM, we used Field L as a landmark. For MLd we used the tectal ventricle, which appears as a dark line in the fresh frozen tissue. We determined the concentration of 5-HIAA by reversed-phase HPLC with electrochemical detection (Kilts, Breese, & Mailman, 1981). This method has been precisely described elsewhere (see Sockman & Salvante, 2008). We also measured the protein content of each sample by dissolving the remaining sample pellet in 0.2 N NaOH (100 μl) and performing the Bradford protein-dye binding assay (Quick Start Bradford Protein Assay, Bio-Rad) with bovine serum albumin as a standard (Bio-Rad) on a μQuant microplate spectrophotometer (BioTek) (Bradford, 1976). Concentrations of 5-HIAA were normalized by dividing 5-HIAA levels by protein content of each sample.

Statistical Analysis—For each dependent variable, we plotted histograms and performed Shapiro-Wilk tests (SPSS) to determine whether the distribution of the data was normal. In the event of significant deviation ($P < 0.05$), we performed a square-root transformation to normalize the distribution. The transformation was necessary and sufficient to normalize the distributions for 3 of the 11 variables: 5-HIAA in NCM and MLd, and SERT-immunoreactivity in MLd. The other 8 variables did not require transformation. In all cases, visual inspection of the histogram confirmed the necessity of transformation and its effectiveness.

We looked for effects of treatment on 5-HIAA concentration and the estimated density of SERT-ir fibers using a mixed-effects linear model (Stata), which uses restricted maximum likelihood to estimate parameters. For each of the 11 dependent variables (concentration of 5-HIAA in CMM, NCM, and MLd and estimated density of SERT-immunoreactivity in CMM, aNCM, rdNCM, rvNCM, cNCM, Ov, MLd, and HA), hormone treatment was a predictor and bird was nested as both a random intercept and random coefficient on treatment (Schiezelth & Forstmeier, 2009). Effect sizes were estimated using Cohen's d on untransformed data.

Experiment Two: Rapid effects of hearing song on serotonin and 5-HIAA in auditory regions

In order to determine whether the serotonin system is rapidly engaged during sound presentation, we conducted a playback study. We treated 12 female white-throated sparrows, collected and housed as for Experiment 1, with E2 as described above and presented recordings of male song seven days later. The sound stimuli and playback protocol have been published previously (Maney et al., 2006, 2007; 2009; Sanford et al., 2010). On the afternoon prior to sound presentation, we isolated each female in a sound attenuation booth equipped with a video camera, microphone and speaker. The following morning approximately 1 h after lights-on, we played male white-throated sparrow songs at 70 dB *via*

the speaker. Each female heard either 30 min of song (n = 4), 15 min of song (n = 4), or no song (n = 4). The stimulus presentations contained songs from a variety of males (five males in the 15 min presentations and ten males in the 30 min presentations), with the identity of the singer changing to a new male every three minutes in order to prevent habituation to the stimulus (Maney et al., 2006). Each female heard the five or ten males in a unique order. We made audio and video recordings of all birds during the stimulus presentation (the birds hearing no song were recorded for 30 min).

We rapidly decapitated the birds and harvested the brains immediately following playback, with time of day balanced across stimulus treatments. As in Experiment 1, we carefully bisected each brain into hemispheres, flash froze them in powdered dry ice, and performed HPLC analysis using one hemisphere from each brain (left or right balanced across stimulus treatments). 5-HIAA was assayed in CMM, NCM, and MLd as described for Experiment 1. At the time of Experiment 2, we had revised our method and used punch tools of smaller diameter (0.5 mm in diameter for CMM and MLd, and 1.0 mm in diameter for NCM) in order to more accurately sample the regions of interest. We had also developed a method to detect serotonin in micropunches; we therefore assayed serotonin in these samples as well. We used the behavioral recordings to quantify the number of vocalizations during the period 30 min prior to sacrifice. We counted the number of trills, which are associated with copulation solicitation displays, as well as chip-ups, pinks, seets, and songs (see Maney et al., 2009 for descriptions of these vocalizations).

Statistical Analysis—In some cases, we had difficulty getting reliable protein measurements from the smaller punches. As a result, we had a complete set of protein measurements only for NCM; for CMM and MLd we were missing the protein measurement for at least one sample. We therefore normalized for protein content only for NCM. The data from CMM and MLd were not normalized. Since 5-HIAA concentrations were compared only between treatment groups and not regions of interest, this discrepancy did not pose a problem for the data analysis.

To analyze the effects of song duration on the concentrations 5-HIAA and serotonin, we used a separate general linear model (Stata) for each compound and each brain region. For each model, the response variable was the mean concentration from the two punches for that region (in pg/mg protein for NCM and pg for CMM and MLd; see Methods) and the predictor song duration was expanded into a dummy-variable set to model the contrast between 0 and 15 minutes and the independent contrast between 0 and 30 minutes.

In order to rule out the birds' own vocalizations as a factor influencing 5-HIAA and serotonin concentrations in CMM, NCM, and MLd, we ran Spearman correlation tests to look for relationships between the number of vocalizations (total number as well as the number of each type) and the level of each compound for each region.

