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Effects of endocrine state on sound-induced monoamine release in the auditory system and reward pathway of a seasonally-breeding songbird

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Lic. Pontifica Universidad Católica del Ecuador, 2011

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An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of

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Abstract

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By Carlos A. Rodríguez Saltos

In seasonally breeding species, gonadal hormones modulate behavioral responses to social stimuli. For example, breeding levels of plasma estradiol are necessary for female white-throated sparrows to engage in proceptive sexual behaviors when they hear male song. To produce these changes, hormones may affect the auditory and reward pathways in the brain. Past research showed that genomic responses in both of these brain systems are selective for song over behaviorally irrelevant sounds only when plasma estradiol reaches breeding-like levels. I hypothesized that the effects of hormones on these brain systems are mediated by monoamines. I mimicked a breeding endocrine state in female white-throated sparrows by photostimulating them and administering estradiol. I then exposed them to male conspecific song, a control sound (artificial tones), or silence and used HPLC to measure the resulting monoamine release in the auditory and reward pathways. Sound-induced dopamine release in the auditory forebrain was selective for song over tones, but that selectivity did not depend on endocrine state; hearing song induced greater dopaminergic activity than did tones also in untreated, non-breeding females. In two areas of the reward pathway, however, the selectivity of the response did depend on endocrine state. Dopaminergic activity in the ventral pallidum and in the medial amygdala was higher in response to song than to tones, but only in the birds in breeding-like condition. Throughout the brain, serotonergic activity was higher in the estradiol-treated birds regardless of the type of stimulus presented. The mechanisms underlying seasonal changes in behavioral responses to courtship signals may depend on the monoaminergic system recruited.

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The behavioral relevance of social signals depends on the internal state of the receiver. A courtship signal, for example, would be relevant only to a receiver in breeding condition. To understand the mechanisms by which the internal state of a receiver influences adaptive behavioral responses to social signals, we need an animal model for which manipulations of internal state are straightforward (Catchpole, 1983). Seasonally-breeding species of songbirds are ideal for this purpose because their endocrine state can be easily manipulated in the lab through administration of hormones or changes to photoperiod regime. Moreover, the social relevance of bird song and how it changes according to endocrine state is relatively well-known (Catchpole & Slater, 2008).

In seasonally-breeding species of songbirds, females perform proceptive behavior in response to male song only during the breeding season (Catchpole, 1983; Moore, 1983). During this season, circulating levels of gonadal steroids such as estradiol are high in females (Wingfield & Farner, 1978). Estradiol, as shown by several laboratory experiments (e.g., Moore, 1983), primes female songbirds to show proceptive behaviors in response to male song. For example, administration of estradiol to non-breeding female white-throated sparrows (*Zonotrichia albicollis*) primed the females to perform copulation solicitation displays (CSD) when hearing male song (Maney, Goode, Lange, Sanford, & Solomon, 2008).

Not only do gonadal steroids change the behavioral responses of females to song, but they also seem to change the motivation of females to seek song (Maney, 2013; Maney & Pinaud, 2011). Several studies suggest that conspecific song has incentive salience to songbirds; that is, songbirds want to hear song. Evidence that song has incentive salience to songbirds first came from experiments in the field, in which female European flycatchers (genus *Ficedula*) were shown to approach speakers playing the songs of conspecific males (Eriksson & Wallin, 1986).

More recent experiments, under controlled conditions in the lab, used operant conditioning to evaluate the preferences of birds to hear song. Using this technique, it was shown that juvenile male zebra finches (*Taeniopygia guttata*) pressed keys during operant conditioning to hear songs that they would later learn to imitate (Adret, 1993). Female zebra finches were then also shown to press keys to hear song (Riebel & Slater, 1998). In our lab, operant conditioning has been used with female white-throated sparrows that were induced into a breeding-like endocrine state through photostimulation and subcutaneous administration of estradiol. Preliminary results showed that females treated in this way pressed keys hundreds of times per day to hear song, but untreated birds seldom pressed the keys. These results suggest that in seasonally -breeding species the motivation to seek song is controlled by gonadal steroids.

The mechanisms by which steroids elicit changes in the motivation to hear song are beginning to be understood. Estradiol modulates auditory responses to song, and by doing so it may increase the salience of conspecific song. Neural responses to song have been mapped in the auditory system by measuring the expression of immediate early genes (IEGs) such as *zenk* (Clayton, 2000) (following conventional practice in molecular biology, I will use lower case italics, e.g., *zenk*, to refer to the gene and upper case regular font, e.g., ZENK, to refer to the product of expression). The magnitude of these responses, meaning the stimulus-induced expression of *zenk*, is often proportional to the behavioral relevance of the stimulus. For example, in the auditory forebrain of zebra finches, ZENK responses have been found to be greater to song than to behaviorally irrelevant tones and greater to conspecific than to heterospecific song (Mello, Vicario, & Clayton, 1992). When the relevance of a sound was enhanced, for example by pairing the sound with foot shock, ZENK responses for the sounds increased (Jarvis, Mello, & Nottebohm, 1995).

If the behavioral relevance of song depends on plasma estradiol, as behavioral studies suggest (e.g., Moore et al., 1983), estradiol treatment should alter the auditory ZENK response to song. In non-breeding, estradiol-treated female white-throated sparrows, ZENK responses in the auditory forebrain were selective for song over tones, but in birds that had low plasma estradiol and were treated with placebo, the response to song was indistinguishable from the response to tones (Maney, Cho, & Goode, 2006; Sanford, Lange, & Maney, 2010). The effects of estradiol on the selectivity of ZENK responses were detected in the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM) (Maney et al., 2006; Maney & Pinaud, 2011), which are thought to be analogous to the mammalian supragranular layers of the auditory cortex (Calabrese & Woolley, 2015; Mello, Velho, & Pinaud, 2004) and auditory association cortex (Tremere, Jeong, & Pinaud, 2009), respectively. These results suggest that increases in plasma estradiol result in auditory tuning to conspecific song. Such tuning may increase discrimination, detection, or preferences for song during the breeding season (Maney & Pinaud, 2011).

The profound effects of estradiol on the motivation to hear conspecific song are unlikely to be explained entirely by actions on auditory responses only. We should also consider brain systems that attribute incentive salience to stimuli. Research in mammals has shown that this attribution is mediated by a network of mesocorticolimbic regions collectively known as the reward system (reviewed by Arias-Carrión, Stamelou, Murillo-Rodríguez, Menéndez-González, & Pöppel, 2010; Berridge, 2006). The reward pathway mediates the ascription of incentive salience to various stimuli, regardless of their modality, including those associated with food, water, and sex (Arias-Carrión et al., 2010; Berridge, 2006). Therefore, the reward system is a good candidate to mediate the ascription of incentive salience to conspecific song.

The reward pathway of female songbirds shows *zenk* responses that are selective for conspecific song over tones (Earp & Maney, 2012). In female white-throated sparrows, the selectivity was detected in estradiol-treated birds only (Earp & Maney, 2012). Estradiol-dependent modulation of *zenk* responses to song was widespread within the reward pathway; the effects were detected in the nucleus accumbens (nAc), hippocampus (Hp), medial amygdala (MeA), and the caudolateral nidopallium (NCL), the latter being the avian analog of the prefrontal cortex (Güntürkün, 2005). Research in mammals has shown that release of the monoamine dopamine (DA), synthesized in the ventral tegmental area (VTA), in regions of the reward pathway is required for incentive salience to be ascribed to stimuli (Berridge, Robinson, & Aldridge, 2009). In rats, administration of DA agonists into the nAC, a primary target of the VTA, increased motivation to work for food, suggesting that the incentive salience of the food was increased by the treatment. In further support of this hypothesis, DA antagonists inhibited this motivation (reviewed by Wise, 2006). Because female songbirds seem motivated to hear song, I hypothesize that exposure to song increases DA levels in the reward pathway. Because the incentive salience of song changes with endocrine state, I also hypothesize that the selectivity of dopaminergic responses to song depends on endocrine state. Song-induced release of dopamine, however, has yet to be detected in female songbirds.

DA and other monoamines, such as serotonin (5-HT) and norepinephrine (NE), have widespread effects throughout the brain to maintain the salience of behaviorally-relevant stimuli. As we have seen for DA, for example, monoamines can act on brain systems that control motivated behaviors (Berridge, Robinson, & Aldridge, 2009). In sensory systems, monoamines can tune sensory responses to behaviorally relevant stimuli (Hurley, Devilbiss, & Waterhouse, 2004). In rats, for example, simultaneously playing a tone and inducing DA release in the brain

through electrical stimulations of VTA increased the size of the cortical representation for the frequency of that tone and diminished the size of the representations for other frequencies (Bao, Chan, & Merzenich, 2001). Monoamines may have enduring effects on sensory tuning, by activating molecular machinery that drives neural plasticity. Monoaminergic activity drives ZENK expression (Velho et al., 2012), which generally occurs along with expression of genes that drive neural plasticity (Knapska & Kaczmarek, 2004). Degradation of noradrenergic fibers in the canary brain (*Serinus canaria*), for example, eliminated the capacity of NCM and CMM to show ZENK expression in response to sound (Lynch & Ball, 2008). In NCM, noradrenergic input is sufficient for expression of ZENK, as the application of these monoamine alone, in the absence of any stimuli, induces ZENK expression (Velho et al., 2012). Because monoaminergic activity enhances the salience of stimuli and can induce ZENK expression, monoamines are good candidates to mediate estradiol-driven changes in the selectivity of sound-induced ZENK responses.

If monoaminergic systems mediate the effects of estradiol on sound-induced neural responses, they should be sensitive to estradiol, as results from several studies indeed show to be the case. For example, performing ovariectomy in macaques to reduce levels of plasma estradiol modulated the density of monoaminergic fibers in the prefrontal cortex. The changes were reversed after peripheral administration of estrogen, showing convincing evidence that estrogens regulate the density of monoaminergic fibers in this brain region (Kritzer & Kohama, 1998, 1999). In non-breeding white-throated sparrows, which have regressed ovaries, systemic administration of estradiol increased the density of monoaminergic fibers in the auditory forebrain and midbrain (Matragrano et al., 2011, 2012b), suggesting that the capacity for release of monoamines in these auditory regions is greater during the breeding season. Although these

results show evidence of the sensitivity of monoaminergic systems to estradiol, a link between estradiol-driven modulation of monoaminergic systems and that of neural responses to song has yet to be established.

Figure 1 shows a hypothetical model (Fig 1) that could explain how monoamines may mediate effects of estradiol on song-induced neural responses in the auditory and reward pathways. According to the model, estradiol may act on monoaminergic systems to increase constitutive release of monoamines during the breeding season. Consequently, monoamine release may cause neurons in the auditory and reward pathways to respond selectively to conspecific song. Studies on mammals have shown that constitutive release of monoamines has neuromodulatory effects on target neurons (Grace, 1991). Alternatively, or complementarily to inducing constitutive release, estradiol may prime monoaminergic systems to release monoamines selectively in response to song (Fig. 1). Under this mode of action, modulation of neural responses may occur on a moment-to-moment basis when song is heard. Monoaminergic modulation of sensory responses on a moment-to-moment basis has been shown in studies on mammals (Bao, Chan, & Merzenich, 2001; Hurley et al., 2004). In birds, monoamines are known to be rapidly released during exposure to song (Matragrano et al., 2012a, 2012b). In estradiol-treated female white-throated sparrows, hearing song rapidly increased immunoreactivity for the active form of tyrosine hydroxylase, a rate-limiting enzyme in the synthesis of dopamine, in the auditory. In these birds, release of dopamine and serotonin in the auditory forebrain also occurred within minutes of hearing song (Matragrano, Beaulieu, et al., 2012; Matragrano, Sanford, et al., 2012). By responding to behaviorally relevant sounds, monoaminergic neurons may convey information about the behavioral relevance of a stimulus to the auditory and reward systems on a moment-to-moment basis (Maney, 2013); in this case, release of monoamines in the

auditory system and reward pathway may be proportional to the relevance of a stimulus. It is not known, however, whether the release of dopamine and serotonin was selective for song over behaviorally irrelevant sounds, because no control sounds were presented in those studies. Also unknown is whether this release depended on the endocrine state of the birds, because song-induced monoamine release has not been investigated in females with low levels of plasma estradiol.

In this study, I tested the hypothesis that changes in endocrine state affect monoaminergic responses altering their selectivity for song. To test for selective monoamine release, I exposed female white-throated sparrows to male song or frequency-matched tones and used HPLC to measure the resulting monoamine release in the auditory system and in the reward pathway. To test whether selective release depended on endocrine state, I performed these experiments using nonreproductive females that were either photostimulated and treated with estradiol to simulate a breeding state, or housed on short days and treated with placebo. I predicted that hearing song would induce greater monoamine release than hearing tones, but only in the birds in a breeding-like endocrine state. Such a result would suggest that increases in estradiol during the breeding season facilitate song-induced monoamine release, which in turn may underlie the increased incentive salience of male song at a time when it is adaptive to approach males.

