The Integration of Song Environment by Catecholaminergic Systems Innervating the Auditory Telencephalon of Adult Female European Starlings

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ABSTRACT: **Mate choice is among the most consequential decisions a sexually reproducing organism can make. In many songbird species, females make mate-choice decisions based, in part, on variation between males in songs that reflect their quality. Importantly, females may adjust their choice relative to the prevalence of high quality songs. In European starlings (*Sturnus vulgaris*), females prefer males that primarily sing long songs over those that primarily sing short songs, and sensitivity of the auditory telencephalon to song length depends on the prevalence of long songs in the environment. Several lines of evidence suggest a role for noradrenergic innervation of the auditory telencephalon in mediating this neuro- and behavioral plasticity. To simulate variation in quality of the song environment, we exposed adult female starlings to 1 week of either long or short songs and then quantified several monoamines and their metabolites in the caudomedial mesopallium and caudomedial nidopallium (NCM) using high performance liquid chromatography. We also used immunocytochemistry to assess these areas for immunoreactive dopamine-β-hydroxylase (DBH-ir), the enzyme that synthesizes norepinephrine. We found that long songs elevated levels of the principal norepinephrine metabolite, the principal dopamine metabolite, and the probability of DBH-ir in the NCM compared to short songs. Song environment did not appear to influence norepinephrine or dopamine levels. Thus, the quality of the song environment regulates the local secretion of catecholamines, particularly norepinephrine, in the female auditory telencephalon. This may form a basis for plasticity in forebrain sensitivity and mate-choice behavior based on the prevalence of high-quality males.** © 2008 Wiley Periodicals, Inc. Develop Neurobiol 68: 656–668, 2008

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INTRODUCTION

Choice of prospective mates is among the most consequential decisions a sexually reproducing organism can make because it affects the genetic constitution of the offspring and, in some species, the quantity or quality of parental care and other resources (Andersson, 1994). Importantly, individuals may be flexible in their mate-choice decisions, adjusting them according to context, condition, and environment in taxa ranging from arthropods to vertebrates (Qvarnström, 2001; van Gossum et al., 2001; Badyaev and Qvarnström, 2002; Hebets, 2003; Lynch et al., 2005; Bleay and Sinervo, 2007). Research on the physiological underpinnings of mate choice has lagged substantially behind that on its ultimate, adaptive components, but recent studies on insects, anurans, and songbirds now provide a foundation for understanding some of the neural mechanisms not only for mate-choice in general but specifically for individual flexibility in mate choice (Ryan et al., 1990;

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Phelps et al., 2001; Sockman et al., 2002, 2005a; Maney et al., 2003; Hoke et al., 2004; Fertschai et al., 2007; Sockman, 2007).

Songbirds have long provided a model system for the study of mate choice, owing largely to the study of song, a learned vocal behavior produced by males that attracts mates (McGregor, 1991; Sockman et al., 2005b). Within many species, variation in male song reflects some aspects of male quality (Duffy and Ball, 2002; Nowicki and Searcy, 2004, 2005) and therefore may provide an honest signal of traits that are relevant to a female making mate-choice decisions (Gil and Gahr, 2002). Thus, females should attend to this variation to maximize fitness, and, how the brain might program this task is one focus of this study.

The caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) of the songbird auditory forebrain contribute to the high-order processing of complex song cues (Chew et al., 1995; Stripling et al., 1997; Ribeiro et al., 1998; Bolhuis et al., 2000; Clayton, 2000; Gentner et al., 2001; Gentner and Margoliash, 2003; Theunissen and Shaevitz, 2006). This important role of the CMM and NCM was discovered by virtue of their robust expression of the immediate-early gene and neural activity-marker ZENK (the avian homologue of and an acronym for zif-268, egr-1, NGFI-A, and Krox-24) in response to conspecific song relative to heterospecific song (Mello et al., 1992). Subsequent studies revealed that ZENK expression in the CMM and NCM depends on song variation that is relevant to the species-specific basis for female choice. For example, in zebra finches (*Taeniopygia guttata*), song similar to the father's is preferred in a mate-choice context by females and elicits greater ZENK expression in the female auditory forebrain than does novel song (Terpstra et al., 2006). In canaries (*Serinus canaria*), song containing the so-called sexy syllable is preferred by females and elicits greater ZENK expression in the female auditory forebrain than does song without the syllable (Leitner et al., 2005). The same occurs in the white-crowned sparrow (*Zonotrichia leucophrys*), for which the song variable relevant to mate choice is its dialect (Maney et al., 2003); in the non-songbird budgerigar (*Melopsittacus undulatus*), for which the song variable relevant to mate choice is its syllable complexity (Eda-Fujiwara et al., 2003); and in the European starling (*Sturnus vulgaris*), for which the song variable relevant to mate choice is its length (Gentner et al., 2001; Sockman et al., 2002, 2005a). In short, the expression of ZENK in the auditory forebrain is sensitive not simply to whether or not the acoustic stimulus is of conspecific origin but particularly to the quality of the conspecific song stimulus, whereby quality depends on the species and is tied to the suitability of the singer as a prospective mate (Ball et al., 2006; Sockman, 2007).

In some systems, constraints imposed by the environment can contribute toward maintaining the honesty of an animal communication signal, such as one used in courtship and mate choice. In such cases, as the environment fluctuates, the prevalence or distribution of high-quality signals can too (K. W. Sockman, unpublished results), leading to situations in which females may face a dearth of such signals (Sockman, 2007). In these instances, females should adjust their mate-choice criteria according to the prevalence of high quality traits, as investigators have discovered for the white-crowned sparrow (MacDougall-Shackleton et al., 2001), the canary (Nagle and Kreutzer, 1997), and the brown-headed cowbird (*Molothrus ater*) (Freeberg et al., 1999).