RESULTS

Experiment 1: Effects of E2 treatment on serotonergic fiber density and 5-HIAA

Distribution of SERT-ir Fibers—The distribution of serotonergic fibers was similar to what has been described in chickens, Japanese quail, and pigeons (Challet et al., 1996; Cozzi, Viglietti-Panzica, Aste, & Panzica, 1991; Kaiser & Covey, 1997; Metzger, Toledo, & Braun, 2002; Zeng et al., 2007). SERT-ir fibers were densely distributed throughout the entire brain. We noted the highest densities in the striatum, diencephalon, and midbrain. The most intensely labeled structures in the brain were the pretectal nucleus, the lateral geniculate nucleus, and the nucleus taeniae of the amygdala (Fig. 4). Basket-like immunoreactive structures appeared to surround neuronal somata in the ventral tegmental

area, substantia nigra, and locus coeruleus. We noted high densities of fibers in the medial septum, lateral bed nucleus, hippocampus, ventromedial hypothalamus, habenula, and periaqueductal gray. The song control nucleus HVC (proper name) stood out as unlabeled compared to the surrounding tissue. Many SERT-ir fibers appeared to contact the intraventricular space, particularly in the lateral ventricles and the cerebral aqueduct. The median eminence also contained labeled fibers, suggesting secretion into the portal vasculature.

With the exception of Ov, within which only the shell region contained appreciable labeled fibers (see also Belekhova et al., 2002; Kaiser & Covey, 1997; Zeng et al., 2007), the auditory structures we focused on for this study were densely innervated (Fig. 5). The distribution of SERT-ir fibers was relatively homogenous across CMM and NCM, with clusters of fibers appearing to surround unlabeled cell bodies in some individuals. Whereas Zeng et al. (2007) reported that MLd in Bengalese finches is nearly devoid of serotonergic fibers, we found that although it was less densely labeled than the surrounding nucleus intercollicularis, it was clearly innervated by SERT-ir fibers (Fig. 5B). We did not detect convincing labeling in nucleus magnocellularis or nucleus laminaris, which are avian homologues of the cochlear nuclei and the medial superior olive, respectively, in mammals (Boord & Rasmussen, 1963).

Effects of E2 on serotonergic fiber density and 5-HIAA—The effects of E2 treatment on SERT fiber density are shown in Fig. 6A. E2 significantly increased the density of SERT-ir fibers in aNCM ($z = 2.95$, $P = 0.003$, $d = 1.546$), rdNCM ($z = 2.14$, $P = 0.032$, $d = 1.186$), and MLd ($z = 4.51$, $P < 0.001$, $d = 1.325$). The effect of E2 treatment in CMM was not significant, but there was a compelling trend ($z = 1.83$, $P = 0.067$, $d = 0.946$). We did not detect an effect of E2 on the density of SERT-ir fibers in rvNCM ($z = 1.02$, $P = 0.306$, $d = 0.449$), cNCM ($z = -1.43$, $P = 0.156$, $d = 0.583$), the shell of Ov ($z = -0.97$, $P = 0.333$, $d = 0.351$), or the non-auditory region HA ($z = 0.07$, $P = 0.942$, $d = 0.038$).

The effects of hormone treatment on the concentration of the serotonergic metabolite 5-HIAA in our regions of interest are shown in Fig. 6B. E2 treatment significantly increased concentrations of 5-HIAA in CMM ($z = 2.86$; $P = 0.004$; $d = 1.241$). We did not detect an effect of treatment in NCM ($z = 1.07$; $P = 0.284$; $d = 0.383$) or MLd ($z = 0.43$; $P = 0.669$; $d = 0.321$).

Experiment 2: Rapid effects of hearing song on serotonin and 5-HIAA in auditory regions

The serotonin metabolite 5-HIAA increased in NCM during song playback (Fig. 7). After 15 min of song presentation, 5-HIAA levels were not significantly higher than at 0 min, but after 30 min the metabolite had increased significantly ($z = 2.25$, $P = 0.024$). Hearing song did not increase 5-HIAA in CMM or MLd, nor did it affect serotonin concentrations in any of the regions of interest. We did not detect any significant relationships between serotonin or 5-HIAA and either the total number of vocalizations or the number of each type, indicating that the increase in 5-HIAA in NCM was unlikely to be driven by self-stimulation.