Methods

Animals

Animals were collected under appropriate local, state and federal permits. Emory University's Institutional Animal Care and Use Committee approved all the procedures that involved the use of animals. Sixty female white-throated sparrows (*Zonotrichia albicollis*) were

trapped with mist-nets around Emory University campus during their fall migration. The birds were initially housed in flight cages at the Division of Animal Resources (DAR) in Emory University. Each flight cage contained up to 18 birds. The birds were fed on a diet of white millet, red millet, thistle, and bits of oyster shell, and provided with water *ad libidum*. The light system of the room containing the flight cages was programmed to simulate a winter photoperiod (9.5L:14.5D) to keep plasma estradiol at low levels before the experiments.

White-throated sparrows occur in two plumage morphs, tan-striped and white-striped (Lowther, 1961). Because birds of different morphs differ in social behavior (Tuttle, 2003), I balanced morph in my experimental design (n = 25 tan-striped, n= 35 white-striped). Morph and sex were determined by performing PCR on a small blood sample (Griffiths, Double, Orr, & Dawson, 1998; Thomas et al., 2008) (see below).

Age was determined by skull and plumage characteristics (Pyle, Howell, DeSante, & Yunick, 1997). Two groups were distinguished according to age: birds that hatched earlier in that year (hatch-years, HY) and birds that hatched in a previous year (after hatch-years, AHY). I balanced age in my experimental design to the extent possible.

The techniques that I used for aging birds are of widespread use among ornithologists. The most reliable character used to age birds was the degree of pneumatization of the skull. The skull of HYs is unpneumatized at the top, while that of AHYs is completely pneumatized (Pyle et al., 1997). Pneumatized bone is porous and filled with air. Seen from outside, the bone appears to have whitish grains embedded over its surface. Those ‘grains’ are points where bone is denser, that is, where no air spaces are found underneath. The ‘grains’ can be easily seen thanks to the semitransparent scalp of birds. Un-pneumatized bone does not have the grainy look, instead, it looks uniform. It also tends to look pinker than pneumatized bone (Pyle et al., 1997).

Some white-throated sparrows, however, complete pneumatization by November of their hatch year (Pyle et al., 1997). Because I caught my birds in November, I needed an additional method of aging to validate the results from inspecting the skull. The additional method was based on plumage features that vary according to age (Kilts, Breese, & Mailman, 1981). Because distinguishing features in the plumage of HYs persist until about one year after the hatching year, those features should have still been present in my HYs, which likely were at most seven months old in November (the oldest HYs are born in May; Falls & Kopachena, 1994). Wing and tail feathers in HYs have sharp tips while those feathers in AHYs have tips that are squared and have smooth edges (Pyle et al., 1997). The identification of such features, however, can be challenging, especially if the feathers have been damaged, which can happen for a number of reasons in the wild even before the bird is collected. When the results of both methods of aging were inconsistent, the birds were assigned to an unknown age group.

Polymerase chain reaction (PCR)

To determine sex and morph, 30-40 uL of blood was extracted from the brachial vein and stored in 400uL blood lysis buffer at room temperature. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). Melting curve analysis was then used, accomplished by Quantitative Polymerase Chain Reaction (qPCR), on appropriate genetic markers. To confirm sex, the gene *P2/P8* was amplified using the primers P2, 5'-TCTGCATCGCTAAATCCTTT-3', and P8, 5'- CTCCCAAGGATGAGRAAYTG-3' (Griffiths et al., 1998). To confirm morph, the gene *DSE* was amplified using the forward primer, GAAGGACTACGTAGGGATCGTG, and the reverse primer, CCTCATCTTCATCTACAGGCAAC (Thomas et al., 2008).

qPCR makes use of a fluorescent dye (i.e., Sybr Green) that intercalates with the DNA bases during replication. The molecular constituents of the dye fluoresce once they have been incorporated into the chain and pair-bonded with bases from the complementary strand. During the melting phase, the bounds between the complementary strands of DNA are disrupted, thereby diminishing the intensity of the fluorescence of the sample. The rate of change in fluorescence during the melting phase, known as the melting profile of the sample, is unique to segments of DNA with the same length and sequence. Thus, different alleles of a same gene have discernable melting profiles.

Behavioral experiments

Manipulation of endocrine state. One month before the presentation of the stimulus, each bird was transferred to an individual cage (38×38×42 cm^3). To prevent stress from isolation, each bird was assigned a companion. Cages of companions were placed next to each other, allowing the birds to see and hear each other. Companions were under the same hormonal treatment and photoperiod regime.

One week before the experiment, a breeding-like endocrine state was induced in half of the birds (n = 30) by subcutaneously implanting a silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI) filled with 17β-estradiol (Steraloids, Newport, RI) and sealed at both ends with silicone adhesive. The rest of the birds (n = 30) were administered blank implants, which consisted of empty sealed capsules. Lidocaine (4%) was applied topically over the implantation site (upper back) before the surgery. After surgery, the incision was closed using GLUture (Abbott Laboratories) surgical glue. To facilitate the induction of a breeding-like endocrine state, the estradiol-treated birds were switched to a summer-like photoperiod (14/24

hours of light) three weeks before the implantation. The blank-treated birds were kept under the winter-like photoperiod regime in which they had been since they were captured.

Stimulus presentation. One day before playback, each bird was isolated from its companion and placed inside a sound-attenuating booth (Industrial Acoustics, Bronx, NY). The booth was equipped with a video camera (Panasonic WV-CP240), a speaker (AudioSource LS-300), and a microphone. Audio and video from the cameras and microphones were recorded using a Digital Video Recorder (Dahua DVR 1604 HF-U), and the stimuli were played from Macintosh computers, equipped with QuickTime (Apple), to speakers located inside the booths.

Birds in each endocrine state (breeding-like or non-breeding) were assigned to one of five stimulus groups (n= 6 in each group): 1) male conspecific song heard for 15 min; 2) male conspecific song heard for 30 min; 3) artificial tones heard for 15 min; 4) artificial tones heard for 30 min; and 5) silence. Age and morph were balanced across each group to the extent possible.

Conspecific male songs used as stimuli were the same as in previous studies (Maney et al., 2006; Matragrano et al., 2012a, 2012b). Briefly, I downloaded fourteen recordings of male song from the Borror Library of Bioacoustics database (Ohio State University). The rationale of presenting more than one song exemplar to each female was to prevent habituation to the stimulus. The songs were concatenated into a single presentation file, in which each song was repeated every 15 seconds for 3 minutes before switching to a different song. I controlled for effects of order of song by creating six different presentations, each one differing only in the order in which the songs were concatenated. The order of the songs in each file was based on a Latin-square design, which prevented having two given songs placed next to each other in more than one presentation. Each presentation was used for one bird only within each group of birds

that heard song. Finally, I clipped the end of each file to yield versions of 15 and 30 minutes in duration, which thus contained either 5 or 10 different songs, respectively. Concatenation of songs into presentation files was made using Audacity (Audacity Team, 2013).

For each song, a sequence of artificial tones was generated to match the dominant frequencies in the song (Maney et al., 2006). The order of the tones within each sequence was randomized and their durations equalized, while keeping the overall duration of the tone sequence equal to that of the song. The tone sequences were automatically generated with a script (available upon request) written in R (R Core Team, 2013), using functions from the software packages seewave (Sueur, Aubin, & Simonis, 2008) and tuneR (Ligges, Krey, Mersmann, & Schnackenberg, 2013). The tone sequences were arranged into stimulus presentations exactly as is described above for the songs, so that for each of the six unique song presentations, there was a matching tone presentation. As was the case for the birds hearing song, each of the six birds per experimental group heard a unique tone presentation.

On the day of the playback, I began recording the behavior of the birds at lights-on. The presentation of the stimulus started at least half an hour after lights-on and lasted for either 15 or 30 minutes. Immediately after playback, the bird was rapidly decapitated. Birds exposed to silence were sacrificed 60-100 min after lights-on, which was the same time interval elapsed between lights-on and sacrifice of the birds that heard sounds.

Immediately after decapitation, the brain was harvested and cut in half along its midline. One hemisphere was frozen in powdered dry ice for later analysis of its content of monoamines, while the other hemisphere was preserved in fixative (acrolein) for immunohistochemistry in follow-up studies. Whether the left or right hemisphere was frozen was balanced across

treatments. After freezing the hemisphere it was wrapped in aluminum foil and stored in a -80ºC freezer.

Measurement of concentration of metabolites

Tissue collection. The frozen hemisphere was mounted in OCT (Optimal Cutting Temperature compound; Tissue-Tek ®), a resin that prevents the brain from moving while it is being sectioned on a cryostat. 300µm thick sagittal sections were made on a Leica CM1850 cryostat at -12ºC. The sections were mounted on a microscope slide by rapid thawing and refreezing. The slides were stored at -80ºC until micropunching.

I extracted tissue by micropunching with the Palkovits technique (Palkovits & Brownstein, 1983). Tissue was extracted from three auditory regions of interest (ROIs): dorsal lateral nucleus of the mesencephalon (MLd), caudomedial mesopallium (CMM), and caudomedial nidopallium (NCM); from five regions from the reward pathway: nucleus accumbens (nAc), ventral pallidum (VP), medial amygdala (MeA), hippocampus (Hp), and caudolateral nidopallium (NCL); and from Area X, a striatal region that responds selectively to conspecific song. Area X is not formally considered part of the reward pathway, but resembles regions of the reward pathway in that it receives extensive dopaminergic projections from VTA and its activity is modulated by social context (Reiner & Wullimann, 2004; Sasaki, Sotnikova, Gainetdinov, & Jarvis, 2006).

The slides with the brain sections were placed on a cold, flat granite slab. The regions of interest (ROIs) were located with reference to major fiber bundles and tracts, following (Nixdorf-Bergweiler & Bischof, 2007; Stokes, Leonard, & Nottebohm, 1974). A pre-cooled micropunch tool (Fine Science Tools), with its plunger retracted, was pressed against the slide and lifted in a single motion to remove a chunk of tissue. The tissue was immediately expelled, by swiftly

pressing the plunger, into a cold empty centrifuge tube. The diameters of the punches were 0.5mm for MLd, CMM, nAc, VP, MeA, and Hp; and 1.0mm for Area X, NCL, and NCM. In most cases two punches were collected, one from each of two consecutive sections, for each ROI. The centrifuge tubes were placed inside a Styrofoam container with dry ice and shipped to the University of North Carolina at Chapel Hill, NC for the HPLC analysis.

HPLC analysis. I prepared 1.5 L of mobile phase solution by mixing citric acid monohydrate (13.26 g), sodium acetate (4.65 g), sodium octyl sulphonate (322.5 mg), and EDTA (7.5 mg) in ultrapure water (1200 ml) and methanol of HPLC grade (300 ml). The mobile phase was loaded into the HPLC analyzer, an HTEC-500 HPLC-ECD detector system (Eicom). I ran the analyzer for 5 hours to create a stable baseline. The mobile phase solution often became depleted after running about 50 samples, in which case I prepared new mobile phase and generated a new baseline.

I prepared external standard solutions of 10 pg/ul and 1 pg/ul for the monoamines DA, NE, epinephrine (E), and 5-HT, as well as for the DA metabolites homovanillic acid (HVA) and 3,4-dihidroxyphenilacetic acid (DOPAC), the NE metabolite 3-Methoxy-4-hydroxyphenylethylene glycol (MHPG), and the 5-HT metabolite 5-Hydroxyindolacetic acid (5-HIAA). An internal standard, Isoproterenol (10 pg/µl), was added to the solutions to control for any degradation of monoamines or metabolites during processing of the sample, such as during sonication.

Working solution was prepared by mixing the mobile phase solution with an internal isoproterenol standard (10 pg/µl). Each sample tube, containing the micropunched tissue, was loaded with 125µl of working solution. The solution was then homogenized by sonicating for 10

seconds. After sonication, the tubes were centrifuged at 16000 *g* for 16 minutes at 4ºC and the supernatant (100µl) was transferred to a cold HPLC tube.

The HPLC system was operated from a personal computer using the software PowerChrom (edaq) and samples were loaded into the system through a MIDAS Autosampler (Spark Holland) with a tray pre-cooled at 4ºC. Analytes were electrochemically detected (Kilts et al., 1981). Data was stored directly into the personal computer. Powerchrom generated a chromatogram displaying the electrochemical peaks of the analytes in the solution and automatically identified the peaks by matching their retention times to the retention times of the peaks of the external standards. An error margin of 5% was allowed for this matching. I manually classified peaks that were not recognized by the program, such as when the concentration of the analyte was low or when overlapping peaks interfered. Concentrations of the analytes were calculated by diving the heights of the peaks of the analytes to the heights of the peaks of the corresponding external standards. The height of all peaks was normalized to the height of the peak of the internal standard.