One mechanism by which the prevalence of high quality traits might modulate choosiness is by modulating neural sensitivity to stimulus quality; if part of the brain is not sensitive to variation in stimulus quality, that part cannot tune behavior accordingly. Consistent with this notion, forebrain ZENK sensitivity to song quality (i.e., the difference between ZENK in response to novel long song-stimuli and ZENK in response to novel short song-stimuli) is greater in female European starlings exposed to an environment of high quality (preferred, long) songs than in those exposed to an environment of low quality (short) songs (Sockman et al., 2002, 2005a). How, exactly, the song environment induces this plasticity in forebrain sensitivity is not known, but the most likely explanation is that song environment does not modulate ZENK levels directly but instead modulates the sensitivity of forebrain ZENK systems to the quality of new, novel songs (Sockman et al., 2002, 2005a). Still, it is possible that, in addition to this indirect effect on ZENK expression, song environment also affects baseline, non-song-induced ZENK expression in the auditory telencephalon. Therefore, one purpose of the present study was to test this explicitly. The primary purpose of the present study was to examine how the female auditory forebrain integrates the quality of the song environment and thus induces this form of neuroplasticity that might contribute to the regulation of context-dependent flexibility in mate choice.

Neuromodulators such as the catecholamines are strong candidates for the context-dependent modulation of forebrain plasticity in immediate-early gene expression and other forms of neuronal activation (Cirelli et al., 1996; Dave et al., 1998; Bao et al., 2001; Cardin and Schmidt, 2004; Cirelli and Tononi, 2004; Castelino and Ball, 2005). The songbird catecholaminergic noradrenergic system sends projections

from the locus coeruleus to the auditory forebrain (Mello et al., 1998; Ribeiro and Mello, 2000; Appeltants et al., 2001, 2004). Chemical lesion of these projections reduces female choosiness for preferred song in canaries (Appeltants et al., 2002) and reduces affiliation with a location broadcasting song in female starlings (Riters and Pawlisch, 2007). Moreover, in the zebra finch auditory forebrain, noradrenergic fibers come into close contact with song-responsive neurons, and pharmacological antagonism of α-adrenergic receptors blocks song-induced ZENK expression (Ribeiro and Mello, 2000). These findings raise the hypothesis that noradrenergic activity in the auditory forebrain mediates the effects of song environment on female forebrain sensitivity to song quality (Sockman, 2007).

We examined how the prevailing song environment affects noradrenergic activity in the auditory forebrain of adult female starlings by quantifying norepinephrine and its major metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) as a proxy for norepinephrine secretion, following a 1-week exposure to one of two song environments, long song (high quality) and short song (low quality). We also examined the effects of song environment on immunoreactivity of the enzyme that synthesizes norepinephrine from dopamine, dopamine-β-hydroxylase (DBH-ir) (Cooper et al., 2003) and on baseline (as opposed to song-induced) ZENK expression.

METHODS

The University of North Carolina at Chapel Hill Institute for Animal Care and Use Committee (protocol 04-241.2) approved all procedures used in this study. Adult European starlings were captured on December 12, 2005, in a food-baited funnel trap on a farm in northwest Pennsylvania, USA (41.75°N 80.35°W). At this time of year, individuals can be sexed with ~80–90% reliability using the coloration of the proximal region of the bill, which tends to be pink in females and blue in males (Kessel, 1951). We housed 36 individuals with pink bills in large cages initially on an 8 h light and 16 h dark (8L 16D) photoperiod, because this was the approximate photoperiod to which they were accustomed at the time of their capture and because it does not drive the birds into a nonreproductive physiological state, as a photoperiod with a longer photophase (e.g., 12L 12D) would (Nicholls et al., 1988). For the entire study, we provided them with food and water *ad libitum*.

Experiment Procedure

Our protocol was similar to that used for the experience treatment in the previous study showing that song environment affects forebrain ZENK sensitivity to song length in female starlings (Sockman et al., 2002, 2005a). In both the previous and this present study, birds were exposed to an approximately week-long (6.5 days in this study compared to 7 in the previous) environment of either long or short songs. In the previous study, this exposure was followed by exposure to a 30-min stimulus of either novel long or novel short songs. In the present study, we did not conduct this 30-min stimulus treatment and thus any ZENK-ir in the present study will reflect baseline levels of ZENK expression, not song-induced levels, which require a novel song or novel song context (Kruse et al., 2004) (see Discussion). Other differences between this and the previous study were the use of a third, no-song treatment (to be excluded from analyses; see below), and the housing in sound chambers of individuals in this study as opposed to female-female pairs in the previous study.

Beginning ~8 h into the birds' photophase on February 5, 2006, we transferred 12 individuals each to one of 12 sound chambers located together in a small room on an 11L 13D photoperiod. This spring-like photoperiod helps stimulate the development of a reproductive condition, but, unlike photoperiods with longer photophases (e.g., 12L 12D), it does so without eventually driving the bird into the nonreproductive state known as photorefractoriness (Nicholls et al., 1988). Each chamber was lined with sound foam and equipped with a cage with two perches, an exhaust fan (and separate air intake) for ventilation, a fluorescent light that maintained an 11L 13D photoperiod within the chamber, and a speaker (Pioneer Corp. TS-G1040R). Beginning at the onset of the photophase the next day, we broadcast through each chamber's speaker one of three acoustic treatments—long song, short song (see *Song Recordings* below), or nothing. We spatially interspersed each of one treatment's replicate chambers with those of the others. We broadcast song for 5.5 h/d at partially randomized 30-min intervals during the photophase only. Broadcasts began at the onset of the photophase each day. No more than two 30-min broadcasts occurred in a row (i.e., without at least one intervening, 30-min silent period), and no broadcast occurred during the last 30 min of the photophase. Thus, we broadcast each song treatment throughout the photophase each day in an unpredictable pattern that, along with the use of an 11L 13D photoperiod, we intended to resemble the environment of free-living females making mate-choice decisions early in the spring. Because chambers attenuated but did not eliminate exposure to outside sounds, we broadcast white-noise in the room to mask interchamber sound, although this masking was, at best, only partially effective. This problem was minimal for individuals exposed to the song treatments, because the songs of one treatment were always broadcast simultaneously with and therefore masked the songs of the other. However, individuals in the no song treatment did not have this additional masking and, to some extent, were exposed to both long and short songs broadcast in other chambers. As a consequence, we excluded the no song treatment from further consideration in this study.