DISCUSSION

The serotonergic system is a prime candidate for mediating the effects of internal state and behavioral context on auditory processing in vertebrates (reviewed by Hurley & Hall, 2011). In this study, we have explored a model wherein E2 regulates both tonic and phasic release of serotonin in auditory areas. In Experiment 1, we showed evidence that E2, which varies in plasma according to reproductive state and alters IEG responses in auditory areas, also affects serotonin markers in the same areas independently of sound stimulation. First, E2

treatment of nonbreeding female white-throated sparrows enhanced the density of SERT-ir innervation of the auditory midbrain as well as the auditory forebrain (Fig. 6A). Because these fibers may release serotonin tonically, an increase in their density suggests a mechanism by which E2 might increase local levels of serotonin, thereby altering the firing properties of local auditory neurons. Second, we found an E2-induced increase in the concentration of the serotonin metabolite 5-HIAA (Fig. 6B), which indicates enhanced synthesis or release of serotonin (Commissiong, 1985; Houdouin, Cespuglio, Gharib, Sarda, & Jouvet, 1991). The females in Experiment 1 were isolated from males and male song during the course of hormone treatment, so any increase in serotonergic innervation or release occurred independently of sociosexual stimuli. In Experiment 2, we tested whether hearing song induces phasic serotonergic responses in auditory areas. Exposure to conspecific male song increased the concentration of 5-HIAA in NCM within minutes (Fig. 7) suggesting either an increase in intracellular serotonin turnover or actual release in response to hearing song. When considered together, the results of Experiments 1 and 2 are thus consistent with a model wherein tonic release of serotonin is enhanced by E2 and phasic release may be triggered by behaviorally relevant sound.

The effects of E2 on serotonergic activity may depend on the region. For example, we show here that E2 treatment enhanced serotonergic innervation of MLd and NCM but not CMM. In contrast, E2 treatment significantly increased levels of 5-HIAA in CMM but not the other regions. The hormone treatment may therefore have very different functional consequences depending on the region. Note however that the evidence of such differences is not particularly strong in this study. For example, although we did not find a significant effect of E2 on SERT-ir in CMM, there was a strong trend in that direction (P = 0.067). Similarly, the trends for 5-HIAA were in the same direction for all regions (Fig. 5B). Thus, although it is tempting to speculate that E2 may have site-specific effects on serotonergic activity in the auditory pathway, a more detailed analysis will be necessary to draw those contrasts with confidence.

A major goal of the current study was to map the effects of E2 on serotonergic innervation of the auditory system and to determine the extent to which those effects overlap anatomically with the effects of E2 on the ZENK response. In a previous series of studies, E2 induced ZENK expression and increased the selectivity of the ZENK response in the auditory midbrain (Maney et al., 2006) and the rostral domains of the auditory forebrain (CMM, aNCM, rdNCM, and rvNCM; Maney et al., 2006; Sanford et al., 2010). In contrast, we have consistently failed to detect an effect of E2 on the ZENK response in the caudomedial area of NCM (cNCM; Maney & Pinaud, 2011; Sanford et al., 2010). In the latter region, the ZENK response was selective for song over synthetic tones no matter the endocrine state of the listener. In the present study, the anatomical distribution of E2 effects on serotonergic innervation overlapped exactly with previously demonstrated effects on the ZENK response. E2 treatment enhanced serotonergic fiber density in the auditory midbrain and in the rostral but not the caudal areas of the auditory forebrain. The anatomical match between the effects of E2 on the ZENK response and serotonergic fiber density thus supports our hypothesis that E2-dependent plasticity in the ZENK response may be mediated by the serotonin system.

E2-dependent plasticity in the auditory system may involve not only serotonin, but also catecholamines. In a separately published study using material from the same animals, we reported that E2-treatment of nonbreeding female white-throated sparrows increased the density of fibers immunopositive for tyrosine hydroxylase or dopamine beta-hydroxylase in the auditory midbrain as well as part of the rostral but not the caudal auditory forebrain (Matragrano et al., 2011). Thus, the E2-induced changes in catecholaminergic innervation that we previously reported map closely onto the changes in serotonergic innervation that we

report here. The effects of E2 on catecholaminergic and serotonergic innervation were not identical however, particularly with regard to their distribution within rostral NCM. Whereas E2 treatment affected the density of catecholaminergic fibers similarly in the dorsal compared with the ventral domain (rdNCM and rvNCM; Fig. 3), SERT-ir fibers increased in the dorsal domain only (Fig. 6A), suggesting the possibility of a functional distinction between the two domains.

The practice of dividing NCM into dorsal and ventral domains was introduced by Gentner et al. (2001) and the majority of published studies have since followed this convention (*e.g.,* Avey, Kanyo, Irwin, & Sturdy, 2008; Eda-Fujiwara, Satoh, Bolhuis, & Kimura, 2003; Hernandez and MacDougall-Shackleton, 2004; Lynch and Ball, 2008; Maney et al., 2003; Sockman and Salvante, 2008; Sockman, Gentner, & Ball, 2005; Velho and Mello, 2008). Pinaud, Fortes, Lovell, & Mello (2006) argued that based on connectivity, electrophysiological responses, and neurochemical markers, NCM may be better divided into rostral and caudal domains. Sanford et al. (2010) demonstrated that whereas ZENK responses in the rostral domains are both induced and made more selective by E2 treatment, ZENK responses in the caudal domain are unaffected by E2. Matragrano et al. (2011), after finding similar effects of E2 treatment on catecholaminergic innervation of rdNCM and rvNCM, combined them into one domain (rNCM). In the current study, we detected a strong effect of E2 treatment in rdNCM but not rvNCM and, given that other researchers have reported that dorsal and ventral NCM differ in their sensitivity to and selectivity for a variety of experimental stimuli (Avey et al., 2008; Eda-Fujiwara et al., 2003; Gentner et al., 2001; Maney et al., 2003; Phillmore, Bloomfield, & Weisman, 2003; Sockman & Salvante, 2008), we could not justify combining them. Perhaps our current findings will ultimately contribute toward a more detailed understanding of the functional neuroanatomy of this large, heterogeneous region.