Statistical analysis

Tests for song-induced monoaminergic responses. Matragrano et al. (2012a, 2012b) reported that hearing song increased monoaminergic activity in the auditory forebrain of estradiol-treated white-throated sparrows. I hypothesize that these monoaminergic responses are selective for behaviorally relevant sounds. In this study I aimed to test this hypothesis; but because this study follows from results of Matragrano and collaborators I first aimed to confirm those results. Thus, I tested the hypothesis that hearing song increases monoaminergic activity in the auditory system (Hypothesis 1, H1, in the auditory system). Because song has incentive salience to estradiol-treated birds (Maney, 2013), I also tested whether hearing song increased

monoaminergic activity, specifically that of DA, in the reward pathway of estradiol-treated females (Hypothesis 1, H1, in the reward pathway). Matragrano et al. (2012a) tested their hypothesis in the auditory system only; thus testing for song-induced dopaminergic responses in the reward pathway is an original contribution of this study.

Using t-tests, I compared monoaminergic activity in ROIs of the auditory system and reward pathway between estradiol-treated birds exposed to silence and estradiol-treated birds that heard song. My proxy for monoaminergic activity was monoamine turnover (Kilts et al., 1981). I estimated turnover by dividing the concentration of each metabolite by the concentration of its monoamine precursor (i.e., DOPAC/DA, HVA/DA, MHPG/NE, or 5-HIAA/5-HT). I used each of the four measures of monoamine turnover as dependent variables in separate t-tests. Before running the tests, data were squared-root transformed to fit the statistical assumption of normality for all of the tests. I considered H1 to be supported if I found a significant ($p < 0.05$) or large ($d > 0.80$ or eta-squared > 0.20) effect of hearing song on monoamine turnover.

If H1 was supported in any given ROI, I then proceeded to test whether in that ROI monoaminergic responses were selective for song over tones (Hypothesis 2, H2). Using t-tests, I compared monoamine turnover between E2-treated birds that heard song and E2-treated birds that heard tones. I considered H2 to be supported if I found a significant ($p < 0.05$) or large ($d > 0.80$ or eta-squared > 0.20) effect of type of playback stimulus on monoamine turnover.

Finally, if H2 was supported, I tested whether the selectivity for song depended on endocrine state (Hypothesis 3, H3). I ran a 2X2 ANOVA with type of playback stimulus (song or tones) and endocrine state (estradiol or blank) as the independent variables. I looked for interactions between these two variables. If I detected an interaction, I then did a *post hoc* t-test to test whether in blank-treated birds selectivity for song was absent or whether it was present

but was weaker than the selectivity in estradiol-treated birds. All analyses were done in the R statistical platform (R Core Team, 2013). Power analyses were done for each test using the PWR extension package for R (Champely, 2012).

Compared with an approach in which every hypothesis is tested in every ROI, the step-wise approach that I used here had a major advantage. My approach reduced the number of statistical comparisons, because ROIs in which a hypothesis was not supported were discarded for the following analyses. By reducing the number of statistical comparisons, I also reduced the probability of Type I error, which is the probability of falsely rejecting the null hypothesis.

Estradiol-depended increases in constitutive activity of monoamines. Alternatively or in addition to modulating sound-induced monoaminergic release, estradiol may increase constitutive release of monoamines in the auditory system and the reward pathway. I tested for an effect of endocrine state on monoamine turnover in the auditory system and reward pathway. I used t-tests in which either DOPAC/DA, HVA/DA, MHPG/NE, or 5-HIAA/5-HT in each ROI was the dependent variable, and endocrine state was the independent variable.

Behavioral observations

To rule out CSDs as a source of variation in monoaminergic activity I tested for a correlation between frequency of CSD-related events and monoaminergic activity. CSD-related events were scored from videos recorded during playback. Only data from estradiol-treated birds were used, because blank-treated birds are not known to perform CSDs and none did in this study. I included data from estradiol-treated birds exposed to song, tones, and silence. The rationale behind scoring birds that heard tones and silence was that birds in the lab occasionally solicit to tones as well as spontaneously, in the absence of stimuli.

An observer blind to experimental condition scored the behavior of the birds. I assessed the proficiency of the observer in scoring CSD-related events through inter-rater reliability tests between the observer and me. The CSD-related events that were scored were wing quivers and tail lifts. A wing quiver was a rapid fluttering of the wings. A tail lift was any lifting of the tail above horizontal not accompanied by defecation. The videos used for the reliability tests were produced in previous studies(Matragrano et al., 2012a). I used a Spearman rho analysis to test whether the number of CSD-related events was correlated with monoaminergic activity in each ROI.

Results

Song-induced monoaminergic responses: overview

I looked for sound-induced monoaminergic responses in the auditory system and the reward pathway of female white-throated sparrows. Based on the studies of Matragrano *et al.* (2012a, 2012b), I predicted that in estradiol-treated females hearing song would induce higher monoaminergic activity in the auditory system and reward pathway than would hearing tones, and that this selectivity for song would depend on endocrine state. First, I aimed to replicate the results of Matragrano *et al.*; that is, I tested the hypothesis that hearing song increases monoaminergic activity in estradiol treated birds (H1). If I found support for H1 in a given ROI, I then tested whether in E2-treated birds the monoaminergic responses in that ROI were selective for song over tones (H2). Finally, if H2 was supported, I tested whether the selectivity for song was contingent on endocrine state (H3). The results of each of these tests are discussed below in turn.

Song-induced monoaminergic responses in the auditory system

Test of H1: Effects of song playback on monoaminergic activity in estradiol-treated birds. In estradiol-treated white-throated sparrows, hearing song increased dopaminergic activity in the forebrain regions NCM and CMM. I detected significant effects of song playback ($p < 0.05$) on DOPAC/DA in NCM, and HVA/DA in CMM and NCM (Figure 2; Table 1, 2). In contrast, hearing song did not seem to affect turnover of DA in MLd (Table 1, 2). Neither did it modulate the turnover of other monoamines in any of auditory ROIs (Figure 2; Table 3, 4).

Test of H2: Effects of type of playback stimulus on monoaminergic activity in estradiol-treated birds. In the next step of my analysis, I proceeded to test whether song-induced increases in dopaminergic activity in CMM and NCM were selective for song over tones. In estradiol-treated birds, the type of playback stimulus had large effects on HVA/DA in CMM and NCM ($\delta = 0.957$ for CMM, $\delta = 0.917$ for NCM) (Figure 3, Table 2). In both regions, hearing song resulted in higher values of HVA/DA than did hearing tones. Sound-induced dopaminergic responses in these regions were thus selective for song over tones. This selectivity was not detected using DOPAC/DA, however (Figure 3). I therefore proceeded to test H3, whether selectivity for song was contingent on endocrine state, using data on HVA/DA only.

H3: Effects of interactions between endocrine state and type of playback stimulus on monoaminergic activity. I did not find support for the hypothesis that the selectivity of dopaminergic responses in the auditory pathway depends on endocrine state. No significant or large effects of an interaction between endocrine state and stimulus type were found in a 2X2 ANOVA (Figure 4, Table 5). Overall, my results show that hearing song induces more dopaminergic activity in CMM and NCM than does hearing tones, but this selectivity cannot be attributed to endocrine state.

Song-induced monoaminergic responses in the reward pathway

Hearing male song is clearly rewarding to female songbirds in breeding condition (Riebel & Slater, 1998; see Earp & Maney, 2012 for review). I therefore predicted that hearing song would increase dopaminergic activity in the reward pathway of estradiol-treated female sparrows, that this response would be selective for song over tones, and that this selectivity would depend on endocrine state. To test these predictions, I employed the three-step sequential approach described above.

Test of H1: Effects of song playback on dopaminergic activity in estradiol-treated birds. In E2-treated birds, hearing song increased dopaminergic activity in most ROIs in the reward pathway. I detected large effects ($\delta > 0.80$) of song playback on both HVA/DA and DOPAC/DA (Figure 5; Table 1, 2). These effects were detected mostly in birds that heard song for 15 minutes. Area X and nAc were the only ROIs in which I did not detect any effect of song playback on dopaminergic activity. I therefore proceeded to test in all other ROIs of the reward pathway whether sound-induced dopaminergic responses were selective for song over tones.

Test of H2: Effects of type of playback stimulus on dopaminergic activity in estradiol-treated birds. Among estradiol-treated birds, dopaminergic activity in Hp, VP, and MeA was higher in birds that heard song than in birds that heard tones. The type of playback stimulus that was presented to the birds affected the values of DOPAC/DA in Hp and HVA/ DA in Hp, VP, and MeA (Figure 6; Tables 1, 2). These results suggest that sound-induced dopaminergic responses in these three ROIs are selective for song.

H3: Effects of interactions between endocrine state and type of playback stimulus on monoaminergic activity. I next tested whether selectivity of sound-induced dopaminergic

responses in MeA and VP depended on endocrine state (Figure 7, Table 5). I found a significant effect ($p < 0.05$) of an interaction between stimulus type and endocrine state on HVA/DA in both regions. Using *post-hoc* t tests, I found that in blank-treated birds the values of HVA/DA in MeA were higher in birds that heard tones than in birds that heard song; in VP the values of HVA/DA did not depend on the type of stimulus presented (Figure 7). The pattern of sound-induced dopaminergic responses in MeA was not consistent with my predictions that dopaminergic activity in blank treated birds would be indistinguishable between birds that heard song and birds that heard tones; but nonetheless, my results suggest that selectivity for song occurs only in birds in breeding condition.

Main effects of endocrine state on monoamine turnover

I tested the hypothesis that monoaminergic activity was higher in estradiol-treated birds than in blank-treated birds, regardless of which stimuli was presented. Higher activity in estradiol-treated birds would suggest that estradiol induces sustained released of monoamines during the breeding season; such sustained release, I believe, might prime monoaminergic targets to show neural responses that are selective for song over behaviorally irrelevant sounds.

Manipulations of endocrine state significantly ($p < 0.05$) affected monoamine turnover in the auditory system and in the reward pathway. Using ANOVAs (Table 7) I detected a significant main effect of endocrine state on HVA/DA in NCM (Figure 8), on MHPG/NE in NCM, and on 5HIAA/5HT in CMM and NCM (Figure 9). In the reward pathway, ANOVAs revealed significant main effects of endocrine state on 5-HIAA/5-HT in almost every ROI (Figure 10, Table 7). No main effects of endocrine state on dopaminergic or noradrenergic activity were detected in the reward pathway.

Correlation between behavior and monoaminergic activity

I tested for correlations between the frequency of events related to copulation solicitation display and the level of monoaminergic activity in estradiol-treated birds, regardless of whether they heard song, tones, or were exposed to silence. I included data on birds that heard tones and on birds exposed to silence because estradiol-treated birds are known to solicit to tones and to even solicit spontaneously in the absence of a stimulus.

I did not find significant correlations ($p < 0.05$) between CSD-related events and monoaminergic activity. Spearman rho estimates were low overall, between -0.240 and 0.194 for all tests (Figures 16, 17). When the video recordings were analyzed it was found that only two birds exhibited most of the CSD's that were recorded in the study. The sum of their CSD events was 479, which was 95% of the sum of the events from all birds. Out of the 29 estradiol-treated birds that were scored, 23 (79%) did not solicit at all. My ability to detect relationships between monoamine release and behavior was therefore limited. Overall, I found no evidence that the level of monoaminergic activity correlates with the proceptive response to hearing male song.

Discussion

In seasonally breeding species, the behavioral relevance of sociosexual signals depends on endocrine state. In this study I tested whether sound-induced monoaminergic responses in the brain of a seasonally breeding bird, the white-throated sparrow, also depends on endocrine state. I replicated a major finding of Matragrano *et al* (2012a): hearing song increased dopaminergic activity in the auditory forebrain of female estradiol-treated sparrows. Moreover, I found that dopaminergic activity in both NCM and CMM was greater in birds that heard song than in birds

that heard tones, suggesting that sound-induced dopaminergic responses in the auditory forebrain are selective for conspecific song. This selectivity, however did not seem to depend on endocrine state; I detected no interactions between the effects of endocrine state and stimulus type on monoamine turnover. . Therefore, monoaminergic activity in the auditory forebrain may not participate in the encoding of seasonal changes in the behavioral relevance of song.