At 4 h into the photophase on the 7th day of broadcast, we ceased broadcasting and began dissecting individuals'

brains after rapid decapitation, starting with the individual in Chamber 1 and progressing every 15 min through that in Chamber 12. One hemisphere we rapidly froze on pulverized dry ice (alternating left and right with each bird), and the other we agitated in a solution of 5% acrolein (Polysciences, Warrington, PA, Cat. No. 107-02-8) and 95% phosphate buffered saline (pH 7.5; PBS) for 5 h. We then rinsed fixed hemispheres three times in PBS (one quick rinse and two 30-min rinses), saturated them in 30% sucrose (in PBS) for cryoprotection, rapidly froze them on pulverized dry ice, and stored them frozen until sectioning in the sagittal plane at 40 μm on a cryostat. We determined the sex of each individual by inspecting the gonads.

Once we had removed all individuals and cleaned the chambers, we reassigned each chamber's song environment, assigned one new individual to each of the chambers, and repeated the procedures described above. We concluded with a third session of birds the following week, making a total of 36 individuals, divided among 12 replicates for each of three song environments that we counterbalanced over three sessions among the 12 chambers. Of the 24 individuals in the short- or long-song treatments, we excluded three from analyses because they were males.

Song Recordings

We used the same recordings of long and short song that were used in the experience treatment of the previous study showing that song environment affects forebrain ZENK sensitivity to song length in female starlings (Sockman et al., 2002, 2005a). Briefly, a large library of complete songs was recorded from a laboratory-housed male directing song at a female. From these songs, 12 exemplars were selected, which, based on length, were divided into two sets of six: a long-song set and a short-song set, with mean song lengths of 55.2 and 26.0 s, respectively. Song sets were repeated in each file and enough silence inserted between songs to ensure that neither total song nor total silence duration differed between the long- and short-song sets. Because we did not replicate each song environment with recordings from multiple males, we cannot extend our conclusions to long and short songs in general (Kroodsma et al., 2001; Wiley, 2003). However, because a previous study showed that females in a mate-choice context preferred this exact long-song set to this exact short-song set (Gentner and Hulse, 2000), results are based on responses to stimuli known to differ in their attractiveness to females.

We broadcast the set of short songs simultaneously with the set of long songs, and we randomized the order of songs within each set each time it was played. To do this, we used the software Garage Band (version 2.0.2, Apple, Cupertino, CA) to pan each long-song file entirely to the left channel and each short-song file entirely to the right channel, the shuffle and play list features of iTunes (Apple) to randomize song order in each song environment, and a feature of the Macintosh 10.3 operating system (Apple) that allows the simultaneous playing of multiple iTunes play lists. Because we panned the songs for one song environment left and the songs for the other song environment right, we could assign a song environment to a chamber based on the channel to which we connected the chamber's speaker. We powered speakers by a daisy chain of eight monoblock amplifiers to which we patched an audio-computer interface. These files played continuously; however, we regulated the birds' exposure to the sounds by controlling power to the amplifiers with a digital timer programmed at the predetermined, partially randomized on and off points.

Quantification of Monoamines, Metabolites, and Protein

We determined the concentration of some monoamines and their metabolites from the brain regions described below by reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection (Kilts et al., 1981). The chromatographic system consisted of a SM-909 isocratic HPLC pump (ANSPEC), Basic+ Marathon type 816 Autosampler (Spark, Holland), Model 400 potentiostat (EG&G Princeton Applied Research), and TurboChrom software Version 4.1 (Perkin Elmer) running on a PC. We separated compounds using a Monochrom C18 3 μm column (100 mm $\times$ 4.6 mm, MetaChem) 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 1 L with double-distilled, deionized water, with pH lowered to 3.9 with hydrochloric acid. We filtered the mobile phase through a 20 μm filter (Kontes Scientific Glassware and Instruments) before use, and flow rate was 0.8 mL/min. We maintained 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 (isoproterinol, Sigma) and variable amounts of each of the seven external standards: dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); norepinephrine and its metabolite, 3-methoxy-4-hydroxy-phenylglycol (MHPG); epinephrine; and the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Sigma). We included a five-point standard curve in each assay (5 $\times$ 50 μL injections), and used linear regression to fit a line through the standard curve points ($R^2 > 0.99$ for each of the seven components in each assay).

We sectioned the frozen, nonfixed hemispheres at 10°C in the sagittal plane at 300 μm on a cryostat, thaw mounted them onto glass microscope slides, and rapidly refroze them on pulverized dry ice. From each of three consecutive sections starting at the midline and using a chilled 1-mm (i.d.) custom-made, thin-walled stainless steel spring-loaded punch, we micropunched a region of the CMM, a caudodorsal region of the NCM (NCMd), and a ventral region of the NCM (NCMv), each for which the anatomical boundaries have been described (Sockman et al., 2002) (see Fig. 1). We expelled punches into 1.9-mL polypropylene microcentrifuge tubes, froze them on dry ice, and stored them at -80°C until assay. Immediately before assay, we added

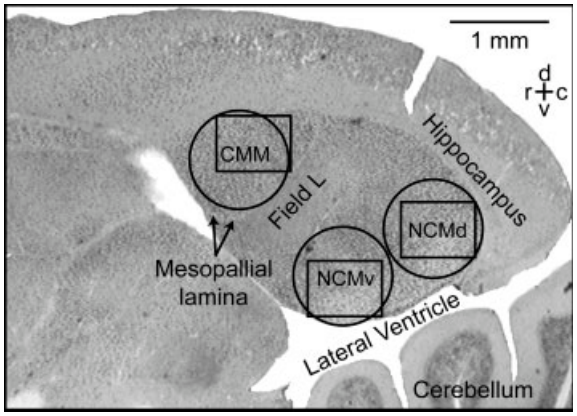

Figure 1 Photomicrograph of the female European starling auditory telencephalon. Rectangles (for ZENK quantification) and circles (for monoamine and metabolite quantification) indicate the three sampling locations. CMM, caudomedial mesopallium; NCMd, caudo-dorsal part of the caudomedial nidopallium; NCMv, ventral part of the caudomedial nidopallium; r, rostral; c, caudal; d, dorsal; v, ventral.

