Title: Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain.

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Abstract

Recent evidence shows that brain-derived steroids such as estrogens (‘neuroestrogens’) are controlled in a manner very similar to traditional neurotransmitters. The advent of in vivo microdialysis for steroids in songbirds has provided new information about the spatial and temporal dynamics of neuroestrogen changes in a region of the auditory cortex, the caudomedial nidopallium (NCM). Here, experiments using in vivo microdialysis demonstrate that neuroestradiol (E2) fluctuations occur within the auditory NCM during presentation of naturalistic auditory and visual stimuli in males, but only to the presentation of auditory stimuli in females. These changes are acute (within 30 min) and appear to be specific to the NCM, because similar treatments elicit no changes in E2 in a nearby mesopallial region or in circulating plasma. Further experiments coupling in vivo steroid microdialysis with extracellular recordings in NCM show that neuroestrogens rapidly boost auditory responses to song stimuli in females, similar to recent observations in males. We also find that the rapid actions of estradiol on auditory responses are fully mimicked by the cell-membrane impermeable estrogen biotinylestradiol, consistent with acute estrogen actions at the neuronal membrane. Thus, we conclude that local and acute E2 flux is regulated by convergent multi-modal sensory input, and that this regulation appears to be sex-specific. Secondly, rapid changes in local E2 levels in NCM have consequences for the modulation of auditory processing in females and males. Lastly, the rapid actions of neuroestrogens on NCM auditory processing appear to be mediated by a non-classical, membrane-bound estrogen receptor.

Keywords: Aromatase, Birdsong, Nongenomic, Multimodal
Introduction

The interconnected forebrain nuclei that control singing and song learning in the zebra finch are largely sexually dimorphic. The enlargement of these forebrain nuclei in males as compared to females was the first notable sexual dimorphism identified in the vertebrate brain (Nottebohm and Arnold 1976). The mechanisms that give rise to this striking dimorphism appear to be a blend of the actions of steroid hormones like estrogens (Holloway and Clayton 2001) and direct chromosomal effects (Agate et al. 2003). In contrast to the sexual dimorphism of the song pathway(s), the ascending circuits that process auditory signals (i.e., the cochlear nucleus (CN), midbrain mesencephalicus lateralis dorsalis (MLd), thalamic nucleus ovoidalis (Ov), and cortical nuclei Field L, NCM, and CMM) are largely similar in size and composition in adult males and females (Bailey and Wade 2005; Braun et al. 1991; Pinaud et al. 2006). In the absence of a pronounced anatomical dimorphism in auditory pathways, auditory processing demands that may differ between males and females could depend on the sex-specific actions of neuromodulators.

Estrogens are a candidate group of intrinsic cortical modulators, since they can be produced within brain circuits independent of the circulation (Remage-Healey et al. 2008; Schlinger and Arnold 1992). Aromatase, the enzyme that synthesizes estrogens, is expressed and active within presynaptic terminals in some neuronal circuits (Naftolin et al. 1996; Peterson et al. 2005; Rohmann et al. 2007; Saldanha et al. 2000). Alongside estrogen production in brain, novel actions have been identified in which estrogens rapidly modulate ion channel receptors on neuronal membranes. Estrogens can therefore act within extremely fast timescales to change the activity of neurons in the hippocampus, hypothalamus, hindbrain, and cortex (Herbison 2009; Kelly and Ronnekleiv 2009; Kuo et al. 2010; McEwen 2002; Meitzen and Mermelstein 2011; Remage-Healey and Bass 2007; Woolley 2007). These findings have bolstered the view
that some neural estrogen actions are in fact neuromodulatory rather than classically endocrine 
(Balthazart and Ball 2006; Garcia-Segura 2008; Remage-Healey et al. 2011b; Saldanha et al. 2011).

A current gap in the understanding of estrogens as neuromodulatory agents is how and whether they fluctuate in brain circuits in the manner of traditional neurochemicals. The vertebrate brain is capable of fast changes in estrogen production, as indicated by rapid shifts in enzymatic activity of the aromatase protein in brain tissue explants in response to social and/or sexual encounters (reviewed in Balthazart et al. 2006; Black et al. 2005; Cornil et al. 2005). A direct method to measure estrogen fluctuations in the forebrain has recently been developed and validated for songbirds (Remage-Healey et al. 2008). This method employs in vivo microdialysis coupled to sensitive ELISAs to allow direct monitoring of changes in neuroestradiol (E2) in awake, behaving animals engaged in behavioral tasks.

Recent experiments using in vivo microdialysis have shown that E2 in the auditory cortex of male zebra finches can change quickly in social contexts. Experiments focused on the secondary auditory cortex (caudomedial nidopallium or ‘NCM’) in adult males have demonstrated that E2 is elevated (<30 min) during brief social interactions with females as well as during acoustic playback of zebra finch songs (Remage-Healey et al. 2008). Furthermore, in vivo microdialysis coupled to extracellular recordings from neurons within the NCM has shown that auditory processing of zebra finch song is enhanced by the local production of neuroestrogens in adult males (Remage-Healey et al. 2010). These latter electrophysiological findings were simultaneously and independently confirmed by Tremere and Pinaud, using slightly different methods in NCM (multiunit array recordings coupled to microinjections; Tremere et al. 2009; Tremere and Pinaud 2011). Together, these studies indicate that estrogens are produced within the auditory cortex during auditory stimulation in males, and
appear to rapidly enhance the neural processing of auditory signals. The extent to which this neuromodulatory system in auditory NCM is activated in females and/or in other sensory contexts is unclear.