Manipulations of endocrine state affected activity of DA, NE, and 5-HT in the auditory forebrain, regardless of the type of stimulus presented in playback experiments. In NCM and CMM, increased levels of monoamine turnover were detected in estradiol-treated birds compared with blank-treated birds. This increase may reflect release that occurs independently of external stimuli, attained by paracrine release or tonic firing of monoaminergic neurons, and sustained during the breeding season. Increased background levels of monoamines may selectively increase the responsiveness of the auditory system to socio-sexual stimuli. In mammals, sustained monoaminergic activity is known to set the excitability and responsiveness of forebrain neurons (Bubar & Cunningham, 2006; Grace, Floresco, Goto, & Lodge, 2007; Reader, Ferron, Descarries, & Jasper, 1979). Monoamines do not, however, increase responsiveness to all stimuli; the effects of monoamines seem targeted towards increasing responses to behaviorally relevant stimuli (Hurley, Devilbiss, & Waterhouse, 2004; Hurley & Hall, 2011). For example, application of serotonin in the auditory system of rodents selectively increases responsiveness to conspecific vocalizations (Hurley & Hall, 2011). The selective effects of serotonin seem to rely both on the density of serotonin receptor expression and the receptor subtype. In mice, neurons tuned to conspecific vocalizations express a type of serotonin receptor that upon binding of serotonin activates signaling pathways that increase the excitability of the neuron (Hurley & Hall, 2011; Hurley, Tracy, & Bohorquez, 2008). In seasonally-breeding birds, similar

mechanisms may facilitate dopamine or serotonin-driven increases in the responsiveness of auditory neurons to conspecific song.

Previous studies have shown that in female white-throated sparrows, administration of estradiol increases monoaminergic fiber density in NCM and the auditory midbrain (Matragrano et al., 2012b; Matragrano, Sanford, Salvante, Sockman, & Maney, 2011). In NCM, this increase in fiber density may cause larger quantities of MAs to be released constitutively during the breeding season. In the midbrain, however, that explanation may not apply since my results did not show that monoaminergic activity increased in birds in a breeding-like endocrine state. This difference between NCM and the auditory midbrain suggests that constitutive release of MAs depends not only on priming of monoaminergic neurons by estradiol, but also on local regulatory mechanisms that may differ across auditory regions. Although in my study I did not detect effects of hearing song on monoaminergic activity in the auditory midbrain, possibly because of low statistical power, Matragrano et al. (2012a) reported that hearing song decreased rather than increased dopamine turnover in the auditory midbrain. The mechanisms that explain differences in local regulation of MA release between auditory regions, as well as the relevance of these differences to the modulation of neural responses to song, are as yet unclear

My results in the auditory forebrain suggest that endocrine state affects monoaminergic activity there by modulating background concentration of monoamines, and apparently not by modulating the selectivity of monoaminergic responses to song. However, I caution against concluding from my analysis that such a modulation of selectivity for song does not exist in the auditory forebrain. The absence of support for an interaction between endocrine state and stimulus type on monoamine turnover in the auditory forebrain may have been due to the low power of the tests for the interaction. Low power was a general issue among my tests, including

pairwise comparisons and tests for interactions.. It is normal for tests of interactions to have lower power than pairwise comparisons because to test for interactions any statistical procedure must first estimate main effects, which comes at a cost of statistical power (Wahlsten, 1991). Therefore, if the interaction is revisited in future studies, I recommend using larger sample sizes than the ones used here.

Manipulations of endocrine state modulated background serotonergic activity not only in the auditory forebrain, but also in several regions of the reward pathway. The auditory forebrain and reward pathway may receive the same sources of serotonin, because axons of serotonergic neurons are known to show extensive collateralization (Berridge and Waterhouse, 2003); a single neuron may therefore innervate not only the auditory forebrain but one or more regions of the reward pathway. Estrogen receptors on the cell bodies of serotonergic neurons, located for example in the dorsal raphe nuclei (Hurley & Hall, 2011), may enable estradiol to modulate neural responses to conspecific song throughout the brain. A variety of processes, governed by different brain regions, that result in increased salience of song may be coordinated by a single modulatory system; in the auditory system, serotonin-mediated effects of estradiol may tune neurons to conspecific vocalizations (Hurley & Hall, 2011), whereas in the reward pathway, serotonin may interact with dopaminergic systems to modulate the ascription of incentive salience to stimuli. In fact, studies in mammals have suggested that adequate dopamine function requires interaction between serotonin and proteins that regulate the release of dopamine (Daw, Kakade, & Dayan, 2002; Sasaki-Adams & Kelley, 2001; Sora et al., 2001).

When the brain ascribes incentive salience to stimuli, it does so during stimulus-induced release of dopamine in the reward pathway (Berridge *et al.*, 2009). Therefore, I hypothesized that in the reward pathway of estradiol-treated female sparrows, dopaminergic responses are selective

for song. Because the incentive salience of song depends on endocrine state (reviewed by Maney, 2013), I also hypothesized that the selectivity of dopaminergic responses to song depends on endocrine state. In estradiol-treated birds, hearing song induced greater dopaminergic activity in VP and MeA than did hearing tones. This selectivity depended on endocrine state; in blank-treated birds, selectivity for song was not detected in either region.

The mechanism by which manipulations of endocrine state affected selectivity, however, seemed different between the two brain regions; whereas in VP, selectivity was absent in blank-treated birds, in MeA, dopaminergic responses were selective for tones over song (Figure 7). Pharmacological studies in mammals have shown that dopaminergic activity in VP is crucial for ascribing incentive salience to stimuli (reviewed by Smith, Tindell, Aldridge, and Berridge, 2009). Enhancing dopaminergic transmission in VP through local injection of psychostimulants activated eating behavior in rats (reviewed by Smith *et al.*, 2009) and induced place preference (Gong, Neill, and Justice, 1996). Moreover, administration of DA antagonists into VP inhibited food intake (Shimura, Imaoka, Yamamoto, 2006), and lesions of VP blocked conditioned place preference (Gong, Neill, and Justice, 1997). When interpreted in light of these studies, my results suggest that levels of sound-induced dopaminergic activity in VP may be related to the valence of song. Considerable evidence suggests that song has positive valence for estradiol-treated birds but not for blank-treated birds (Maney, 2013); in my experiments, hearing song induced higher levels of dopaminergic activity in VP of estradiol-treated birds, but not of blank-treated birds, than did hearing tones (Figure 7). Future studies should test whether DA activity in VP has a causal role in the attribution of incentive salience; a good strategy would be to administer DA agonists and antagonists into VP and evaluate preferences for song by using an operant testing device.

A role in ascribing incentive salience to stimuli has also been described for MeA. In mammals, MeA is the primary relay in the forebrain for pheromonal information processed by the vomeronasal organ. In rats, exposure to sex pheromones induced expression of immediate early genes in MeA (Bressler & Baum, 1996). Integrity of MeA is necessary for the expression of proceptive behavioral responses to sex pheromones. In male rats, for example, lesions of MeA decreased the probability that female odor contained in urine and feces would induce non-contact erections (Kondo & Sachs, 2002). A link between MeA and the processing of sociosexual stimuli has also been established in birds. In male Japanese quail (*Coturnix coturnix*) lesions of MeA increased latencies to approach females and reduced the number of cloacal movements in anticipation of copulation (Thompson, Goodson, Ruscio, & Adkins-Regan, 1998), suggesting that arousal was decreased. Feeding and drinking was not disrupted by MeA lesions in rats or quail, therefore the lesions probably did not affect motor function. Rather, the lesions may have impaired categorization of the stimuli as sexually arousing. Dopamine released in the presence of behaviorally relevant, sociosexual stimuli may facilitate the responses of MeA to those stimuli. In my study, hearing song selectively increased dopaminergic activity in this region in estradiol-treated birds, suggesting that dopamine may have a role in facilitating the responses of MeA to sociosexual stimuli.

Behavioral responses to sociosexual signals depend on steroid activity in MeA. Application of estradiol into MeA in castrated male hamsters, for example, can restore behavioral responses to the presence of females (Wood, 1996). Results from my study suggest that estradiol could recruit dopaminergic systems to modulate activity in MeA. In my study, manipulations of endocrine state altered sound-induced dopaminergic responses in MeA. In blank-treated birds, dopaminergic activity was higher in response to tones than in response to

song. This result, which persisted even after elimination of an outlier (data not shown), suggests that dopaminergic activity in MeA may not occur only in response to stimuli with positive valence. Studies in mammals have shown that MeA is important for processing stimuli other than those with positive valence; MeA also plays a role in adaptive behavioral responses to stimuli that signal a threat, such as predator odor (Li, Maglinao, & Takahashi, 2004). A plausible explanation of my results may be that the increased dopaminergic activity in response to tones is related to negative rather than positive valence, if blank-treated birds found tones more aversive than did estradiol-treated birds. Studies in which the valence of song and tones is assessed, and how this valence changes with endocrine state, will be required to interpret my results in MeA. Operant conditioning is an ideal technique to be used in such studies to test whether a sound has positive or negative valence; if songbirds are willing to work in an operant cage to start the playback of a sound, the sound probably has positive valence, and if the birds work to stop the playback, then the sound probably has negative valence.

A region in the reward pathway in which it was intriguing to not detect dopaminergic responses to song was nAc. A vast number of studies in mammals highlights dopaminergic activity in nAc as a key mediator of the attribution of incentive salience to stimuli (Berridge, 2007). Little is known about the avian nAc, however, for example whether DA is released there during exposure to stimuli with incentive salience, and if so, exactly where within the structure that release occurs. Earp and Maney (2012) showed that in female white-throated sparrows, hearing song induced a greater ZENK response in nAc than did hearing tones. That effect was seen only in birds in breeding condition, suggesting that some form of hormone-dependent neuromodulation is acting in that region. The avian nAc has been the subject of relatively few neuroanatomical investigations (Abellan & Medina 2009; Balint & Csillag 2007, Husband &

Smizu 2011). The punch location used in this study was based on the most recent information available (Husband & Shimizu, 2011), but we still need to learn more about this area in the songbird brain, and as we do so it will become possible to more accurately measure DA responses to sensory stimuli.

The effects of my experimental treatments on dopaminergic activity, whether manipulations of endocrine state or playback of a sound stimulus, were detected primarily on HVA/DA rather than DOPAC/DA. HVA/DA may indeed be a better indicator of DA release than DOPAC/DA, as suggested by studies of dopamine metabolism in the nigrostriatal dopaminergic neurons of mammals (Meiser, Weindl, & Hiller, 2013). In such neurons, DOPAC is synthesized either from DA that has been recaptured after release into the extracellular space or DA that has leaked into the cytoplasm from storage vesicles (Meiser, Weindl, & Hiller, 2013). Synthesis of DOPAC from the latter source of DA may therefore mask synthesis from released and then recaptured DA. HVA, in contrast, cannot be synthesized in the cytoplasm of nigrostriatal dopaminergic neurons; the neurons lack the enzymes required (Meiser, Weindl, & Hiller, 2013). Neuroglia surrounding the dopaminergic neurons synthesize HVA (Meiser et al., 2013), and to do so they must acquire DA by capturing it from the extracellular space. Therefore, HVA/DA is more sensitive to DA release than is DOPAC/DA. Whether this pattern for HVA and DOPAC synthesis holds in the reward pathway is unknown. Studies using techniques that show unequivocal evidence of DA release, such as microdialysis, will help to test relationships between DOPAC/DA or HVA/DA and DA release.

To rule out CSDs as a source of variation in monoaminergic activity across my birds, I tested for correlations between number of CSD-related events and monoaminergic activity. In no ROI, however, was monoaminergic activity correlated with the frequency of CSD events,

therefore ruling out the possibility that frequency of CSDs explains monoaminergic variation across my birds. Moreover, CSD-related events were scarce among my birds, with only two birds performed more than 90% of all scored events. Many birds did not perform CSDs, yet they showed monoaminergic activity in response to song or treatment with estradiol.

Overall, my results show that manipulations of endocrine state affect monoaminergic activity in the reward pathway and in the auditory forebrain. I presented evidence that a breeding-typical endocrine state increases background levels of monoamines in the reward pathway and auditory system. Such an increase in background levels of monoamines may prime neurons to respond selectively to behaviorally relevant stimuli, as suggested by studies in mammals (Hurley et al., 2004). I also presented evidence that two regions in the reward pathway showed dopaminergic responses that are consistent with my model of rapid DA release in response to song (Figure 1). In these regions, hearing song induced rapid release of DA but only in birds in a breeding-like endocrine state. This pattern of dopaminergic responses to song resembles that of sound-induced ZENK responses in some regions of the reward pathway (Earp & Maney, 2012).