225 μL of mobile phase containing 30 ng isoproterinol to each 1.9-mL tube containing the microdissected nuclei. We sonicated the samples and then centrifuged them at 16,000*g* for 15 min at 4°C. We aspirated the supernatant and injected 50 μL into the HPLC system. We calculated monoamine and metabolite concentrations by comparing the peak heights of the seven compounds within each sample to those obtained from the standard curves and correcting the peak heights for percent recovery of the internal standard (i.e., the height of the isoproterinol peak) in each sample.

We measured 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 (Bio-Rad) as a standard on a μQuant microplate spectrophotometer (BioTek).

Immunocytochemistry

We performed immunocytochemistry for the protein ZENK and for DBH as previously described by Sockman et al. (2002) except, for our DBH primary antibody, we used rabbit antibovine-DBH (Immunostar, Hudson, WI, Cat. No. 22806) diluted 1:16,000. Briefly, this immunocytochemistry procedure involved initially treating the acrolein-fixed tissue with a sodium borohydride solution, blocking endogenous peroxides with a hydrogen peroxide solution, and suppressing endogenous avidin and biotin binding activity with a blocking kit, before incubating for 40–48 h at 4°C in the primary antibody. We followed this with an incubation with biotinylated goat anti-rabbit secondary antibody to which an avidin-biotin horseradish peroxidase complex bound and which we colored with a nickel-enhanced diaminobenzidine tetrahydrachloride solution. We washed tissue in solutions of PBS or PBS with Triton-X-100 detergent (Thermo Fisher Scientific, Waltham, MA, Cat. No. BP151-500) between steps. For DBH, we processed all tissue in a single immunocytochemistry batch (eliminating the possibility for between-batch variation) and in 12 mesh-bottom 24-well plates (the three brains from each chamber per plate), which bathe the contents of each well in the common solution of a single plate. We did the same for ZENK in an alternate set of tissue, except we processed the tissue in three immunocytochemistry batches, counterbalancing the song-environment treatment between them.

We have previously validated the specificity of the ZENK antibody (Sockman et al., 2002) but not the DBH antibody. Therefore, we performed immunocytochemistry on another series of tissue collected from one starling, incubating some tissue in buffer with no primary antibody, some with the primary antibody exactly as we did for the experimental tissue, and some with antibody that we had first preabsorbed with the protein (10 μg/mL of 1:16,000 primary antibody solution) against which the antibody was raised (Immunostar, Hudson, WI). We observed specific staining in the tissue incubated with antibody only but not in the no-antibody or the antibody-with-protein incubations.

We quantified ZENK-ir in every third-cut section of tissue from the midline to $\sim$ 1080 μm lateral. Using Köhler Illumination on a Leica DM4000 Digital Research Microscope with Leica DFC480 color digital camera connected by firewire to an Apple Macintosh G5 dual-processor computer, we collected digital brightfield microscope images at 200× magnification of the CMM, NCMd, and NCMv (see Fig. 1). Using ImageJ (version 1.34s, National Institutes of Health), we determined the proportion of pixels in 8-bit gray-scale images that were above a single threshold assigned to all specimens of a single well-plate.

We assessed DBH-ir in the CMM, NCMd, and NCMv of every third brain section from the midline to $\sim$1080 μm laterally over a range of magnifications, from 50–400×. Several individuals had no robust DBH-ir in any section (see Results). Because precise quantification of DBH-ir would have resulted in a high enough proportion of 0 values (no DBH-ir) to violate the assumptions of any statistical analysis requiring a continuous response, we treated DBH-ir in the auditory forebrain as a dichotomous response and, blind to experiment treatment, scored each individual as either having or not having robust DBH-ir in at least one CMM, NCMd, or NCMv section (see Fig. 2).

Statistical Analyses

Because we removed males from the analysis and were missing some values (see Results), our dataset was unbalanced. Furthermore, it consisted of a hierarchical combination of fixed and random effects (song environment as a fixed effect randomly assigned to females nested within chamber as a random effect), which may differ from one another in their correlation structure. Therefore, we analyzed these data in a mixed, multilevel modeling frame-

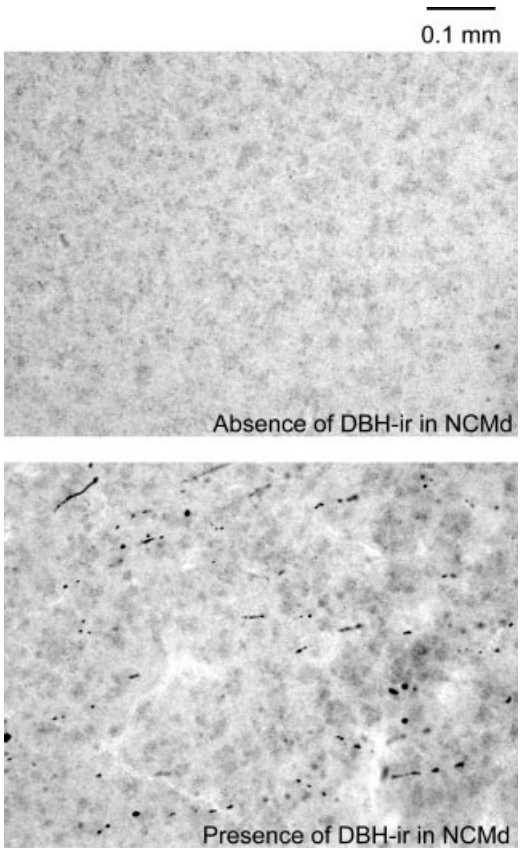

Figure 2 Photomicrographs of the caudo-dorsal part of the caudomedial nidopallium (NCMd) (see Fig. 1) of the female European starling, depicting interpretation of absence and presence of immunoreactivity for dopamine-β-hydroxylase (DBH-ir), the enzyme that synthesizes norepinephrine from dopamine.