The literature on estrogen-dependent regulation of the mammalian serotonin system is vast and reflects a predominantly positive effect of E2 on serotonin markers in the dorsal and median raphe. E2 treatment of ovariectomized rats, for example, increased firing rate (Robichaud & Debonnel, 2005), serotonin content (Cone, Davis, & Goy, 1981), and the expression of SERT and tryptophan hydroxylase-2 mRNA (Bethea et al., 2000; Donner & Handa, 2009; Hiroi, McDevitt, & Neumaier, 2006). These effects may be direct, as many dorsal raphe neurons contain estrogen receptors (Gundlah, Lu, Mirkes, & Bethea, 2001; Lu, Ozawa, Nishi, & Kawata, 2001; Lu et al., 1999; Sheng et al., 2004). The reported effects of E2 treatment on the projections of these neurons, however, vary widely and appear to be highly region- and species-specific. For example, whereas ovariectomy decreased the concentration of serotonin in the prefrontal cortex of rats (Inagaki, Gautreaux, & Luine, 2010), it increased the density of serotonergic fibers in the same region of rhesus macaques (Kritzer & Kohama, 1999). In mountain spiny lizards, ovariectomy reduced and E2 treatment restored 5-HIAA concentrations in the lateral septum, but other regions were not affected (Woodley, Matt, & Moore, 2000). In ovariectomized rats, E2 treatment decreased serotonergic fiber density, serotonin, and 5-HIAA in the ventromedial hypothalamus and medial preoptic area but not the lateral septum (Lu, Yuri, Ito, Yoshimoto, & Kawata, 1998) and increased serotonin turnover in the hippocampus and nucleus accumbens whereas many other regions were not affected (Pandaranandaka, Poonyachoti, & Kalandakanond-Thongsong, 2009). The expression of SERT, as measured by the binding of $[^3H]$-paroxetine, increased in the lateral septum, basolateral amygdala, and ventromedial hypothalamus but decreased in the periaqueductal gray (McQueen, Wilson, & Fink, 1997; see also Bertrand et al., 2005). In ovariectomized rhesus macaques, E2 treatment increased the density of SERT-ir fibers in the zona incerta but not the lateral tuberal nuclei (Lu, Eshleman, Janowsky, & Bethea, 2003). McQueen et al. noted that in rats, each of the regions in which SERT is upregulated by E2 contains high levels of estrogen receptor, and suggested that local effects

on posttranslational processing, rather than effects in the dorsal raphe, may contribute toward E2-evoked changes in reuptake. Clearly, the effects of E2 manipulation on serotonergic innervation cannot be predicted by a simple rule for all brain areas in all species. Just as the receptors are remarkably diverse, serotonin release and reuptake appear to be regulated by heterogeneous mechanisms that depend on the local environment.

We interpret the increases in the density of SERT-ir fibers as an increase in the density of serotonergic axons. In other words, we are interested primarily in a hormone-dependent structural change. The well-known trophic effects of E2, for example on axonal outgrowth in culture and *in vivo* (reviewed by de Lacalle, 2006) are observable without IHC by using tract tracing or Golgi impregnation (e.g. Vanderhorst, Terasawa, & Ralston, 2002). In this study, however, because we labeled a marker that can be regulated independently of structural plasticity, we must consider alternative explanations. It is possible that E2 treatment altered the abundance of SERT protein, affecting our ability to detect immunolabeled fibers. Regardless of whether the increase in SERT-ir fiber density reflects structural plasticity, it likely indicates an increase in serotonin reuptake activity. Studies using *in vitro* and cell culture preparations have demonstrated that E2 inhibits SERT activity (Chang & Chang, 1999; Koldzic-Zivanovic, Seitz, Watson, Cunningham, & Thomas, 2004), which would lead to serotonin accumulation in the synapse and compensatory upregulation of SERT (see also Charoenphandhu, Teerapornpuntakit, Nuntapornsak, Krishnamra, & Charoenphandhu, 2011). As noted above, E2 increases serotonin synthesis (Bethea et al., 2000; Donner & Handa, 2009; Hiroi et al., 2006), which could also result in compensatory upregulation of SERT (see also Attali, Weizman, Gil-Ad, & Rehavi, 1997). Thus, the increase in SERT-immunoreactivity that we observed in auditory structures could have multiple explanations and implications. We hope eventually to confirm and extend our findings using additional markers such as immunoreactivity for serotonin itself.