Monoamines have been shown to regulate the expression of ZENK (Knapska & Kaczmarek, 2004; Velho et al., 2012). Selective ZENK responses to song occur in the auditory and reward pathways, and this selectivity has been shown to depend on endocrine state (Earp & Maney, 2012; Maney et al., 2006). Sound-induced dopaminergic responses in regions such as VP and MeA may explain the selectivity of ZENK responses of the reward pathway to song. Selectivity of ZENK responses to song, however, has not been described in VP (Maney & Rodriguez-Saltos, in review). Selective ZENK responses to song and effects of endocrine state on modulating this selectivity have been described, however, in nAc, NCL, and Hp, regions in

which I did not detect modulatory effects of endocrine state on the selectivity of dopaminergic responses to song (Hp) or effects of hearing song on dopaminergic activity (nAc, NCL). Dopamine released in response to song may indirectly regulate ZENK expression in distant brain regions, through indirect signaling. Song-induced dopaminergic activity in VP and MeA may explain ZENK responses even in the auditory system. Once the reward system has ascribed incentive salience to song, it may send a signal to the auditory system to modulate neural responses to song (Figure 1). In other words, to maximize the acquisition of a reward, the reward system may modulate sensory systems to enhance the salience of that stimulus, as suggested by theoretical models (Berridge et al., 2009; Hickey, Chelazzi, & Theeuwes, 2010). Research in songbirds may provide an exciting and unique opportunity to investigate neural interactions between the reward pathway and sensory systems.

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Tables

Table 1

Effects of song playback (tests of H1) and of type of playback stimulus (tests of H2) on DOPAC/DA

	Song vs. Silence (H1)						**Song vs. Tones (H2)**					
	15 min			**30 min**			**15 min**			**30 min**		
Region	***t***	***p***	***δ***	***t***	***p***	***δ***	***t***	***p***	***δ***	***t***	***p***	***δ***
CMM	0.183	0.859	0.111	0.676	0.516	0.409	-	-	-	-	-	-
NCM	-2.963	**0.014***	**1.711**	-2.547	**0.029***	**1.471**	0.381	0.711	0.220	-0.938	0.370	0.542
MLd	-0.048	0.963	0.029	-0.690	0.508	0.418	-	-	-	-	-	-
Area X	-0.297	0.772	0.172	0.121	0.906	0.070	-	-	-	-	-	-
nAc	-0.965	0.357	0.557	-0.483	0.640	0.279	-	-	-	-	-	-
VP	-2.912	**0.016***	**1.681**	-2.211	0.054	**1.339**	1.331	0.213	0.768	1.111	0.299	0.703
Hp	-2.377	**0.041***	**1.439**	-1.253	0.239	0.723	1.435	0.185	**0.869**	-	-	-
MeA	-1.826	0.098	**1.054**	-0.746	0.473	0.431	1.337	0.211	0.772	-	-	-
NCL	-0.056	0.956	0.034	-1.639	0.132	**0.946**	-	-	-	-0.643	0.535	0.371

* $p < 0.05$
All birds were in a breeding-like endocrine state.
No test had power above 0.75.
Values of effect size written in **bold** indicate large effect sizes.

Table 2

Effects of song playback (tests of H1) and of type of playback stimulus (test of H2) on HVA/DA

	Song vs. Silence (H1)						**Song vs. Tones (H2)**					
	15 min			**30 min**			**15 min**			**30 min**		
Region	***t***	***p***	***δ***	***t***	***p***	***δ***	***t***	***p***	***δ***	***t***	***p***	***δ***
CMM	-1.600	0.141	**0.924**	-2.449	**0.034***	**1.414**	0.646	0.534	0.391	1.657	0.129	**0.957**
NCM	-1.848	0.094	**1.067**	-0.778	0.455	0.449	1.588	0.143	**0.917**	-	-	-
MLd	0.075	0.942	0.050	0.691	0.521	0.527	-	-	-	-	-	-
Area X	0.748	0.472	0.432	0.843	0.419	0.487	-	-	-	-	-	-
nAc	0.182	0.860	0.105	0.914	0.382	0.528	-	-	-	-	-	-
VP	-2.256	**0.047***	**1.303**	-1.710	0.118	**0.987**	2.584	**0.027***	**1.492**	0.833	0.426	0.505
Hp	-2.170	0.062	**1.372**	-1.229	0.254	0.777	1.954	0.086	**1.236**	-	-	-
MeA	-2.210	0.052	**1.276**	-0.891	0.394	0.515	2.225	0.050	**1.284**	-	-	-
NCL	-1.341	0.210	0.774	-0.982	0.349	0.567	-	-	-	-	-	-

* $p < 0.05$
All birds were in a breeding-like endocrine state.
No test had power above 0.75.
Values of effect size written in **bold** indicate large effect sizes.

Table 3

Effects of song playback (tests of H1) on MHPG/NE in the auditory system

	Song v. Silence (BR)					
	15 min			30 min		
Region	***t***	***p***	***δ***	***t***	***p***	***δ***
CMM	-0.468	0.651	0.283	-0.196	0.849	0.119
NCM	0.775	0.456	0.447	0.407	0.693	0.235
MLd	-0.551	0.594	0.318	-1.251	0.239	0.722

* $p < 0.05$

No test had power above 0.75.
All birds were in a breeding-like endocrine state.

Table 4

Effects of song playback (tests of H1) on 5-HIAA/5-HT in the auditory system

	Song v. Silence (BR)					
	15 min			30 min		
Region	***t***	***p***	***δ***	***t***	***p***	***δ***
CMM	0.682	0.511	0.394	-1.317	0.217	0.760
NCM	0.116	0.910	0.067	-1.292	0.225	0.746
MLd	-0.271	0.792	0.156	-0.061	0.953	0.035

* $p < 0.05$
All tested birds had a breeding-like endocrine state
No test had power above 0.75

Table 5

Interactions between endocrine state and type of playback stimulus (tests of H3) on HVA/DA

	15 min			30 min		
Region	***F***	***p***	$\widehat{\omega}^2$	***F***	***p***	$\widehat{\omega}^2$
CMM	-	-	-	1.994 (1. 19)	0.174	0.042
NCM	2.205 (1. 20)	0.153	0.039	-	-	-
MLd	-	-	-	-	-	-
Area X	-	-	-	-	-	-
nAc	-	-	-	-	-	-
VP	5.665 (1. 20)	**0.027***	0.149	-	-	-
Hp	0.403 (1. 17)	0.534	0.000	-	-	-
MeA	4.612 (1. 19)	**0.045***	0.140	-	-	-
NCL	-	-	-	-	-	-

* $p < 0.05$
No test had power above 0.75.

Table 6

Main effects of endocrine state on DA turnover

	DOPAC/DA			HVA/DA		
Region	***F (df)***	***p***	$\widehat{\omega}^2$	***F (df)***	***p***	$\widehat{\omega}^2$
CMM	0.015 (1, 55)	0.903	0.000	1.864 (1, 57)	0.178	0.015
NCM	3.615 (1, 59)	0.063	0.043	9.832 (1, 59)	**0.003****	0.131
MLd	0.094 (1, 56)	0.761	0.000	1.051 (1, 39)	0.312	0.002
Area X	0.035 (1, 58)	0.852	0.000	2.035 (1, 58)	0.159	0.018
nAc	0.124 (1, 59)	0.726	0.000	0.824 (1, 59)	0.368	0.000
VP	4.610 (1, 57)	**0.036***	0.060	0.311 (1, 58)	0.579	0.000
Hp	0.031 (1, 56)	0.862	0.000	0.868 (1, 50)	0.356	0.000
MeA	0.005 (1, 57)	0.944	0.000	0.210 (1, 57)	0.649	0.000
NCL	0.385 (1, 56)	0.537	0.000	0.165 (1, 59)	0.686	0.000

* $p < 0.05$, ** $p < 0.01$

Table 7

Main effects of endocrine state on NE and 5-HT turnover

	MHPG/NE			5-HIAA/5-HT		
Region	***F (df)***	***p***	***w***	***F (df)***	***p***	***w***
CMM	0.020 (1, 56)	0.889	0.000	21.297 (1, 58)	*******	**0.260**
NCM	4.940 (1, 59)	**0.030***	0.063	32.701 (1, 59)	*******	**0.350**
MLd	1.041 (1, 57)	0.312	0.001	2.473 (1, 58)	0.122	0.025
Area X	2.767 (1, 58)	0.102	0.030	19.515 (1, 58)	*******	**0.242**
nAc	0.002 (1, 58)	0.961	0.000	2.773 (1, 59)	0.102	0.029
VP	1.210 (1, 57)	0.276	0.004	3.799 (1, 58)	0.056	0.046
Hp	0.022 (1, 57)	0.883	0.000	9.628 (1, 56)	**0.003****	0.134
MeA	0.228 (1, 59)	0.635	0.000	17.608 (1, 59)	*******	**0.220**
NCL	0.000 (1, 59)	0.991	0.000	24.592 (1, 59)	*******	**0.286**

* $p < 0.05$, ** $p < 0.01$
Values of effect size written in **bold** indicate large effect sizes

Figures

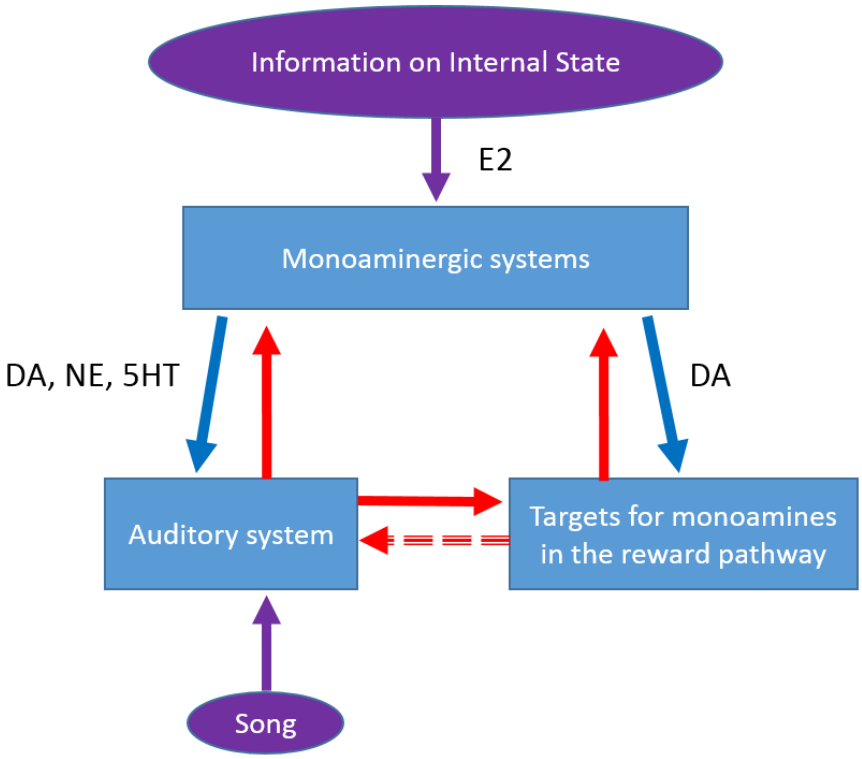

Figure 1. Hypothetical model that explains how monoaminergic systems may integrate information on internal state to modulate song-induced neural responses in seasonally-breeding songbirds. Plasma estradiol may bind to monoaminergic cells to increase constitutive release of MAs, which in turn prime the auditory and reward pathways to respond selectively to conspecific song. Alternatively, or complementarily, estradiol may prime monoaminergic systems to release MAs selectively in response to conspecific song. Monoaminergic targets may then show selective responses to song as a result of song-induced MA release. In both cases, high levels of plasma estradiol facilitate MA release, but in the second case MA release depends also on hearing song. Signals encoding song (red arrows) originate in the auditory system, after song is processed by auditory neurons, and are then relayed to the reward pathway, in which incentive salience is ascribed to the song. The reward pathway may also send feedback signals to the auditory system (dashed red arrow) to further modulate auditory and monoaminergic responses to song in the latter.