work (Stata/IC 10.0 for the Macintosh, Stata Corporation, College Station, TX), which readily accommodates unbalanced, hierarchically structured combinations of fixed and random effects (Burton et al., 1998; Goldstein et al., 2002; Rabe-Hesketh and Skrondal, 2005). For a detailed description of the rationale for and approach to mixed, multilevel modeling frameworks, see Sockman et al. (2008). For the dichotomous response in our DBH-ir analysis, we used a logit link transformation $[\ln(p/(1-p))]$, where p is the probability of an outcome (in this case, presence of DBH-ir), to allow the probability to be bounded between 0 and 1 and to depend linearly on the predictor song environment (Krackow and Tkadlec, 2001). We modeled responses in the CMM, NCMd, and NCMv separately. In an effort to model responses as simply as possible, initial constructions nested individual female within chamber number as a random intercept. Because chamber number correlates perfectly with the additional nuisance variables sacrifice time and immunocytochemistry-tray number, we were able to simultaneously control for all of these nuisance variables with the inclusion of only a single parameter (chamber) in models. For some responses in some brain areas, these initial constructions failed to converge on a solution. In these instances, we tried modeling chamber as a random intercept and as a random coefficient on song environment or only as a random coefficient on song environment. One of these three approaches always yielded a solution. We then estimated the statistical reliability of the parameter coefficient from a Wald statistic with a chi-squared distribution. We provide further details of model formulation in "Results." Additionally, to best illustrate the effect of interest (song environment), figures depict standardized residuals of models that have removed the effects of variables that are not of interest (protein content and chamber).

RESULTS

ZENK Immunoreactivity in the Auditory Telencephalon

We noticed no pattern of change in ZENK-ir with respect to the lateral position of the section and therefore used the mean of an individual's nine sections for analyses. To determine whether the song environment affected baseline (non-song-induced) ZENK-ir in the auditory telencephalon, we nested the individual's mean value for one area within chamber as a random intercept (21 females in 11 chambers) and included song environment as a predictor. We observed no reliable effect of song environment on ZENK-ir in any of the three telencephalon areas (each area: $p > 0.2$) (see Fig. 3).

Monoamines and Metabolite Concentrations in the Auditory Telencephalon

The primary focus of this study was the investigation of how song environment affects noradrenergic activity in the auditory telencephalon of females (Table 1). Therefore, we begin this section with analyses of how song environment affected the levels of norepinephrine and its major metabolite, MHPG, using protein content as a covariate to control for differences in the size of tissue punch. For the CMM (19 females in 11 chambers) and NCMd (21 females in 11 chambers), we observed no effect of song environment on either MHPG or on norepinephrine levels (each response: $p > 0.2$) (see Fig. 4). However, for the NCMv (21 females in 11 chambers), females exposed to the long-song environment had higher levels of MHPG than those exposed to the short-song environment ($p = 0.007$) (see Fig. 4). Song environment did not appear to affect norepinephrine concentrations in the NCMv (21 females in 11 chambers; $p > 0.2$) (see Fig. 4).

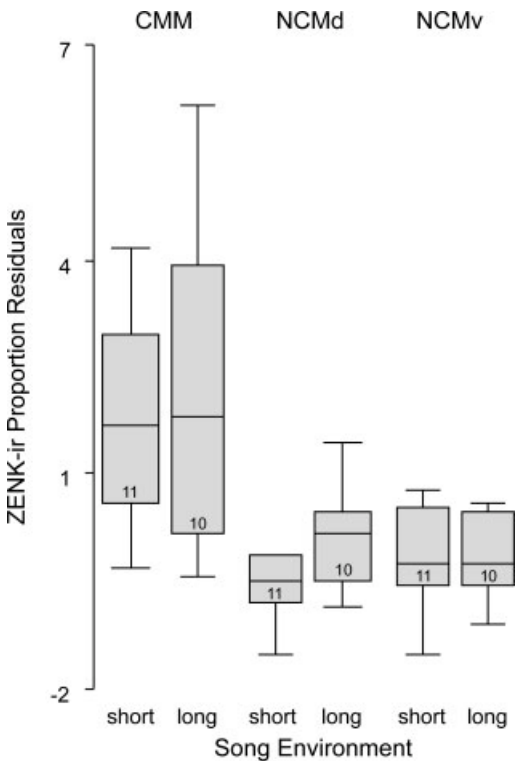

Figure 3 Baseline expression of the immediate early gene ZENK in the auditory telencephalon of female European starlings exposed to a 1-week acoustic environment of either short male songs or long male songs. ZENK levels are the standardized residual immunoreactive proportions of images from a mixed model including the random effect of sound chamber. Individual box plots show the middle half of the data (rectangle), the median (horizontal line), the upper and lower adjacent values (whiskers), and the number of females in the sample. Outside values are not plotted. CMM, caudomedial mesopallium; NCMd, caudo-dorsal part of the caudomedial nidopallium; NCMv, ventral part of the caudomedial nidopallium. Only *p*-values less than 0.2 are indicated (there were none for these ZENK-ir results).

We also measured dopamine, the dopamine metabolites, DOPAC and HVA, epinephrine, and the serotonin metabolite 5-HIAA (Table 1). For DOPAC, we found no effect of song environment in either the CMM (19 individuals in 11 chambers; $p > 0.2$) or NCMv (21 individuals in 11 chambers; $p > 0.2$), but, in the NCMd (18 individuals in 10 chambers), the long song environment induced greater DOPAC concentrations than did the short song environment ($p = 0.04$) (see Fig. 5). Any effect of song environment on HVA, dopamine, 5-HIAA, and epinephrine levels in the CMM, NCMd, and NCMV was, at best, only marginally reliable (each test: $p \geq 0.08$). Details of these results are shown in Figures 5 and 6.

Dopamine-β-Hydroxylase Immunoreactivity in the Auditory Telencephalon

None of the individuals had clear DBH-ir in any CMM section at any magnification; 13 had robust DBH-ir in at least one section of the NCMd; and 7 had robust DBH-ir in at least one section of the NCMv. Therefore, we restricted our analysis of the auditory forebrain to the NCMd and NCMv.