In Experiment 2, we showed that hearing conspecific male song increases the concentration of the serotonin metabolite 5-HIAA in NCM, suggesting release of serotonin (Commissiong, 1985; Houdouin et al., 1991). Sound-induced release has been demonstrated in several species of rodents (Cransac et al., 1998; Hall et al., 2010; Stark & Scheich, 1997); presentation of broadband noise increases serotonin concentrations in the inferior colliculus within 5 min (Hall et al., 2010) and 5-HIAA concentrations in the cochlear nuclei and the auditory cortex within 45 min (Cransac et al., 1998). Sound-induced serotonin transmission in auditory areas may modulate auditory responses by conveying information about internal state of the animal, in that it is modulated by stress (Stark & Scheich, 1997) and possibly by other internal and external factors (Hall et al., 2010; Hurley & Hall, 2011). Because the serotonin system is already widely understood to be affected by endocrine state, we hypothesize that seasonal changes in plasma E2 may underlie plasticity in auditory responses to the conspecific signals that are important for breeding. In this study, since all of the animals in Experiment 2 were treated with E2, we do not know whether the sound-induced increase in 5-HIAA we observed in NCM depends on breeding-typical plasma levels. Because we played only song, neither do we know whether sound-induced serotonin release in NCM depends on the type or behavioral relevance of the stimulus. Future experiments will explore the selectivity of sound-induced serotonin release and how it may be modulated by endocrine state.

In this study, we have shown that an increase from nonbreeding to breeding-typical levels of plasma E2 enhances serotonergic innervation of auditory centers. Our results thus support a model wherein seasonal increases in plasma E2 may induce changes in auditory tuning such that selectivity for courtship signals is increased (Fig. 1). This study has addressed only the first step in this model, however, since we do not know the physiological effects of serotonin on auditory responses in songbirds. When iontophoretically applied to areas of the auditory

pathway in mammals, serotonin inhibits or enhances sound-evoked activity in a pattern probably related to the distribution of receptor subtypes (e.g. Ebert & Ostwald, 1992; Hurley & Pollak, 1999; Ji & Suga, 2007). Serotonin-induced changes in the responses to species-specific vocalizations have been demonstrated in the inferior colliculus of bats (*e.g.,* Hurley & Pollak, 2005) but not other vertebrates. Future studies should investigate whether increased serotonergic activity in the auditory pathway alters auditory tuning in songbirds.

Acknowledgments

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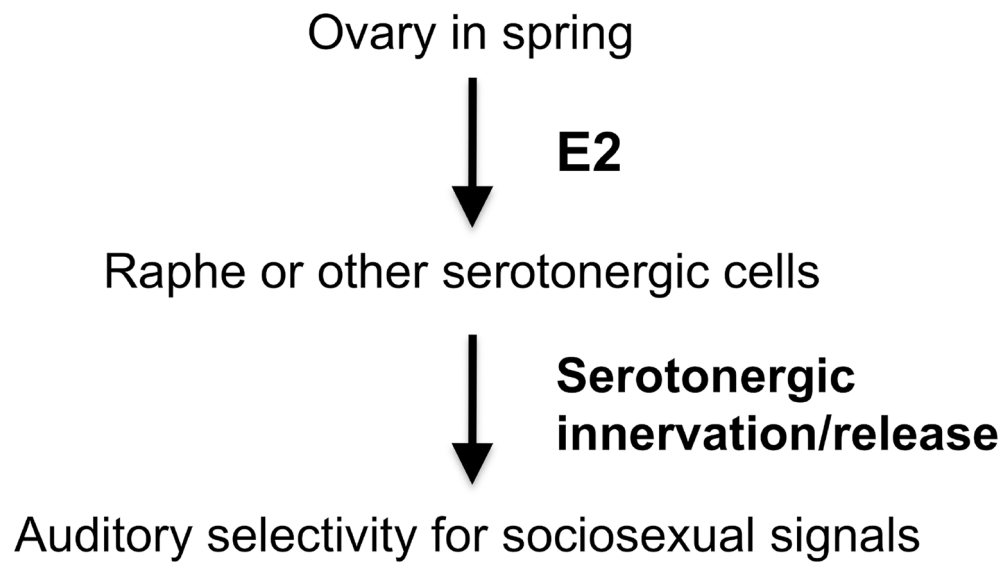
Ovary in spring