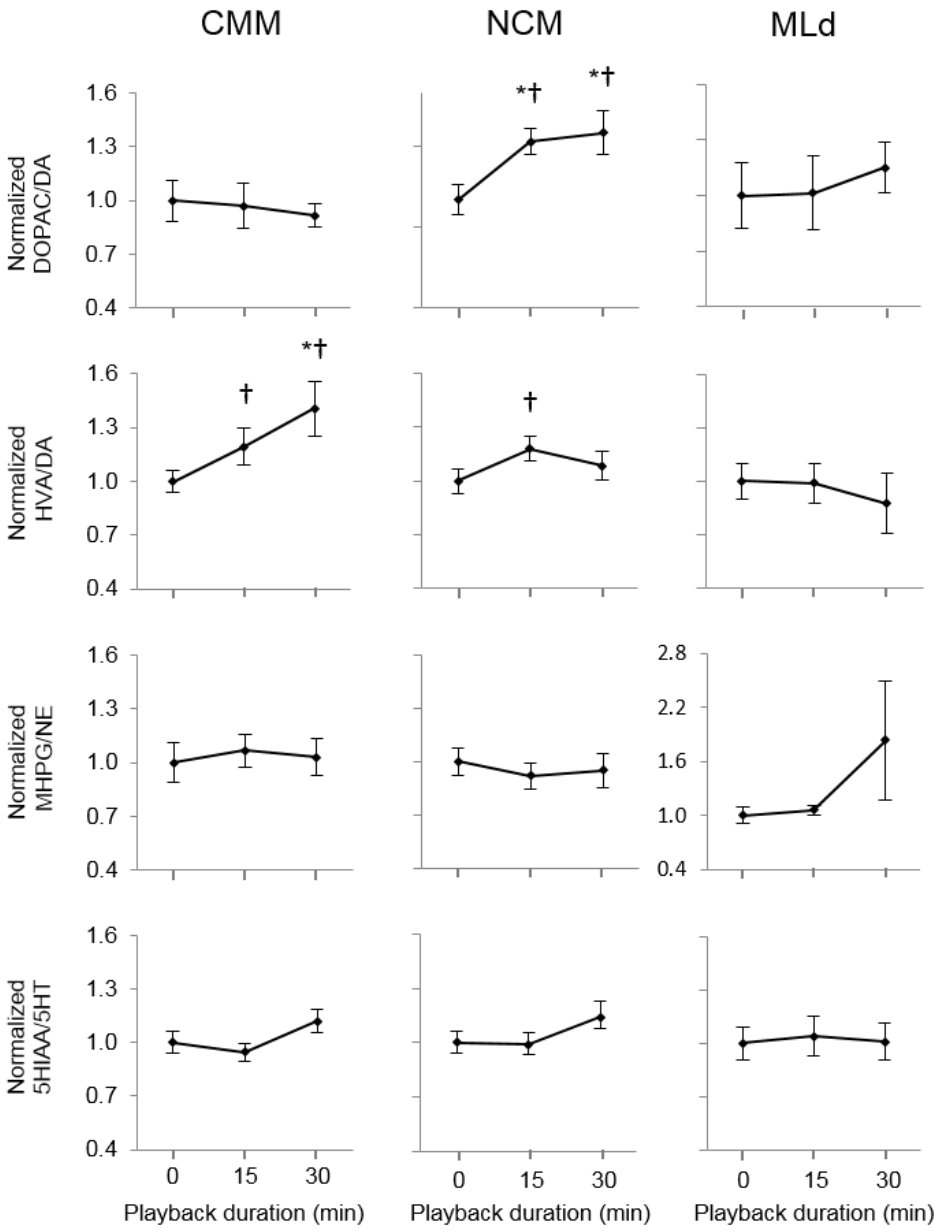

Figure 2. Effects of song playback on monoaminergic activity in the auditory system of estradiol-treated birds (tests of H1). Hearing song increased monoaminergic activity in the auditory forebrain regions NCM and CMM. * Significantly different ($p < 0.05$) from the mean of the silence group (0 min); † large effect ($\delta > 0.8$) of song playback. Data are normalized to the mean of the silence group. The dotted, horizontal line in each panel marks the normalized mean of the silence group (1.0).

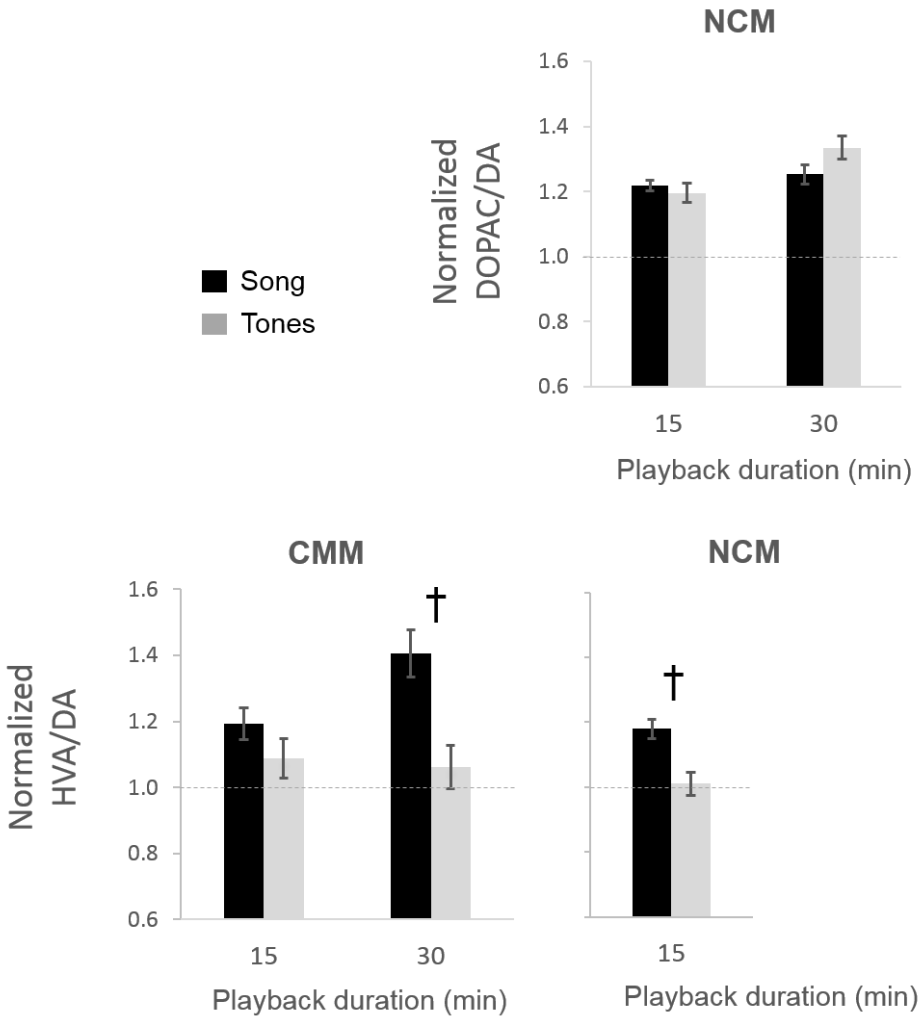

Figure 3. Effects of type of playback stimulus on monoaminergic activity in the auditory system of estradiol-treated birds (tests of H2). This test was done only in ROIs in which H1 was supported (Fig. 2) and thus in which hearing song increased monoaminergic activity in E2-treated birds. Dashed boxes outline comparisons that provided support for H2. In CMM and NCM, HVA/DA was higher in the birds that heard song than in those that heard tones.

† $\delta > 0.8$. Data are normalized to the mean of the silence group. The dotted, horizontal line in each panel marks the normalized mean of the silence group (1.0).

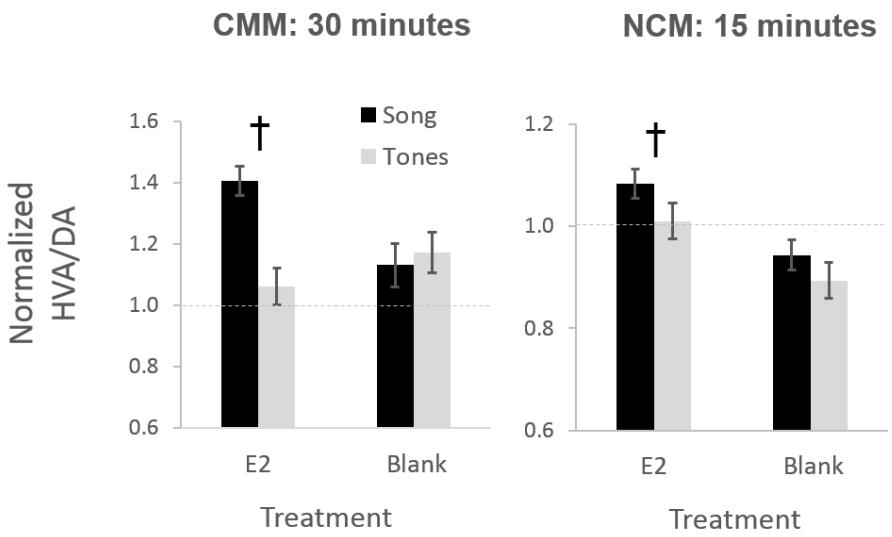

Figure 4. Interactions between endocrine state and type of playback stimulus on monoaminergic activity in the auditory system (tests of H3). This test was done only in ROIs in which I found support for H2 (Fig. 3), and thus in which monoaminergic activity in E2-treated birds was higher in response to song than in response to tones. No significant or large effects of the interaction were found. Data are normalized to the mean of the silence group. The dotted, horizontal line in each panel marks the normalized mean of the silence group (1.0). E2, estradiol-treated birds; B, blank-treated birds.

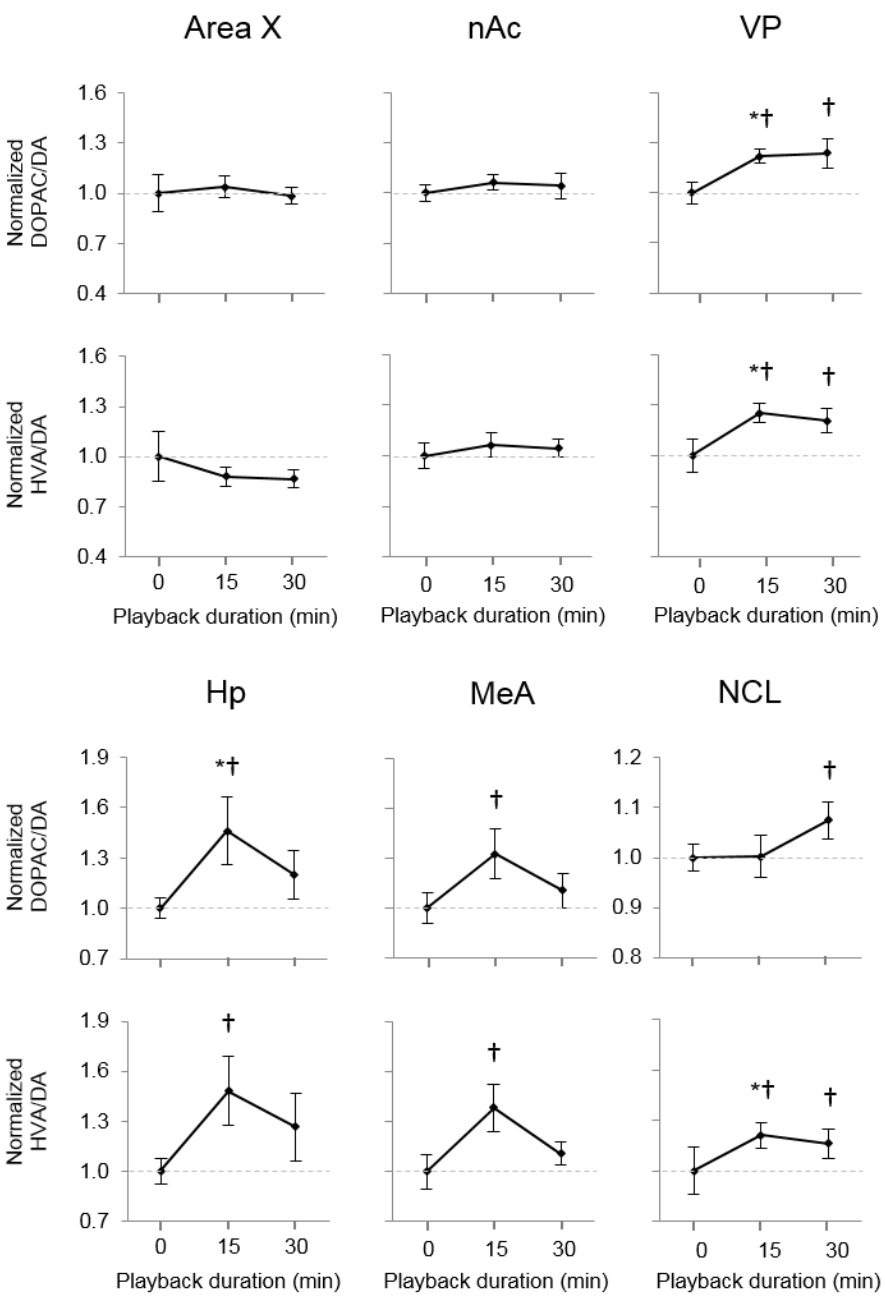

Figure 5. Effects of song playback on dopaminergic activity in the reward pathway of estradiol-treated birds (tests of H1). Hearing song increased dopaminergic activity in VP, Hp, MeA, and NCL. * Significantly different ($p < 0.05$) from the mean of the silence group (0 min). † Large effect ($\delta > 0.8$) of song playback. Data were normalized to the mean of the silence group. The dotted, horizontal line in each panel marks the normalized mean of the silence group (1.0).