Song environment affected the probability of DBH-ir in the NCMd (21 females in 11 chambers; $p = 0.01$). Females exposed to 1 week of long songs were more likely to show DBH-ir in the NCMd than were females exposed to 1 week of short songs (see Fig. 7). In the NCMv, the effect of song environment on probability of DBH-ir was not particularly reliable ($p = 0.15$).

DISCUSSION

The quality of the prevailing song environment affected noradrenergic activity in the auditory telencephalon of female European starlings. This was reflected in the elevation of both MHPG levels and the probability of DBH-ir in the auditory telencephalon of female European starlings after a 1-week exposure to long songs relative to short songs. These findings support the hypothesis that the noradrenergic system plays a central role in integrating information about the quality of the prevailing song environment, which, in some species, can influence mate-choice decisions (Sockman, 2007). Modulation of norepinephrine secretion, which we presume is reflected in the levels of MHPG itself, might then drive the modulation of the enzyme DBH, which synthesizes norepinephrine from dopamine. Unexpectedly, we found that the quality of the song environment also influenced levels of the dopamine metabolite DOPAC in the auditory telencephalon, revealing a previously unrecognized possibility for dopaminergic integration of this important social factor. We noticed no effect of song environment on baseline (non-song-induced) levels of ZENK expression in any of the three areas we analyzed in the auditory telencephalon.

It is not surprising that we found no effect of song environment on ZENK-ir in any of our three focal areas of the auditory telencephalon (see Fig. 3), given that we repeatedly exposed individuals to a single set of song stimuli for 5.5 h/day over an $\sim$1-week duration. The songbird auditory telencephalon is well known for its genomic and electrophysiological habituation to familiar stimuli (Chew et al., 1995, 1996; Mello et al., 1995; Stripling et al., 1997), an

Table 1 Concentrations [pg/mg Protein ± 95% C.I. (*n*)] of Monoamines and Metabolites in Three Areas of the Auditory Telencephalon of Female European Starlings After One-Week Exposure to Short or Long Male Songs

	Song Environment	
Compound	Short	Long
CMM		
MHPG	1597.54 ± 436.00 (9)	1644.30 ± 485.69 (10)
Norepinephrine	3600.38 ± 823.75 (9)	3611.28 ± 767.73 (10)
DOPAC	320.77 ± 133.46 (9)	469.44 ± 254.17 (10)
HVA	1430.17 ± 504.94 (9)	2324.46 ± 1754.26 (10)
Dopamine	1683.11 ± 311.42 (9)	3446.51 ± 3720.37 (10)
5-HIAA	3289.69 ± 759.34 (9)	4281.13 ± 1624.68 (10)
Epinephrine	324.00 ± 68.13 (9)	385.97 ± 222.76 (10)
NCMd		
MHPG	727.38 ± 168.13 (11)	684.01 ± 236.34 (10)
Norepinephrine	2807.35 ± 455.65 (11)	3115.67 ± 706.44 (10)
DOPAC	378.11 ± 140.52 (10)	548.25 ± 234.33 (8)
HVA	750.22 ± 412.34 (10)	711.10 ± 162.03 (10)
Dopamine	1882.83 ± 531.13 (11)	2346.46 ± 540.67 (10)
5-HIAA	1800.51 ± 502.85 (11)	2099.88 ± 700.55 (10)
Epinephrine	320.29 ± 89.21 (11)	391.11 ± 191.18 (10)
NCMv		
MHPG	841.08 ± 212.87 (11)	1060.08 ± 279.00 (10)
Norepinephrine	3931.49 ± 883.11 (11)	4062.86 ± 1233.65 (10)
DOPAC	435.11 ± 308.69 (11)	413.68 ± 326.42 (10)
HVA	1305.01 ± 493.38 (11)	1160.11 ± 604.56 (10)
Dopamine	2938.59 ± 1408.03 (11)	2362.59 ± 877.80 (10)
5-HIAA	3059.03 ± 1031.09 (11)	3087.54 ± 827.49 (10)
Epinephrine	249.05 ± 80.74 (11)	285.30 ± 94.61 (10)

CMM, caudomedial mesopallium; NCMd, caudo-dorsal part of the caudomedial nidopallium; NCMv, ventral part of the caudomedial nidopallium; MHPG, 3-methoxy-4-hydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindole acetic acid.

effect possibly mediated by activation of CB1 cannabinoid receptors in the caudal telencephalon (Whitney et al., 2003). As long as song stimuli and the context in which they are presented (Kruse et al., 2004) do not change, ZENK expression in the CMM and NCM show rapid decrements in their response to familiar song, and these decrements can last 24 h or more (Mello et al., 1995).

Despite this well-established property of the songbird auditory telencephalon, the environmentally induced plasticity in forebrain ZENK sensitivity to song quality shown previously (Sockman et al., 2002, 2005a) is unlikely to be a result of habituation alone (Sockman, 2007). This is because when stimulus songs were most likely to have familiar elements, instead of leading to the lowest ZENK responses, they either led to the highest or to an intermediate response level. The mechanistic basis for this form of neuroplasticity is not known. Although these previous studies demonstrate that the week-long song environment alone has no discernable effect on song-induced ZENK (but instead interacts with the novel song stimulus to influence ZENK expression), it remained possible that song environment influences baseline (non-song-induced) levels of ZENK expression. However, the results from the present study indicate that this is not the case (see Fig. 3) and that forebrain sensitivity to the quality of novel songs, as measured by differential expression of ZENK, must be modulated by the quality of the song environment through other means, such as the innervation of forebrain areas by catecholaminergic systems sensitive to song environment.