Here, we examine the sex-specificity of endogenous neuroestrogen fluctuations and neurophysiological actions in the zebra finch NCM, employing in vivo microdialysis technology. This study formally tests two hypotheses. First, because social interactions caused local $E_2$ elevation even in the absence of singing by focal males (see Remage-Healey et al. 2008), we tested the hypothesis that either visual or auditory stimulation alone can elicit acute $E_2$ fluctuations in the NCM of adult males and females. Second, because the results of this first experiment showed that auditory stimulation caused rapid elevations in NCM $E_2$ levels in females, we tested the hypothesis that the auditory-evoked activity of NCM neurons in females is modulated by fluctuating neuroestrogens, and that this modulation depends on neuronal membrane-specific actions in NCM.

**Materials and Methods**

**Subjects**

Adult zebra finches (> 130 days post-hatch) were taken for this study from breeding colonies at UCLA and UMass Amherst (14 L:10 D light cycle), and all animal care and use protocols were approved by the respective institutional animal care and use committees. In vivo microdialysis for changing neuroestrogen levels has been previously optimized in adult males of this species (Remage-Healey et al., 2008; 2010; 2011a). Animals recover quickly from surgery and are observed engaging in normal social and maintenance behaviors when tethered to the microdialysis apparatus.
Microdialysis

All procedures followed those validated previously, including cannula implantation (Remage-Healey et al. 2008; 2010; 2011a). Birds were anesthetized with equithesin and stabilized in a custom stereotaxic device with a head-angle of 45 degrees (Herb Adams Engineering) and kept warm with a heating pad. Lidocaine (2% in ethanol) was injected under the scalp and feathers were removed to expose the skull area dorsal to NCM. A CMA-7 microdialysis cannula (CMA Microdialysis) was then implanted into NCM and cemented in place with alternating layers of cyanoacrylate and dental cement (Perm Reline/Repair Resin; Coltene-Whaledent). The scalp incision was sealed with cyanoacrylate to the cement, and birds were returned to individual sound-attenuation chambers for post-surgical recovery (approx. 5 days).

Prior to an experimental session, the microdialysis probe, swivel, and FEP tubing were rinsed with 70% ethanol and ddH2O, and pre-filled with aCSF (199 mM NaCl; 26.2 mM NaHCO₃; 2.5 mM KCl; 1.0 mM MgSO₄; 2.5 mM CaCl; 11.0 mM glucose; 1% bovine serum albumin; pH = 7.4). Microdialysis probes were implanted at least 12 hr prior to an experimental sampling session to allow behavioral adaptation and for implantation-induced phenomena to subside. A CMA-7 microdialysis probe that had been pre-filled with aCSF at a flow rate of 2.0 μl/min (Harvard Apparatus 22 syringe pump) was implanted and birds were connected to the microdialysis system. All samples in this study were collected at 30-min intervals. At the end of each sampling period each sample was immediately sealed and stored at -80 °C. A previously-optimized ELISA protocol (Remage-Healey et al. 2008) using a commercially-available ELISA kit for 17β-estradiol (E₂; Cayman chemical) was used to determine fluctuating levels in plasma and dialysate samples in response to visual and acoustic stimuli. At the end of each experimental session birds were euthanized via isoflurane overdose and perfused through the heart with 0.9% PBS followed by 4% paraformaldehyde. Cannulae and probe were removed after...
perfusion to preserve the implantation site. Brains were cryoprotected, sectioned at 40-50
micron thickness, and sections were mounted onto gel-subbed superfrost slides (Fisher
Scientific), air dried, stained with thionin and photographed under light microscope to verify
probe placement. Histological analysis confirmed that probes were within the NCM region for all
birds in this study (with the exception of mesopallium controls, see below).