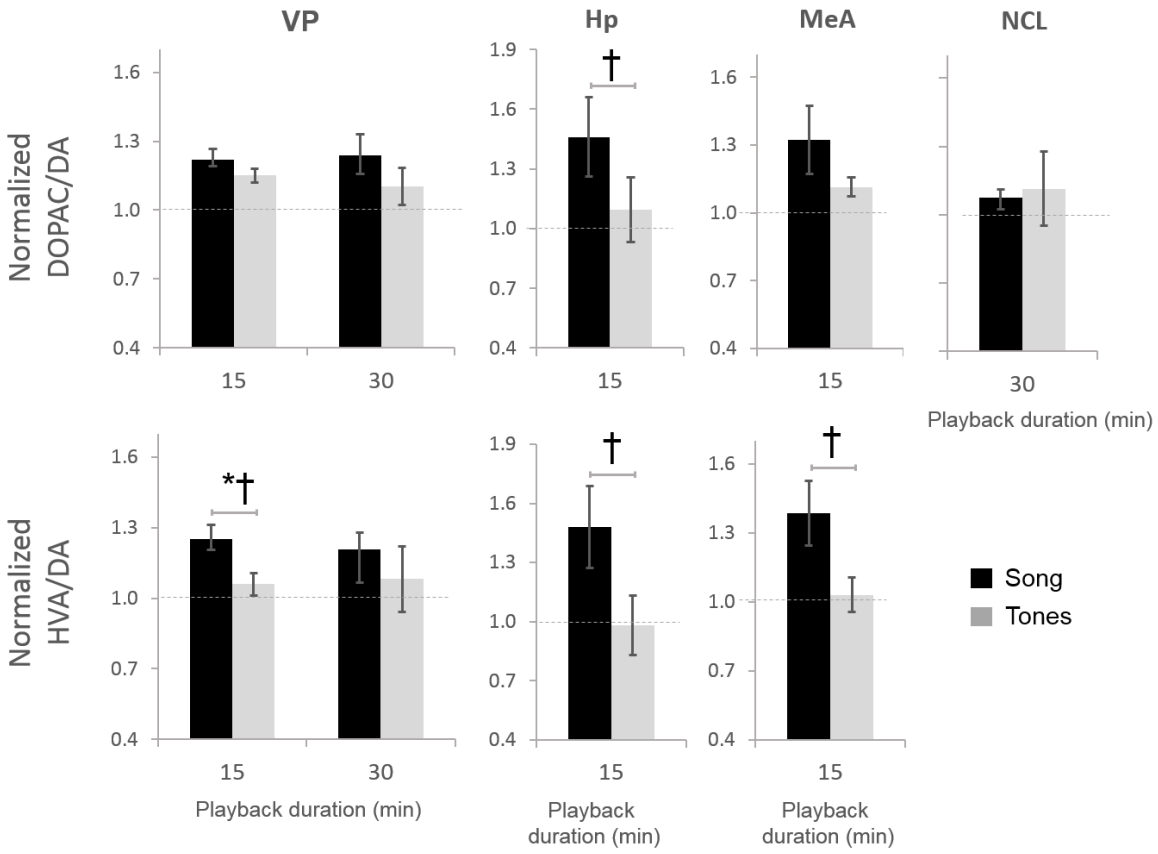

Figure 6. Effects of type of playback stimulus on dopaminergic activity in the reward pathway of estradiol-treated birds (tests of H2). This test was done only in ROIs in which H1 was supported (Fig. 5) and thus in which hearing song increased dopaminergic activity in E2-treated birds. In VP, HP, and MeA, dopaminergic activity was higher in response to song than in response to tones. * $p < 0.05$; † $\delta > 0.8$. Data are normalized to the mean of the silence group. The dotted, horizontal line in each panel marks the normalized mean of the silence group (1.0).

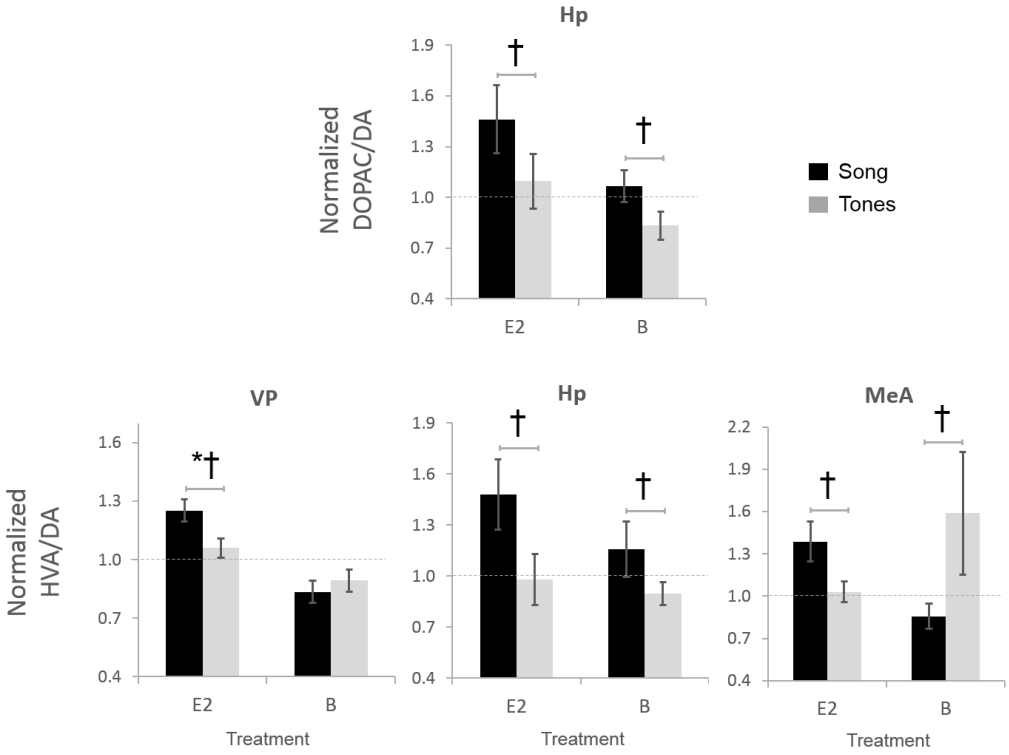

Figure 7. Interactions between endocrine state and type of playback stimulus on dopaminergic activity in the reward pathway (tests of H3). This test was done only in ROIs in which H2 was supported (Fig. 5), and thus in which dopaminergic activity in E2-treated birds was higher in response to song than in response to tones. H2 was not supported using birds that were exposed to stimuli for 30 minutes (Figure 6), and hence H3 was not tested using these birds. Significant effects of the interaction were found on HVA/DA in VP and MeA (gray, dashed boxes). In blank-treated birds, values of HVA/DA in VP were undistinguishable between birds that heard song and birds that heard tones; values of HVA/DA in MeA, in contrast, were greater in response to tones than in response to song. * $p < 0.05$; † $\delta > 0.8$. Data are normalized to the mean of the silence group. The dotted line in each panel marks the normalized mean of the silence group (1.0). E2, estradiol-treated birds; B, blank-treated birds. In all cases, the stimuli were presented for 15 minutes.

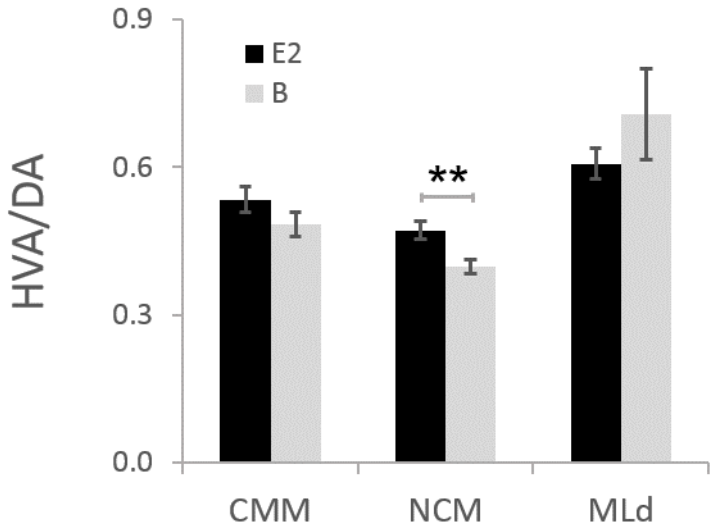

Figure 8. Effects of endocrine state on constitutive dopaminergic activity in the auditory system. HVA/DA in NCM was higher in estradiol-treated birds (E2) than in blank-treated birds (B). ** $p < 0.01$.

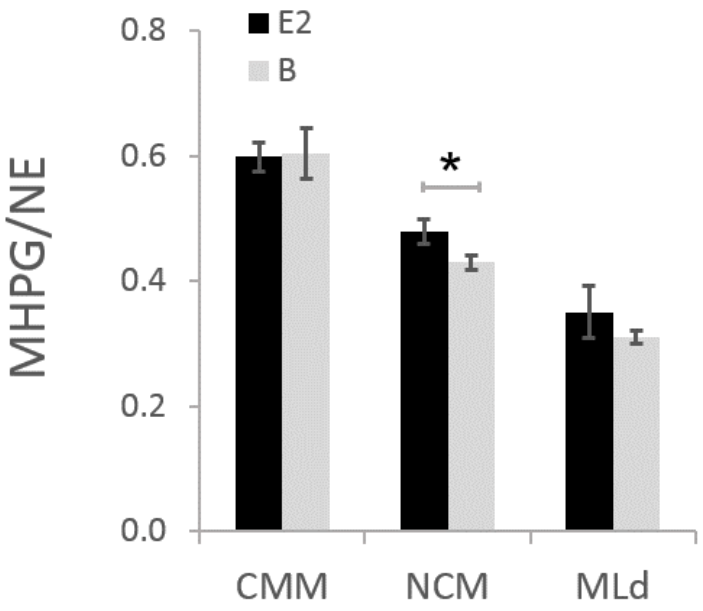

Figure 9. Effects of endocrine state on constitutive noradrenergic activity in the auditory system. MHPG/NE in NCM was higher in estradiol-treated birds (E2) than in blank-treated birds (B). ** $p < 0.01$.

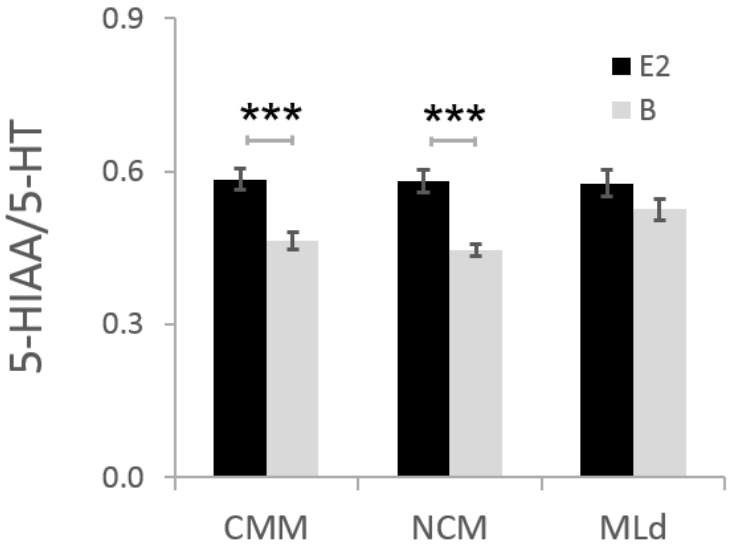

Figure 10. Effects of endocrine state on serotonergic activity in the auditory system. 5-HIAA/5-HT in CMM and NCM was higher in estradiol-treated birds (E2) than in blank-treated birds (B). * $p < 0.05$, *** $p < 0.001$.

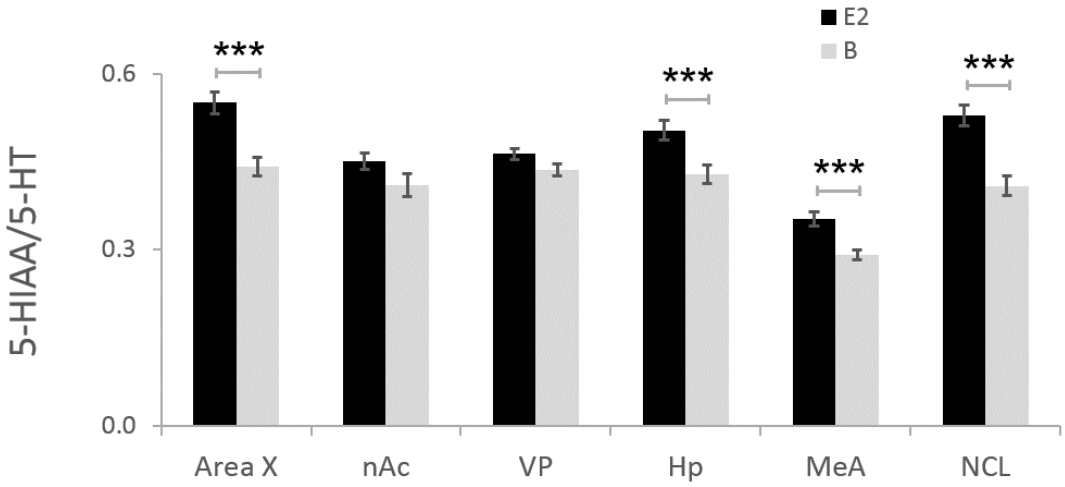

Figure 10. Effects of endocrine state on constitutive serotonergic activity in the reward pathway. 5-HIAA/5-HT was higher in Area X, Hp, NCL, and MeA in estradiol-treated birds (E2) than in blank-treated birds (B). *** $p < 0.001$.