↓ **E2**

Raphe or other serotonergic cells

↓ **Serotonergic innervation/release**

Auditory selectivity for sociosexual signals

Fig. 1.
We hypothesize that ovarian steroids associated with breeding act *via* serotonergic systems in the brain to enhance the processing of auditory sociosexual signals. In Experiment 1 of this study, we tested whether manipulating plasma estradiol (E2) affected the density of serotonergic innervation and serotonin release in auditory areas (top arrow).
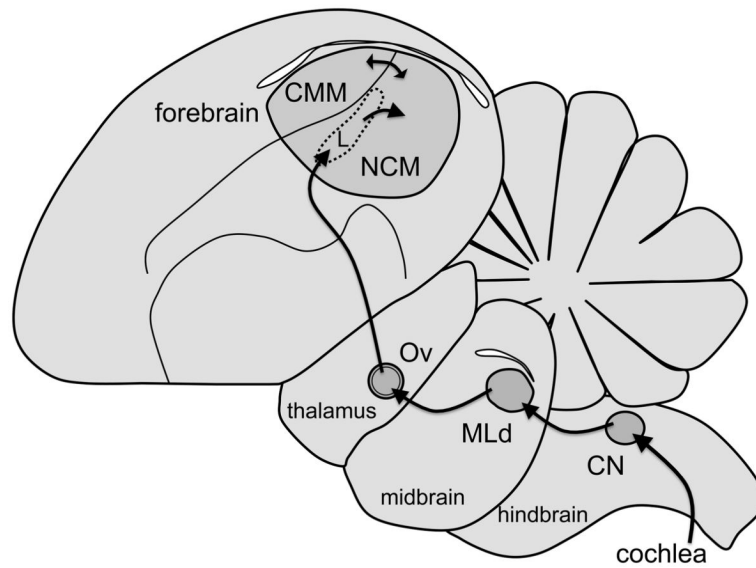
Fig. 2.
Diagram showing a parasagittal view of the auditory pathway in songbirds. The auditory nerve enters the brainstem and arrives at the cochlear nucleus (CN, also called nucleus magnocellularis) which projects to the auditory midbrain (the dorsal portion of the lateral mesencephalic nucleus, or MLd). MLd projects to the core region of the auditory thalamus, also called nucleus ovoidalis (Ov). The core of Ov is relatively devoid of serotonergic fibers but is surrounded by a serotonin-rich shell region. The Ov core projects to the thalamorecipient region of the auditory forebrain, Field L, which then projects to the caudomedial nidopallium (NCM). NCM is reciprocally connected to 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).
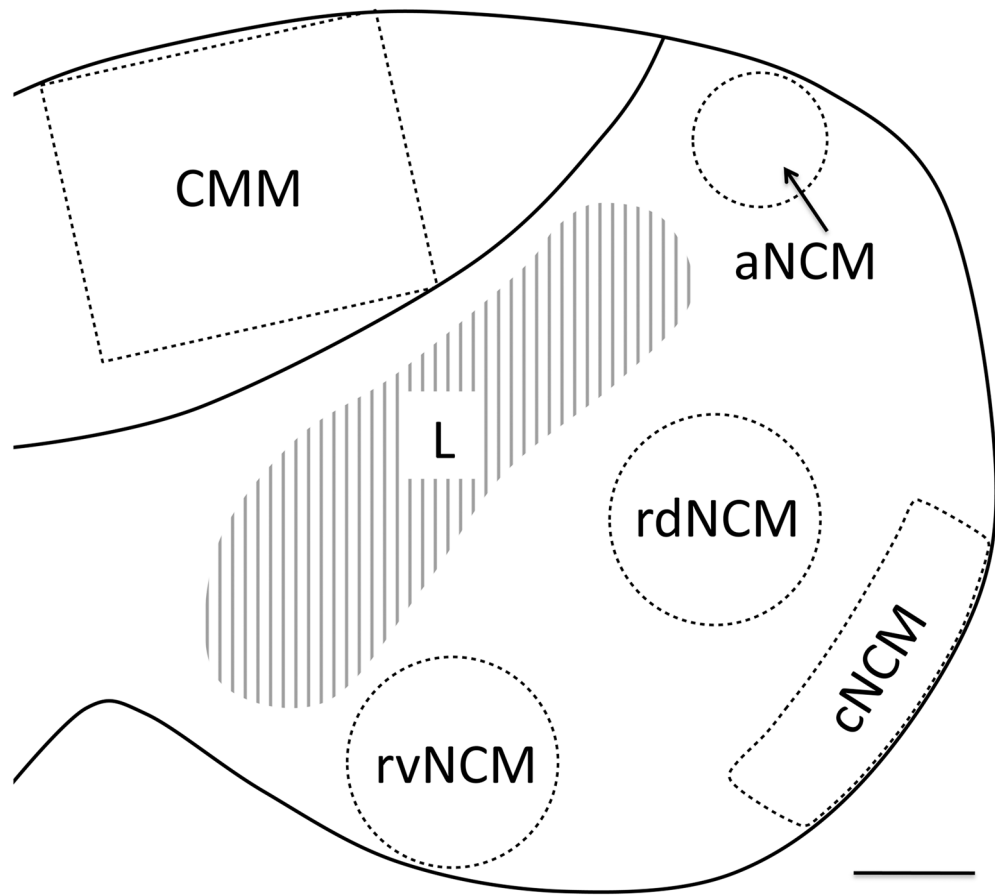
Fig. 3.