Auditory stimulation

Previous study of male zebra finches showed that acoustic playback of male song specifically
induced a rapid and robust elevation in E₂ levels within NCM (Remage-Healey et al. 2008).
Because the NCM of females also expresses aromatase (Peterson et al. 2005; Rohmann et al.
2007), we therefore tested whether the acute effects of song on NCM E₂ levels was sex-
specific. Dialyzed females were housed individually in sound attenuation chambers and
dialysate was collected for two 30-min silent periods prior to playback to establish baseline
conditions (‘pre 1’ and ‘pre 2’). Dialysate was then collected for 30 min from females exposed to
acoustic playback of either male song (1-min recording of intermittent male song from three
individual males, repeatedly looped 30x; n = 8) or white noise (1-min intermittent white noise
pulses matched to the duration and inter-stimulus interval of male song, repeatedly looped 30x;
n = 7). Stimuli were band-pass filtered (1 kHz-12kHz, CoolEdit Pro; identical to procedures in
Remage-Healey et al., 2008) and were played back through a calibrated speaker inside each
chamber at ~ 70 dB. After the playback period dialysate was again collected for two 30-min
silent periods (‘post 1’ and ‘post 2’). All trials were videotaped through a one-way glass partition
and behaviors were monitored offline. Blood samples were drawn from a subset of females (n =
6) at the end of ‘pre 1’ and ‘male song’ playback periods, respectively, to determine effects of
song on circulating E₂ levels.
Auditory playback elicits acute changes in NCM E2 levels, in both males (Remage-Healey et al. 2008) and females (present study). In addition, social interactions with females, accompanied by singing in some but not all cases by the focal male, caused acute elevations in NCM E2 levels in males (Remage-Healey et al. 2008). Because of the potential multimodal (i.e., visual and auditory) input to NCM (see Avey et al. 2005; Kruse et al. 2004) we therefore tested whether visual stimuli alone can elicit acute changes in NCM E2 levels in both male and female adult zebra finches using in vivo microdialysis. The sampling design of these experiments mirrored the above design for acoustic playbacks. A thin-film transistor (TFT) LCD monitor (refresh rate > 75 Hz; Dell Corp) was inserted into the bird’s chamber prior to lights-on, and an opaque barrier was placed on the monitor to restrict the viewing range to a 4” x 4” window at the center of the screen. Video playback of conspecific and heterospecific stimuli using TFT displays elicits prosocial behaviors in both male and female zebra finches (i.e., social vocalizations and beak-wiping; Campbell and Hauber 2010; Galoch and Bischof 2007), indicating that this paradigm reasonably approximates natural social stimulation. Dialysate was collected for two 30-min periods to establish baseline (‘pre 1’ and ‘pre 2’) during which the TFT display broadcasted a constant black background. During the video playback period, dialysate was collected for 30 min while one of the following video clips (Sony PMB 5.0 software) was presented per experimental subject: 1) one female zebra finch expressing prosocial behaviors (n = 7 female microdialysis subjects; n = 11 male microdialysis subjects); 2) one actively courting male zebra finch (n = 8 female microdialysis subjects; n = 7 male microdialysis subjects); 3) one courting male Bengalese finch (n = 5 male microdialysis subjects). Each video stimulus consisted of 5-min clips of behaving animals interspersed with 1-2 min constant black background, and video stimuli were repeatedly broadcast until the end of the 30 min trial. After the video playback period, dialysate was again collected for two 30-min periods (‘post 1’ and
‘post 2’) during which a constant black background was broadcast through the TFT display. No auditory playback occurred during video presentations. All trials were videotaped through a one-way glass partition and behaviors were monitored offline. All subjects were observed visually scanning the TFT display during video playbacks. Importantly, 7 out of 11 microdialysis males who were exposed to female visual stimuli engaged in limited courtship singing, consistent with earlier work showing that visual stimulation elicits the full complement of courtship singing behavior (Galoch and Bischof 2007). The effects of video presentation on calling and singing behaviors by the microdialyzed males were analyzed to assess the potential contribution of auditory-self stimulation on NCM E2 levels.

Mesopallium control experiment

The spatial specificity of changing NCM E2 levels was determined in a separate set of n = 6 female subjects each for both visual and auditory playback treatments (as above). Cannulae were surgically implanted and directed toward the mesopallium (lacking in aromatase) anterior to NCM and Field L. Histological analyses of all control birds confirmed that probes were targeted to mesopallium in the vicinity of caudomedial mesopallium (CMM) and in some cases the probe lesion extended slightly ventral into the underlying anterior nidopallium (in no case was the probe sampling from NCM in these mesopallium control birds).

Electrophysiology

The auditory playback study with females showed that E2 levels are specifically elevated in the NCM of females during song playback. It has been recently established that both exogenous and endogenous neuroestrogens can exert rapid actions on the firing frequency of NCM neurons (Remage-Healey et al. 2010; Tremere et al. 2009; Tremere and Pinaud 2011). These
combined observations lead to the prediction that estrogens exert rapid modulation of auditory
processing in the NCM of females. This hypothesis was tested using unilateral multiunit
extracellular recordings in NCM combined with ipsilateral reverse microdialysis (retrodialysis) of
either the predominant neuroestrogen E₂ (30 ug/ml; n = 7), an abundant neuroandrogen 5β-
dihydrotestosterone (5βDHT; 30 ug/ml; n = 3), or the estrogen synthesis inhibitor Fadrozole
(FAD; 100 uM; n = 3).