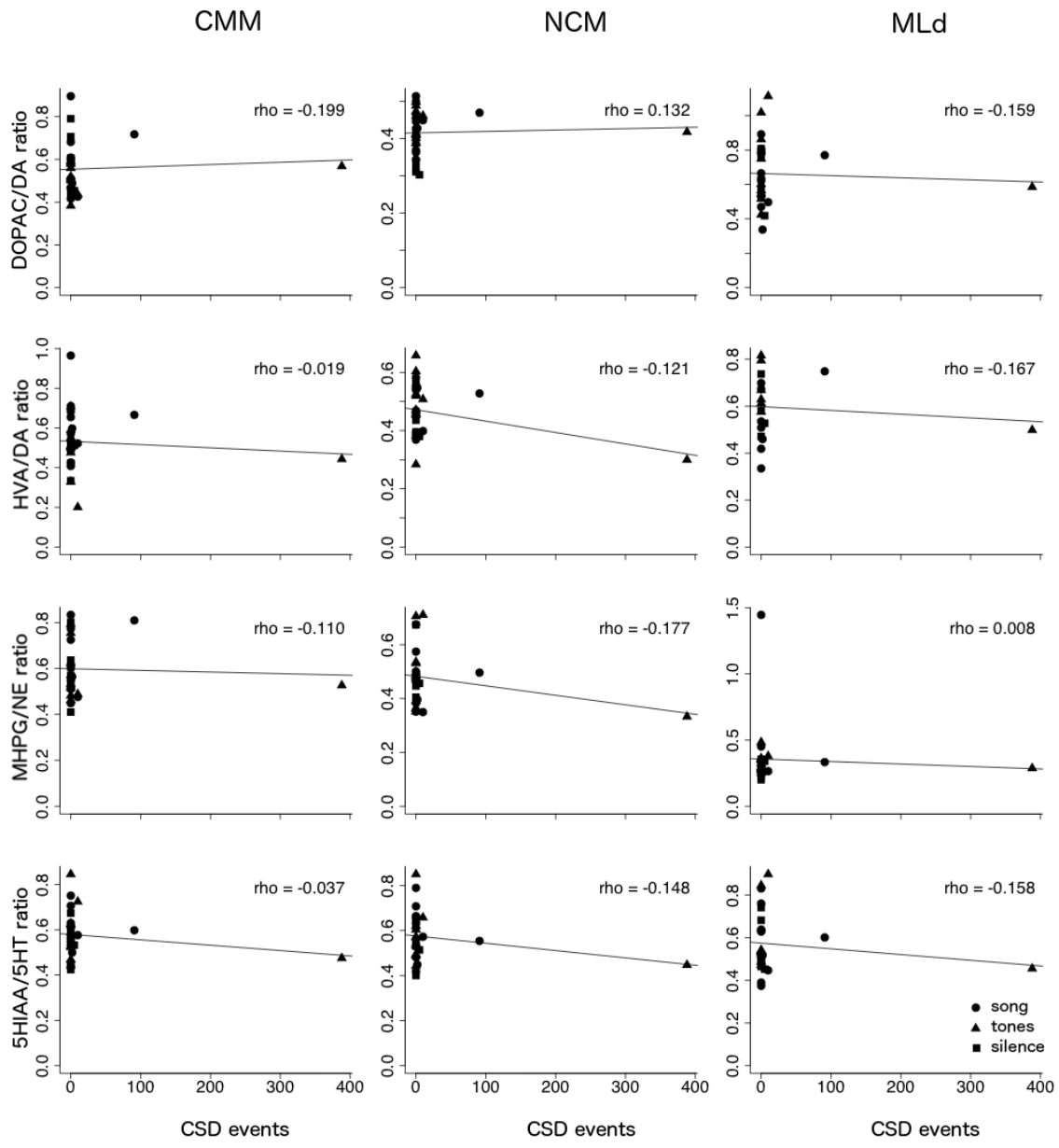

Figure 11. Tests for correlations between copulation solicitation display (CSD)-related events and monoaminergic activity in the auditory system. The number of events related to copulation solicitation display (CSD) given by estradiol-treated birds did not correlate with monoaminergic activity. The x-axis represents the sum of CSD-related visual displays (tail lifts and wing quivers).

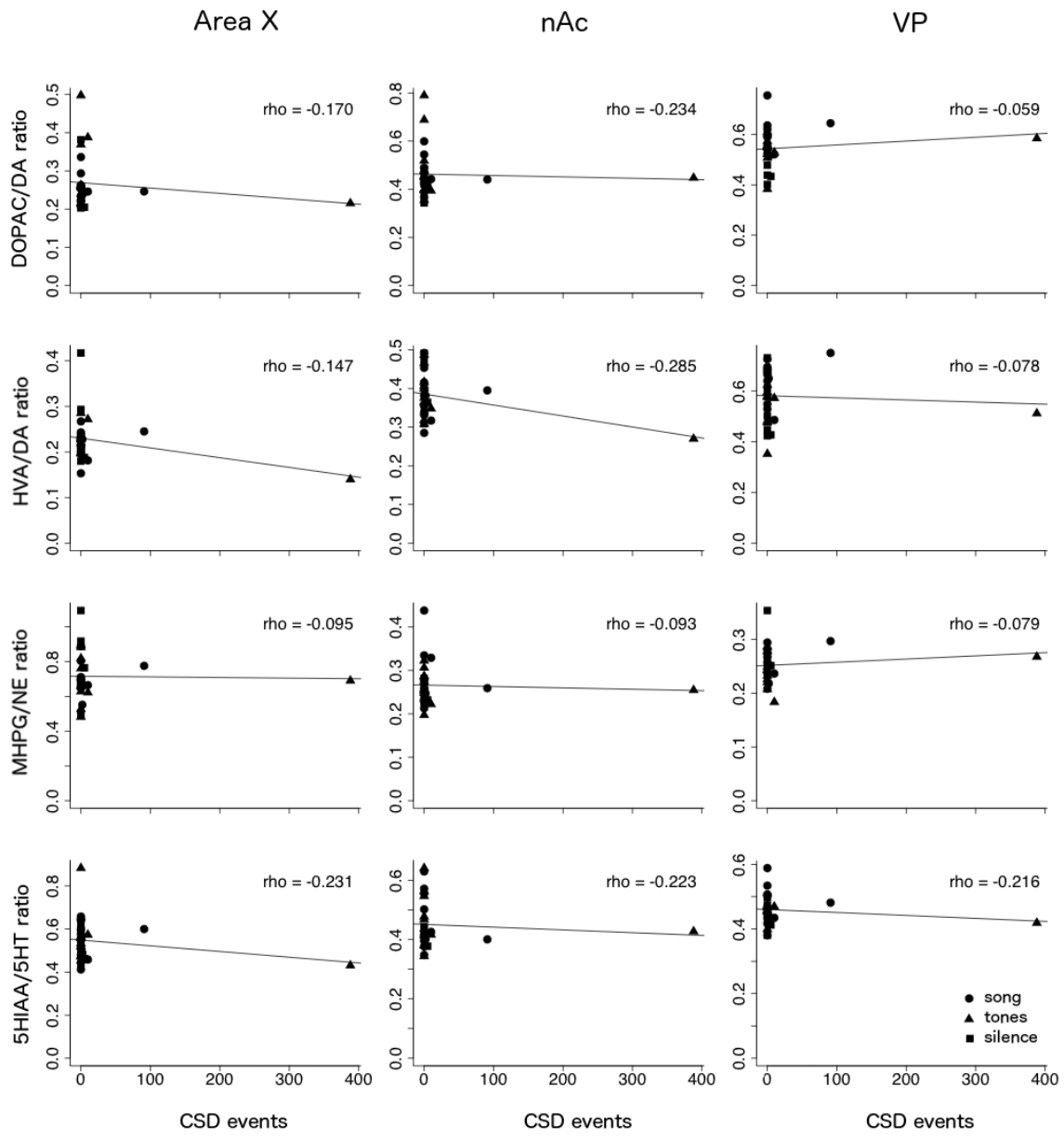

Figure 12. Tests for correlations between copulation solicitation display (CSD)-related events and monoaminergic activity in the reward pathway (see next page for continuation of figure). The number of events related to CSD given by estradiol-treated birds did not correlate with monoaminergic activity. The x-axis represents the sum of CSD-related visual displays (tail lifts and wing quivers).

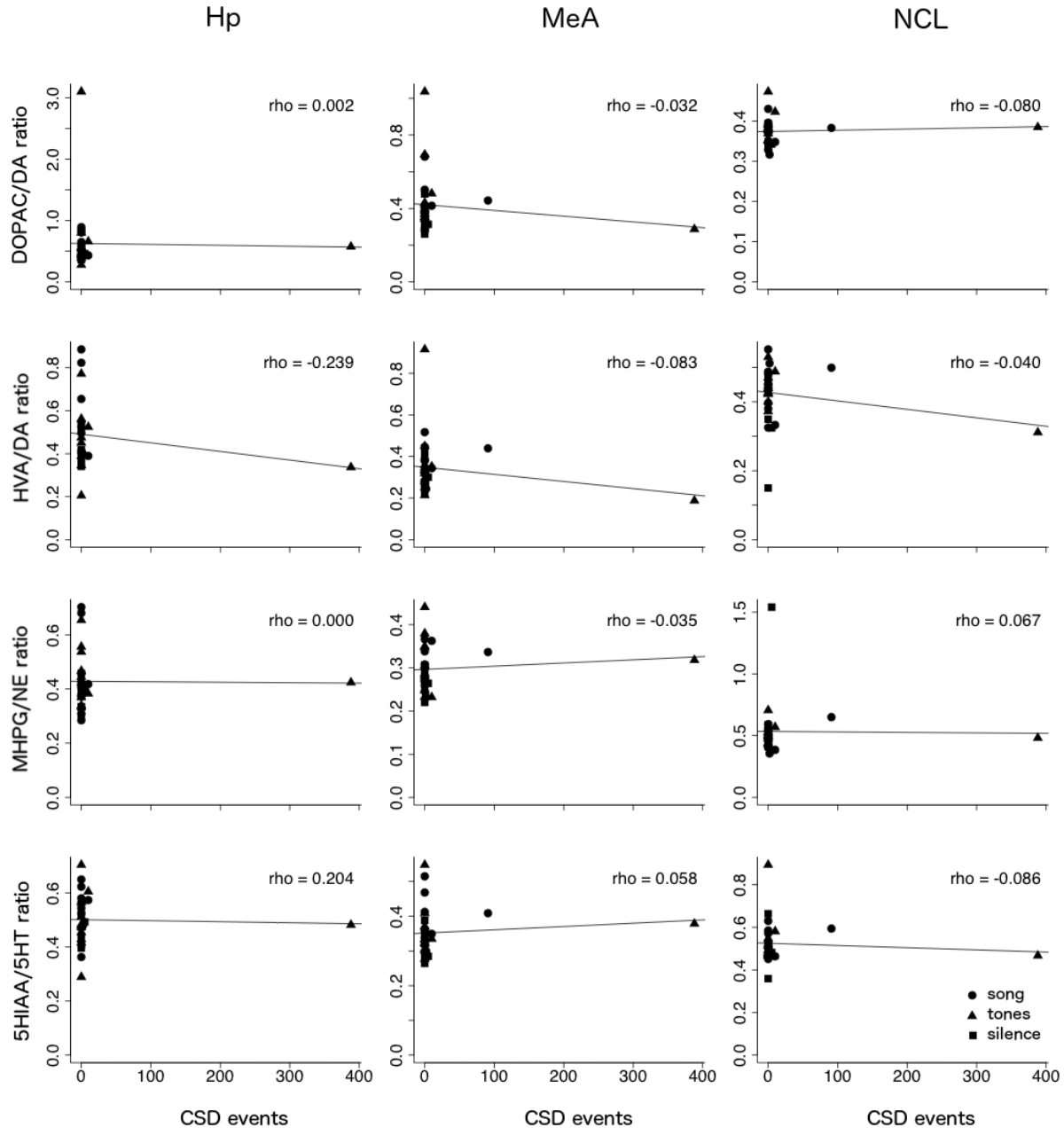

Figure 13 (continued).