Diagram showing a parasagittal view of the auditory forebrain. Dashed lines delineate the areas we sampled when estimating the density of serotonergic innervation. We sampled from within four domains within the caudomedial nidopallium (NCM): apical NCM (aNCM), rostrodorsal NCM (rdNCM), rostroventral NCM (rvNCM), and caudal NCM (cNCM). Rostral is to the left. Scale bar = 300 μm.
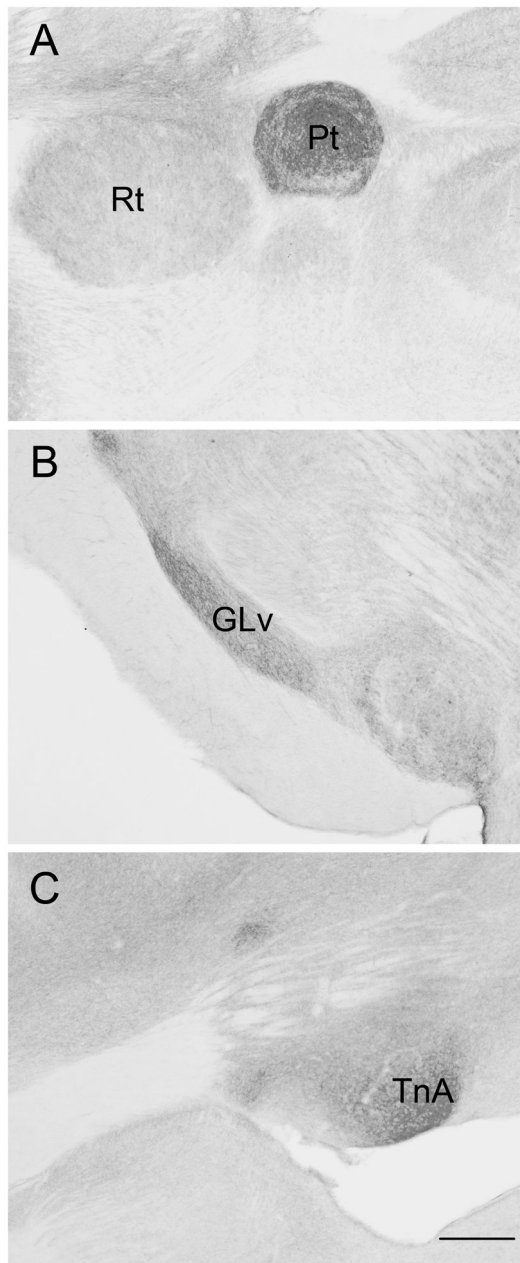
Fig. 4.
Immunoreactivity for serotonin transporter was seen throughout the brain, most notably in the pretectal nucleus (Pt; A), the lateral geniculate nucleus (GLv; B), and the nucleus taeniae of the amygdala (TnA; C). In general, the labeling agreed with the previously reported distribution of serotonin immunoreactivity in birds (see text). Rt, nucleus rotundus. Rostral is to the left. Scale bar = 300 μm.
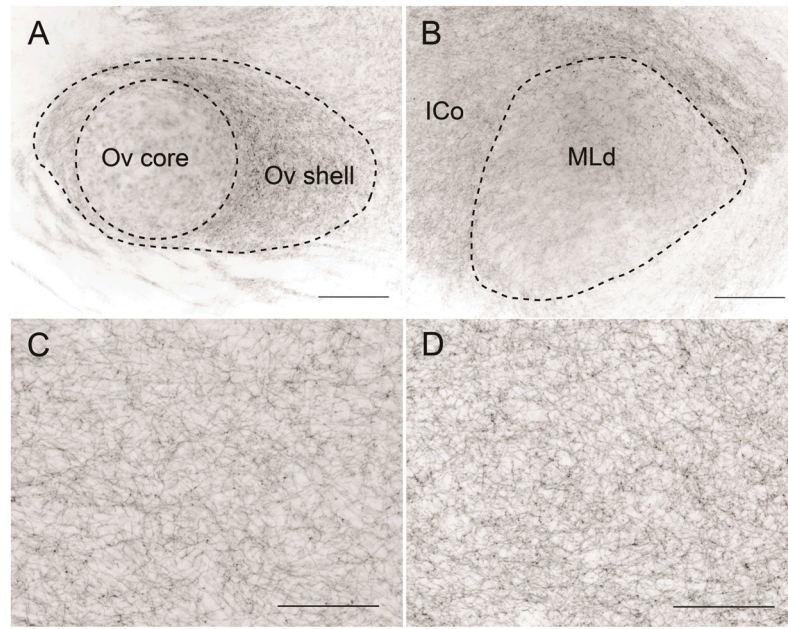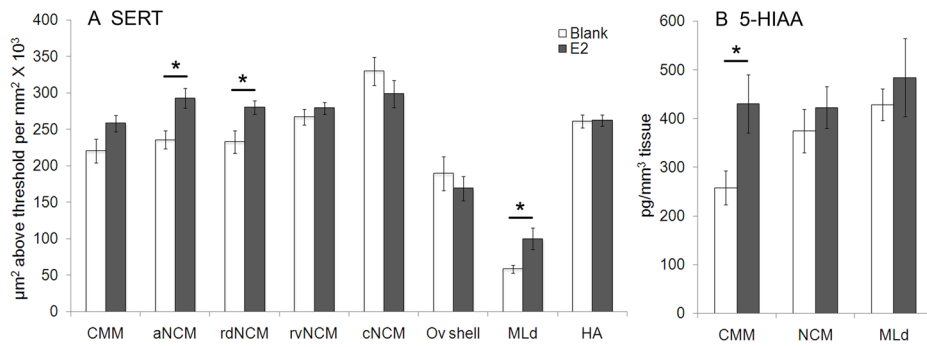
Fig. 5.
Fibers immunoreactive for serotonin transporter in the auditory thalamus (Ov; A), the auditory midbrain (MLd; B), the rostrodorsal caudomedial nidopallium (rdNCM; C), and the apical part of the hyperpallium (HA; D), a visual area, in a female white-throated sparrow. All of the images are from the same female, which had a blank implant (no estradiol). Note that immunolabeling in Ov is limited to the shell region; the core region is relatively devoid of fibers (see also Kaiser & Covey, 1997; Zeng et al., 2007). ICo, nucleus intercollicularis. Rostral is to the left. Scale bar = 150 μm.