All electrophysiology procedures followed a protocol previously established for males (Remage-
Healey et al. 2010). For craniotomy surgery to expose NCM, birds were anesthetized with 20%
urethane via intramuscular injections (3x 30 ul injections over 2hr period). Lidocaine (2% in
ethanol) was injected under the scalp and feathers were removed to expose the skull area
dorsal to NCM. A midline incision and craniotomy exposed the point-of-origin and the location of
NCM was stereotaxically marked using a marking pipette. A stainless steel headpost (Herb
Adams Engineering) was attached to the rostral skull via dental cement/cyanoacrylate. The dura
mater was carefully dissected over the NCM region. The bird was transferred to a custom
headpost stage (Herb Adams Engineering) inside a sound attenuation chamber and kept warm
with a heating pad (FHC Neurocraft). A CMA-7 microdialysis probe pre-filled with aCSF (as
above) was advanced into NCM with a motorized micromanipulator (Warner Instruments)
equipped with a custom mounting clip. During a 60-min stabilization period dialysate was
collected to allow implantation induced phenomena (i.e., neurochemical release and excitability
due to probe implantation) to subside. A hydraulic micromanipulator (Narishige) was then used
to advance a carbon fiber electrode (Carbostar-1, Kation Scientific) into NCM immediately
adjacent to the retrodialysis probe. Neuronal activity was band-pass filtered (0.3-5 kHz),
digitized at 20 kHz (Micro 1401, Cambridge Electronic Design), and stored on a computer using
Spike 2 software. Auditory-responsive NCM neurons were located using playback of filtered,
band-passed white noise stimuli.
To test for steroid-dependent auditory modulation, experimental subjects were exposed to four acoustic stimuli: two distinct conspecific songs (CON1, CON2), a conspecific song played in reverse (REV), and white noise (WN). All stimuli were band-pass filtered (0.5-10 kHz) and were presented in randomized order at an interstimulus interval of 10 ± 2 sec at ~ 70 dB SPL via an audio amplifier connected to a speaker inside the recording chamber. In contrast to triple song used for microdialysis (above), song stimuli used in the electrophysiology experiment were of brief duration (~ 2 sec) to facilitate analysis of changes in auditory-evoked activity of NCM neurons. Electrophysiology experiments were divided into 3 successive 30 min playback periods, such that each playback stimulus was presented 20x within a 15 min block (after an initial 15 min period to allow drug wash-in or wash-out, for a total treatment period of 30 min). During playbacks and recordings aCSF was retrodialyzed for the first 30 min to determine baseline responsiveness, followed by 30 min retrodialysis of either E₂, 5βDHT, or FAD, followed by a 30 min washout period of aCSF alone. Each animal was treated with one compound exclusively. All recordings were analyzed offline by experimenters blind to treatment condition. At the conclusion of each experimental session, the NCM recording site was lesioned (+10 uA for 8 sec) for verification of probe and electrode placement. All recordings in this study were histologically confirmed to be within the NCM.

Bilateral recordings

A separate set of adult females (n = 3) were used for dual-hemisphere recordings and retrodialysis. Surgery, recordings and playback experiments were conducted as above, with the exception that craniotomy exposed the NCM in both left and right hemispheres. Extracellular electrodes were advanced into each NCM hemisphere, and a microdialysis probe was advanced into the left NCM. Following a 60-min stabilization period and baseline aCSF dialysis/playback recordings as above, biotinylated estradiol (E₂-biotin; Steraloids E1360-100;
60 ug/ml in aCSF) was retrodialyzed for 30 min for playback/recordings. Biotinylated estradiol is a membrane impermeable conjugate that has been demonstrated to act specifically at membrane estrogen binding sites (Dewing et al. 2007; Germain et al. 1993) and was used to test for membrane-specific effects of E$_2$.

**Electrophysiology analyses**

The voltage threshold for detecting multiunit responses in NCM was set by the experimenter to exclude low amplitude events (i.e., spikes with less than ~2:1 peak amplitude:background noise ratio) following established protocols (Coleman et al. 2007; Remage-Healey et al. 2010). For each recording session threshold levels were maintained for the entire duration of recordings, so that all sampling periods per experiment (e.g., aCSF, E$_2$, washout) were analyzed with the same voltage threshold. Suprathreshold multiunit activity was then used to compute average response strength (RS) for each 30 min recording. For each playback event, the mean firing rate for 2 sec immediately prior to playback stimulation was subtracted from the mean firing rate for 2 sec during auditory stimulation, resulting in RS. The RS metric therefore accounts for baseline firing rate, and average RS was computed for the entire 20 presentations for each stimulus during each treatment period. Because NCM neurons can habituate to repeated presentation of song stimuli (Chew et al. 1995) the root mean square (RMS) amplitude of NCM multiunit activity was analyzed for all treatment periods. The slope of the decay in RMS amplitude over 20 stimulus presentations is an indication of auditory habituation (Chew et al. 1995; Phan et al. 2006), and was therefore analyzed for all treatment periods for changes in auditory-evoked activity (RMS for 2 seconds prior to the stimulus subtracted from RMS during the 2-sec auditory stimulus).

**Statistical analyses**
Statistical analyses were carried out offline using Statview 4.5 (Abacus) and R (GNU project). Both dialysate samples and multiunit response strength measures (see below) were analyzed using multivariate repeated-measures ANOVAs for within-subject measurements over time with successive sampling periods grouped as a single dependent variable. ANOVAs for dialysate measures were computed for within- and between-subject variability for the treatment period and the preceding sampling period only (e.g., 30 min playback and 30 min pre). Results for all samples including pre1&2 and post1&2 are presented in figures to illustrate time-dependent variability. When overall F-tests were significant for independent treatment effects they were followed by appropriate Wilcoxon signed rank post hoc tests (WSRT) for the effects of treatment (e.g., 30 min male song playback vs. 30 min pre) for a given playback stimulus (e.g., male song, REV, etc.). WSRTs are not robust to sample sizes < 5 (Sokal and Rohlf 1981), so for the electrophysiology experiment with FAD, data were combined from all playback groups for each treatment period using a pooled WSRT (i.e., CON, REV, WN grouped for aCSF vs. FAD). The same procedure for a pooled WSRT for all playback groups was computed for the electrophysiology experiment with E2-biotin.