As mentioned previously, the noradrenergic system does indeed provide rich innervation of the songbird auditory forebrain (Mello et al., 1998; Ribeiro and Mello, 2000; Appeltants et al., 2001, 2004) and influences song-induced ZENK expression (Ribeiro and Mello, 2000) and mate-choice behavior (Appeltants et al., 2002). We therefore hypothesized that the quality of the prevailing song environment might modulate female choice through its influence on norepinephrine secretion in the auditory telencephalon. Although this study did not test the effects of norepinephrine secretion on female choice, we did

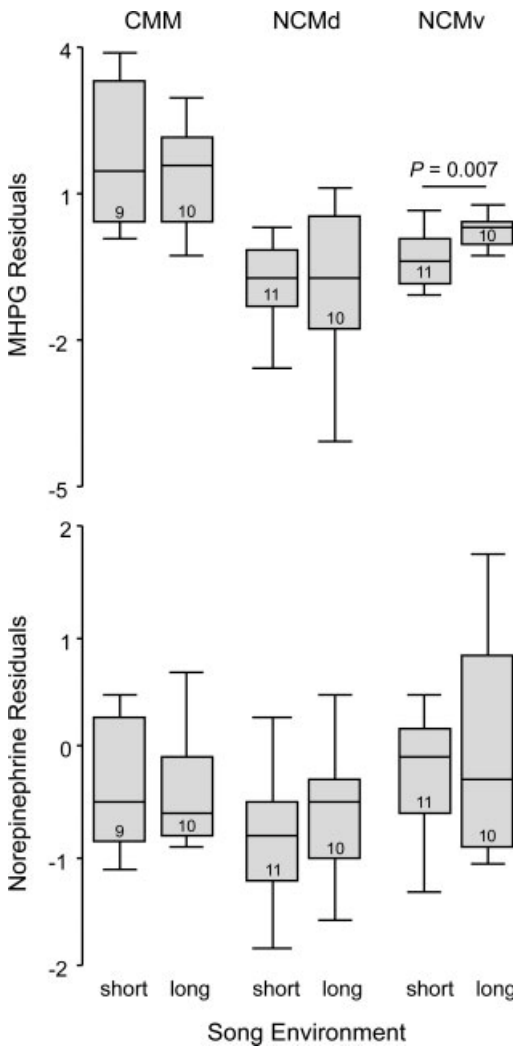

Figure 4 Levels of norepinephrine and its major metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in the auditory telencephalon of female European starlings exposed to a 1-week acoustic environment of either short male songs or long male songs. Levels are standardized residuals from models including protein content of the tissue punch as a covariate and random effect(s) of sound chamber. Conventions follow those in Figure 3.

find support for the prediction that the quality of the prevailing song environment affected norepinephrine secretion. Specifically, a high-quality environment resulted in greater levels of MHPG (see Fig. 4), the primary metabolite of norepinephrine when it is secreted into the synapse and therefore our proxy for norepinephrine secretion. Moreover, this effect of song environment appears to be anatomically specific to the NCMv, as we found no such effect in the other areas of the auditory telencephalon we investigated, including the CMM and NCMd.

We do not know what the mechanism for this effect of song environment might be. However, input from the auditory forebrain or elsewhere modulating the secretion of noradrenergic fibers arising from the locus coeruleus is a viable hypothesis. Auditory representations from many forebrain, midbrain, and hindbrain areas feed directly into the locus coeruleus. Some of these areas include the amygdala, raphe nuclei, and hypothalamus, each of which can show auditory responses in some organisms (Berridge and Waterhouse, 2003). Alternatively, signals from cells

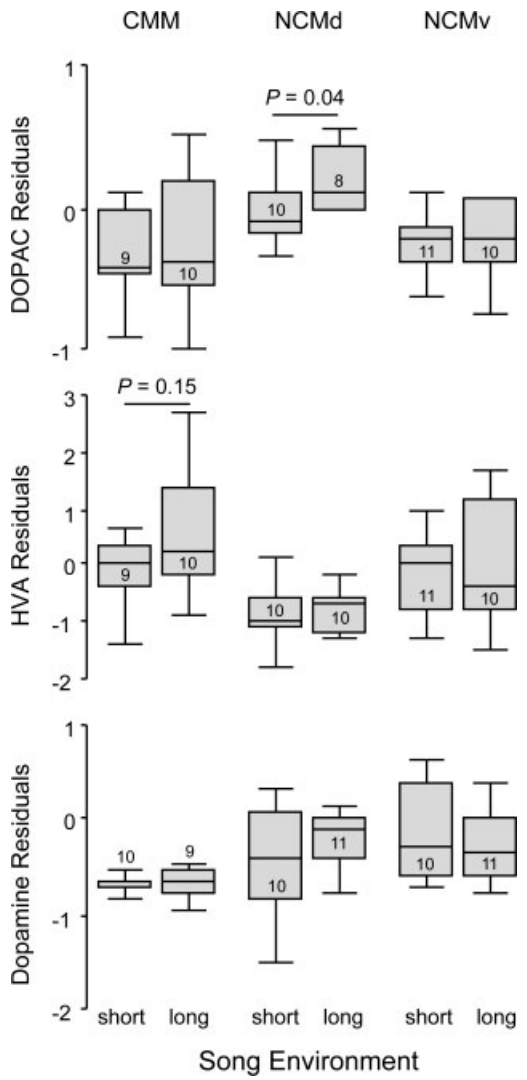

Figure 5 Levels of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in the auditory telencephalon of female European starlings exposed to a 1-week acoustic environment of either short male songs or long male songs. Levels are standardized residuals from models including protein content of the tissue punch as a covariate and random effect(s) of sound chamber. Conventions follow those in Figure 3.