Fig. 6.
The effects of estradiol (E2) on the density of fibers immunoreactive for serotonin transporter (SERT; A) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA; B). E2 treatment increased the density of fibers immunoreactive for SERT in two domains of the caudomedial nidopallium (NCM), aNCM and rdNCM, and the auditory midbrain (MLd; A) and increased the concentration of 5-HIAA in the caudomedial mesopallium (CMM; B). aNCM, apical NCM. cNCM, caudal NCM. rdNCM, rostrodorsal NCM. rvNCM, rostroventral NCM. Ov, n. Ovoidalis. HA, apical part of the hyperpallium. * $P < 0.05$ compared to blank (placebo) condition. See text for P values.
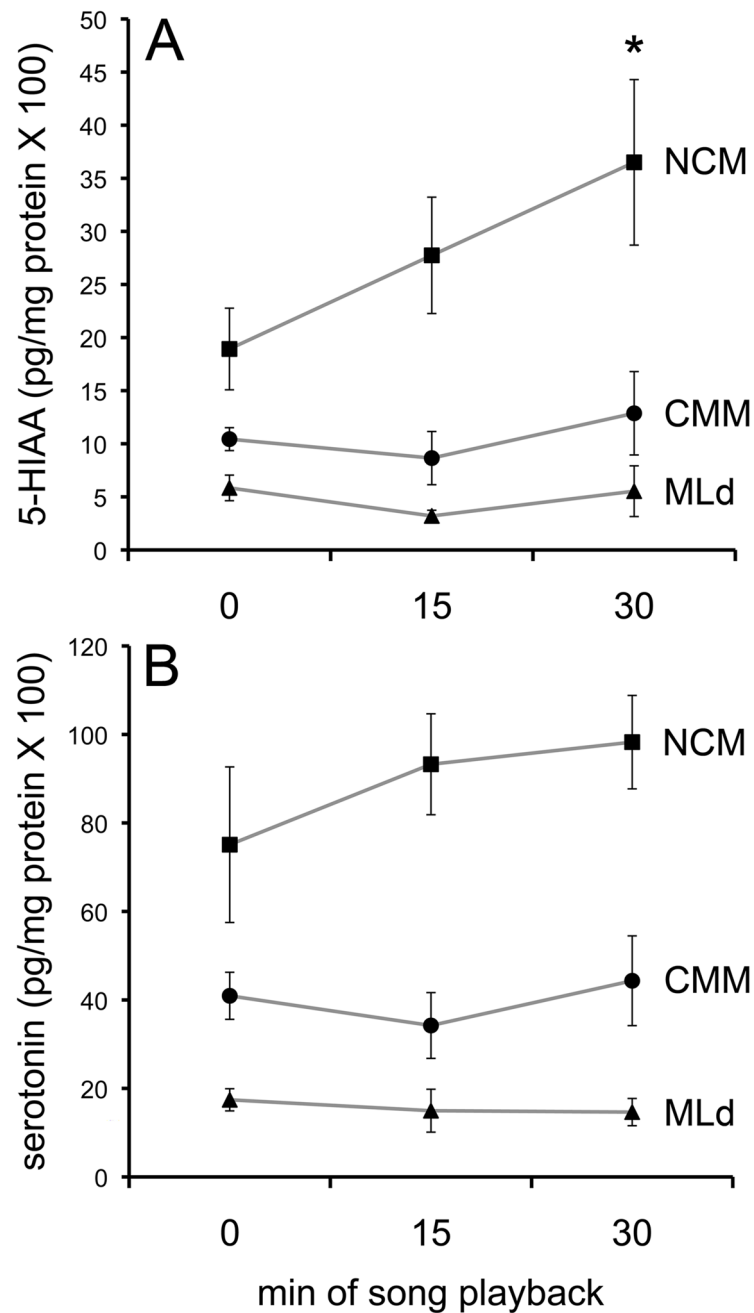
Fig. 7.
5-HIAA (A) and serotonin (B) concentrations in CMM, NCM, and the auditory midbrain, MLd after exposure to 15 or 30 min of conspecific song. Hearing 30 min of song significantly increased levels of 5-HIAA in NCM (A). * significantly different from levels at time 0, P = 0.024. Total protein content was available for all samples only for NCM (see Methods); for consistency in the graphs, plotted values for CMM and MLd have been normalized using the average protein values for those regions.