Results

No sex difference in baseline NCM neuroestrogen levels

A subset of in vivo microdialysate samples were collected from the NCM of both males and females and run simultaneously in the same set of ELISA plates. Analysis of these samples provided the opportunity to test for potential sex differences in neuroestradiol (E2) levels at baseline (i.e., in the absence of any experimental manipulation) in vivo, as predicted by sex differences in the expression of aromatase fibers and synaptic terminals in NCM (Peterson et al. 2005; Rohmann et al. 2007; Saldanha et al. 2000) and reported sex differences in gonadal E2
secretion (Adkins-Regan et al. 1990; Agate et al. 2003; Naguib et al. 2004). In contrast to the circulation, we did not detect a significant sex difference in dialysate E_2 levels from NCM at baseline (males: 13.95 ± 1.68; n = 12; females: 12.29 ± 3.14; n = 10; mean ± SEM; p = 0.65 for unpaired t-test), indicating that baseline levels in NCM are not different between adult males and females. Instead, the presence of sex differences in synaptic aromatase in NCM may be associated with rapid fluctuations in neuroestrogens in the NCM that are sex-specific, as presented below.

Auditory playback elevates NCM neuroestrogens in females

Aromatase is expressed in both neuronal cell bodies and presynaptic terminals in the NCM of adult male and female zebra finches (Peterson et al. 2005) and adult males exhibit acute elevation in NCM E_2 levels in response to 30-min of song playback (Remage-Healey et al. 2008). Because NCM is a site of auditory processing of complex stimuli in both sexes, we tested the hypothesis that auditory activation of NCM in females also elicits acute changes in local E_2 levels. Playback of auditory stimuli to awake, behaving microdialyzed females caused significant changes in local E_2 levels within NCM (Fig. 1; Two-way repeated measures ANOVA, playback effect: F = 10.497; p = 0.007; stimulus effect: F = 0.191; p = 0.669; playback*stimulus interaction: F = 12.719; p = 0.004). Post hoc analysis revealed that local E_2 levels were elevated during the 30-min audio playback period of male song (p = 0.036; relative to the pre2 period, within subject) but not during white noise playback (p = 0.866; relative to the pre2 period, within subject). Plasma E_2 levels in a separate set of n = 6 females were unchanged following male song playback (pre playback: 33.35 ± 8.94 pg/ml; post playback: 29.79 ± 11.40 pg/ml), suggesting that circulating E_2 does not co-vary with fast changes in E_2 levels measured using in vivo microdialysis within NCM (for similar results of NCM vs. plasma E2 levels in male zebra
finches, see Remage-Healey et al. 2008). Therefore, similar to adult males, an acute rise in
NCM E2 levels occurs in females during 30 min of exposure to conspecific song stimuli.

Neither visual nor auditory stimulation alters neuroestrogens in the mesopallium of females

The spatial specificity of changing E2 levels in NCM observed above for females was tested in a
new group of females with probes directed at the mesopallium anterior to NCM (n = 6). Auditory
playback of either male song or white noise caused no significant changes in local E2 levels
within the anterior mesopallium (Fig. 2A; Two-way repeated measures ANOVA; playback effect:
F = 1.231; p = 0.337; stimulus effect: F = 1.232; p = 0.329). Similarly, visual presentation of
either conspecific males or females (as described below) caused no significant changes in local
E2 levels within the anterior mesopallium (Fig. 2B; Two-way repeated measures ANOVA ;
presentation effect: F = 1.065; p = 0.360; stimulus effect: F = 0.385; p = 0.569). Therefore, the
acute changes in local E2 levels observed within NCM of females in response to male song
playback were not accompanied by similar changes in a nearby mesopallial region, indicating
that neuroestrogen changes in response to auditory or visual stimuli are specific to NCM.