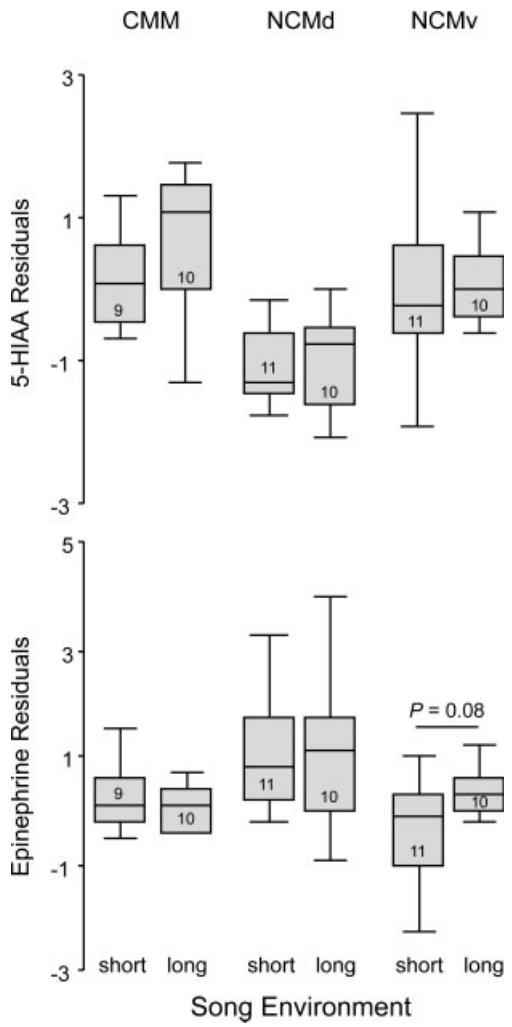

Figure 6 Levels of epinephrine and the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) in the auditory telencephalon of female European starlings exposed to a 1-week acoustic environment of either short male songs or long male songs. Levels are standardized residuals from models including protein content of the tissue punch as a covariate and random effect(s) of sound chamber. Conventions follow those in Figure 3.

locally within the NCMv may influence levels of norepinephrine secretion. Finally, recent studies on white-throated sparrows (*Zonotrichia albicolis*) report that forebrain catecholaminergic innervation (LeBlanc et al., 2007) and ZENK sensitivity to song (not to song quality) (Maney et al., 2006) depend on the presence of estradiol circulating in the female's blood plasma. Although this raises the interesting possibility that quality of the song environment might influence forebrain norepinephrine secretion by modulating circulating estradiol, the evidence for an effect of song quality on plasma estradiol concentrations is limited (e.g., Kroodsma, 1976).

Regardless of which of these or other mechanisms might be at work, there are at least two general means by which a neurosecretory system can alter secretion of neurotransmitter in a specific location. One is by changing the quantity of neurotransmitter secreted in the area and the other is by changing the area's innervation by secretory terminals. Because we observed no effect of song environment on norepinephrine levels (see Fig. 4), it seems unlikely that connectivity of noradrenergic fibers changed appreciably, suggesting that MHPG levels changed as a result of change in secretion of norepinephrine within the NCMv.

As secretion of norepinephrine and therefore norepinephrine metabolism rises, one might assume that norepinephrine levels should decline, unless, of course, norepinephrine synthesis keeps pace with secretion. Although norepinephrine levels did not show marked differences between song environments, we did observe an elevated probability of immunoreactivity for DBH (see Fig. 7), the enzyme that synthesizes norepinephrine from dopamine (Cooper et al., 2003). In combination, these results suggest that the long-song environment did elevate norepinephrine synthesis. Curiously, this change in

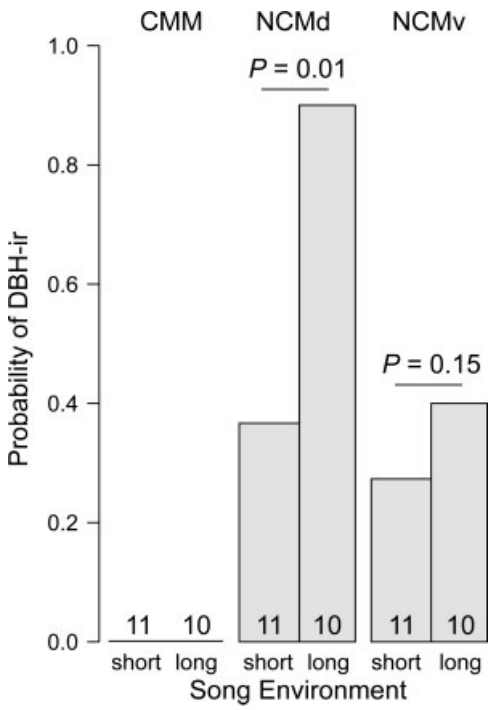

Figure 7 Probability of dopamine-β-hydroxylase immunoreactivity (DBH-ir) in the auditory telencephalon of female European starlings exposed to a 1-week acoustic environment of either short male songs or long male songs. The values for the CMM are both 0. Conventions follow those in Figure 3.

DBH-ir occurred in the NCMd, not in the NCMv, where we observed the change in MHPG. At this point, we do not have a clear explanation for this anatomical disparity, but one possibility is that the noradrenergic fibers involved in the environmental modulation of norepinephrine secretion project through the NCMd before reaching targets in the NCMv. If that were the case, then it is plausible that the norepinephrine secreted in the NCMv is first synthesized more dorsally in noradrenergic fibers, perhaps in the NCMd.

The quality of the song environment also affected levels of DOPAC (see Fig. 5), our proxy for dopamine secretion. We observed this effect in the NCMd, as opposed the NCMv, where MHPG levels changed. As for the noradrenergic system, the mechanism for this dopaminergic modulation was likely based on changing secretion levels within the NCM as opposed to changing connectivity of dopaminergic fibers, due to our observation that dopamine levels showed no substantial variation between song environments (see Fig. 5). We are not aware of previous demonstrations that song cues can modulate dopaminergic activity in the NCM, although dopaminergic systems pervade other regions of the songbird telencephalon (Barclay and Harding, 1988, 1990), including parts of the auditory telencephalon (Harding et al., 1998). Furthermore, dopamine modulates immediate-early gene expression based on context (Sasaki et al., 2006; Hara et al., 2007) and may play a role in female responses to song (Riters et al., 2007) and in partner preferences in mammals (Aragona et al., 2003).

This study adds to the growing body of evidence that catecholaminergic systems, in particular the noradrenergic system, play a central role in mediating a form of behavioral plasticity important in the life-history of birds and possibly other organisms, the context-dependence of mate-choice. Future studies experimentally testing the effects of noradrenergic innervation of the auditory forebrain on behavioral plasticity are essential for a more thorough understanding of the regulation of these processes. By understanding the physiological systems regulating adaptive plasticity in behavior, we can begin to better understand the constraints imposed by these systems on the evolution of behavior and phenotype.

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