Visual playback elevates NCM neuroestrogens in males but not females

Local E2 levels in NCM of males are acutely elevated during social interactions with females,
and in some males this response occurs in the absence of courtship singing (Remage-Healey et
al. 2008). We reasoned therefore that a visual stimulus of a conspecific alone could elicit
changes in local E2 levels within NCM. We tested this hypothesis in both male and female adult
zebra finches with in vivo microdialysis. In males, video playback of conspecific stimuli caused
significant changes in local E2 levels within NCM (Fig. 3A; Two-way repeated measures
ANOVA; presentation effect: $F = 5.203$; $p = 0.034$; stimulus effect: $F = 1.258$; $p = 0.278$). Post hoc analysis revealed that local $E_2$ levels were elevated in response to female video playback ($p = 0.013$; relative to the pre2 period, within subject) but not in response to male video playback ($p = 0.236$; relative to the pre2 period, within subject). During the video presentation of females, 7 out of 11 males vocalized to the stimulus, including courtship song. Because auditory activation of NCM with song stimuli can elicit robust increases in NCM $E_2$ levels in males (Remage-Healey et al. 2008), we tested for the effects of self-stimulation on changing $E_2$ levels. Vocal behavior during trials was analyzed for total vocalizations (number and duration) and for the occurrence and duration of primary vocalization subtypes (tets, long calls, songs, chirps; categories after Zann 1996). There was no significant difference between singing vs. non-singing males in NCM $E_2$ levels during the 30-min female video trial (unpaired t-test: $T = 0.63$; df $= 9$; $p = 0.54$). Variation in individual vocalization data and $E_2$ levels was also tested with linear regression. We analyzed $E_2$ levels both during the female video stimulation for each male and as a function of the transition in $E_2$ levels from baseline to determine the extent of changes in $E_2$ levels that are accounted for by vocal behavior of the microdialyzed male (self-stimulation). All regression tests for the effects of vocalizations (tets, long calls, songs, chirps) on $E_2$ levels during video playback were non-significant (all $p > 0.20$), indicating that any direct effect of self-stimulation (auditory) on NCM $E_2$ levels was negligible.

In microdialyzed females, in contrast to male subjects, video playback of conspecific stimuli caused no changes in local $E_2$ levels within NCM, in response to either male or female video stimuli (Fig. 3B; Two-way repeated measures ANOVA; presentation effect: $F = 0.738$; $p = 0.406$; stimulus effect: $F = 0.007$; $p = 0.935$). Similarly, video playback of a control heterospecific stimulus (Bengalese finch) to male microdialysis subjects did not cause changes in local $E_2$ levels (Fig. 3A inset; $F = 1.241$; $p = 0.333$). Therefore, these results indicate that males exhibit
Electrophysiology

Neuroestrogens rapidly modulate auditory responsiveness in the NCM of females

Because E$_2$ levels are acutely elevated in the NCM of females during auditory playback (above) we reasoned that neuroestrogens rapidly modulate auditory-evoked activity of neurons within NCM. This hypothesis was tested in a new group of females using combined extracellular electrophysiology and retrodialysis of either E$_2$, 5βDHT, or FAD. In anesthetized females, both up- and down-regulation of local neuroestrogen levels produced rapid changes in NCM response strength (RS; see Methods). Retrodialysis of E$_2$ caused a significant change in NCM auditory-evoked response strength (RS; Fig. 4; Two-way repeated measures ANOVA; E$_2$ treatment effect: $F = 19.996; \ p < 0.0001$; playback stimulus effect: $F = 1.128; \ p = 0.357$), and post-hoc analysis revealed that RS was significantly elevated during 30 min of E$_2$ treatment (relative to the ‘aCSF’ period) for both CON1 ($p = 0.028$) and CON2 ($p = 0.042$) playback stimuli. RS during playback of REV ($p = 0.091$) and WN ($p = 0.063$) stimuli were not significantly elevated, but showed similar overall increases in response to E$_2$.

Similarly, when endogenous neuroestrogen levels were suppressed with 30-min treatment with the aromatase inhibitor FAD, RS was significantly and rapidly decreased to all playback stimuli (Fig. 5; Two-way repeated measures ANOVA; FAD treatment effect: $F = 36.247; \ p < 0.0001$; playback stimulus effect: $F = 1.287; \ p = 0.343$; pooled WSRT $p = 0.0037$ for FAD vs. aCSF for all playback stimuli). We note that a pooled post hoc test should be interpreted with caution, and that an overall downward trend in RS that occurred for all treatments in the FAD experiment is
suggestive of response habituation to repeated presentation of the same auditory stimuli.

However, detailed analysis showed that neural habituation was minimal for all stimuli and treatment groups (see below). In summary, up-regulation of local neuroestrogen levels with E$_2$ retrodialysis caused rapid increases in NCM auditory responsiveness, while down-regulation of local neuroestrogen levels with FAD retrodialysis were associated with rapid decreases in NCM auditory responsiveness.

In contrast to the rapid actions of neuroestrogens, there was no effect of a brain-derived androgen similarly derived from testosterone (5βDHT) on RS to any playback stimuli (Fig. 4 inset; Two-way repeated measures ANOVA; 5βDHT treatment effect: F = 0.036; p = 0.854; playback stimulus effect: F = 0.658; p = 0.601). This indicates that the observed acute effects of E$_2$ are steroid-specific. Importantly, analysis of neuronal habituation to repeated presentation of auditory stimuli revealed a shallow habituation rate (as measured by the slope of RMS amplitude vs. stimulus iteration for each sampling period; all slopes < 0.0128). Negligible habituation to the auditory stimuli presented in this study was expected based on the recording site (ventral NCM) and behavioral state (anesthetized; see Chew et al. 1995; Phan et al. 2006) of our study animals (see also Remage-Healey et al., 2010 for similar findings with males). Also, habituation rate did not significantly change in the presence of E$_2$, 5βDHT, or FAD (all p > 0.38 for one-way repeated measures F-tests). Similarly, baseline firing rate (i.e., in the absence of auditory playback stimulation) was not significantly altered by E$_2$, 5βDHT, or FAD treatments (all p > 0.54 for repeated measures F-tests). Therefore, the rapid actions of neuroestrogens on NCM neurons were restricted to the modulation of auditory-evoked activity.

Lateralized, rapid actions of a membrane impermeable estrogen
To test for possible rapid E\textsubscript{2} actions at putative neuronal membrane receptors, the membrane-impermeable biotinylated E\textsubscript{2} (E\textsubscript{2}-biotin) was retrodialyzed unilaterally into NCM in a separate set of anesthetized females. Bilateral electrode placement into each NCM hemisphere allowed recordings from one electrode ipsilateral and one contralateral to the site of local E\textsubscript{2}-biotin delivery. Results of this experiment showed a significant effect of E\textsubscript{2}-biotin on RS (Fig. 6; Three-way repeated measures ANOVA; hemisphere effect: $F = 15.796; p = 0.001$; E\textsubscript{2}-biotin treatment effect: $F = 3.354; p = 0.085$; playback stimulus effect: $F = 1.869; p = 0.175$) and a significant E\textsubscript{2}-biotin treatment*hemisphere interaction ($F = 7.773; p = 0.013$). The pooled WSRT for all treatments revealed that RS was rapidly elevated during E\textsubscript{2}-biotin treatment relative to the aCSF period only in the ipsilateral NCM ($p = 0.018$) and not contralateral hemisphere NCM ($p = 0.272$). Thus, rapid E\textsubscript{2} effects observed in females were fully mimicked by a membrane impermeable biotinylated E\textsubscript{2}, and were limited to the hemisphere NCM that was ipsilateral to the E\textsubscript{2}-biotin delivery site.

**Discussion**

Motor circuits devoted to vocalization present some of the most striking sexual dimorphisms found in the brain of vertebrates, with males typically expressing motor nuclei many-fold larger than females (e.g., Kelley and Bass 2010; Nottebohm and Arnold 1976). By contrast, the circuits that process sensory input, including the auditory NCM of songbirds, are largely similar in structure and composition between the sexes. Even still, sensory regions may be differentially modulated in males vs. females by neurochemicals to aid in sex-specific neural and behavioral tasks. Neuroestradiol may be one such neuromodulator due to its unique chemical properties, its presence at neuronal synapses, and its direct association with sex differences in nervous system structure and function (Balthazart and Ball 2006; Remage-Healey et al. 2011b; Saldanha et al. 2011; Sisneros et al. 2004).
Here, we present evidence that neuroestrogen fluctuations and actions in the auditory cortical nucleus NCM of zebra finches exhibits several properties that are similar between adult males and females, with one notable exception in the context of visual stimulation. Prior work had established that, in males, neuroestradiol (E2) levels are locally elevated within NCM when subjects hear song but not control white noise (Remage-Healey et al. 2008). In this study, this property of NCM is now extended to females, which exhibit the same acute auditory selectivity for song vs. white noise in terms of rapid E2 elevation. Secondly, prior work had established that E2 rapidly boosts (while inhibition of neuroestrogen production rapidly suppresses) the auditory-evoked firing rate of NCM neurons in males (Remage-Healey et al., 2010; see also pooled data for both males and females in Tremere et al. 2009). This property of NCM is also now formally extended to females in this study. Moreover, the same direction of the effects of neuroestrogen up- and down-regulation on the auditory-evoked firing rate of NCM neurons is observed in females as in males. Also, as in males, the rapid effect of E2 on NCM neurons is steroid-specific, because a steroid product similarly derived from testosterone in the brain, 5β-DHT, caused no significant changes in neurophysiological activity. Together this evidence therefore indicates that rapid neuroestrogen fluctuation in NCM (as measured by microdialysis) has a primary role in auditory processing of song in a rapid, neuromodulatory manner (as measured by electrophysiology), regardless of sex. These results further reinforce the primacy that neuroestrogens have in the modulation of auditory information in the songbird brain.

Source of estrogens measured in NCM in females

All of the females used in these studies were gonadally intact, and therefore some of the rapid elevations in E2 measured within NCM in response to male song playback could be of ovarian origin. Indeed, circulating sex steroids secreted from the gonads or other steroidogenic glands can shift rapidly in response to changes in the social environment and/or auditory cues (Harding...
Moreover, in female zebra finches systemic E2 levels are elevated in response to repeated playback of male song, but this was observed over a period of 5 days of exposure (Tchernichovski et al. 1998). Two results from this study are consistent with rapid (> 30 min) fluctuations observed in NCM that are intrinsic to NCM and independent of circulating E2. First, plasma E2 levels were unchanged in females that were exposed to 30 min of male song playback relative to pre-playback. Second, E2 levels in the mesopallium near NCM were unchanged during male song playback. These findings are similar to observations in males, in which both plasma E2 levels and E2 levels outside NCM remained unchanged during male song playback (Remage-Healey et al. 2008). Together, these results are consistent with a local, rapid modulation of neuroestrogen levels intrinsic to the NCM region of both male and female zebra finches.

Aromatase expression (both somal and pre-synaptic) is virtually absent in the mesopallium of female zebra finches (Saldanha et al. 2000), which is consistent with the current observations of generally lower baseline E2 levels and lack of acute changes in E2 in the mesopallium, in comparison with NCM. E2 levels in the mesopallium therefore originate either in the circulation or nearby brain regions, or via a combination of the two. By contrast, the NCM of females appears to be synthesizing neuroestrogens locally and acutely (independent of the circulation), just as has been observed in males. One major difference between males and females, however, is that circulating androgen levels are low in females (Agate et al. 2003; Naguib et al. 2004) and therefore are not as likely to provide a sustainable supply of aromatizable substrate to the female NCM (in contrast to males for whom circulating androgens are abundant). Therefore, in contrast to the traditional view that the female vertebrate brain is thought to respond exclusively to estrogens synthesized in the ovary, the results of this study lead us to the hypothesis that both neuroandrogens and neuroestrogens originate within the female NCM.
itself. Although this hypothesis awaits an explicit experimental test, the critical enzymes for neuroandrogen production are expressed in the zebra finch CNS (London et al. 2003; London et al. 2006) and can be rapidly regulated in the songbird brain (Pradhan et al. 2010; Soma et al. 2004). How these androgen synthetic enzymes coordinate with aromatase in the functional synthesis of neuroestrogens remains an intriguing and active area of research.

Rapid electrophysiological actions of neuroestrogens

Previous work in this species has shown that estrogens modulate auditory-evoked activity of NCM neurons on a fast timescale (i.e., within 5-30 min Remage-Healey et al. 2010; Tremere et al. 2009). This rapid action is confirmed here for females, and is inconsistent with a ‘classical’ genomic mechanism of estrogen action, which depends on gene transcription events and is typically manifest within hours. A multitude of alternative, ‘nonclassical’ modes of estrogen action have been documented in the vertebrate brain, including recruitment of signaling cascades via actions at the neuronal membrane (Meitzen and Mermelstein 2011; Moenter and Chu 2011; Roepke et al. 2011). The current study shows that the rapidity of the actions of estradiol in the NCM can be accounted for by putatively membrane-specific actions. Specifically, the membrane-impermeable conjugate biotinylated- E$_2$ fully mimicked the rapid actions of unconjugated E$_2$ itself on NCM neuronal activity. The receptor-mediated mechanism for this effect is unclear. Although evidence for extranuclear ERs is scant thus far for zebra finches, extranuclear/membrane expression of estrogen receptors appears to be a conserved feature in the vertebrate lineage (Beyer et al. 2003; Milner et al. 2001; Mitterling et al. 2010; Montague et al. 2008; Thomas et al. 2005). Recent in vitro evidence indicates that estradiol in NCM can rapidly alter presynaptic inhibitory potentials (Tremere et al. 2009). The current results implicate a neuronal membrane receptor in NCM that may be coupled to this or a similarly acute mechanism (e.g., Dewing et al. 2007).
Sex-specific effects of visual vs. auditory stimulation

In contrast to the results with auditory stimulation, only males responded to playback of visual stimuli in this study, and the response was restricted to an elevation in NCM E2 levels to the presentation of conspecific females. We considered the possibility that the focal males’ vocalizations in response to the visual stimulus directly caused elevations in their NCM E2 levels (i.e., ‘self-stimulation’ Cheng 1986; Cheng et al. 1998). Our analysis, however, showed that no aspect of the focal male’s behavior, including his vocalizations, accounted for the elevation in NCM E2 levels in response to female video playback. This is consistent with an earlier finding, in which NCM E2 was acutely elevated in males during social interactions with females, regardless of the presence or amount of singing by the focal male (Remage-Healey et al. 2008). Therefore, at least one aspect of social interactions appears to involve a direct, visually-evoked neuroestrogen elevation in the NCM of male (but not female) zebra finches.

This striking sex difference in NCM E2 fluctuations most likely reflects the sensory-specific demands of social interactions in adult male vs. female zebra finches. Given that males court females using both auditory (song) and visual (plumage and postural elements of courtship displays) modalities, it is rather surprising that NCM E2 levels were elevated in females only during auditory playback of male song. Although we presented females with naturalistic video stimuli of actively courting males, we may not have fully captured crucial visual elements of courtship that occur during live social interactions that could lead to changes in NCM neuroestrogens in females, and this possibility awaits further investigation. The current findings predict further that visually-evoked neuroestrogen changes in the NCM should also occur in males of other songbird species, especially those in which visual and auditory displays are used in territorial disputes, such as the song sparrow (e.g., Pradhan et al. 2010; Wingfield 1985).
Rapid elevations in E2 in the NCM of males in response to female video playback (and during social interactions with live females) are suggestive of a modulatory transition to a ‘prepared’ state for auditory processing demands that are likely to ensue. ‘Preparatory’ hormonal changes occur in the general circulation in response to visual cues in the early stages of a social encounter (e.g., plasma androgens: Antunes and Oliveira 2009), and thus may similarly occur within discrete brain circuits to provide local modulatory events. According to this model, when a male zebra finch begins to engage in social interactions with a female, NCM estrogens are elevated rapidly upon visual contact. Thus, enhanced auditory gain provided by estrogens within NCM does not require acoustic stimulation per se, and appears to be driven by multisensory input in males. Resulting E2 elevation could therefore aid in several neural tasks that occur in NCM during social interactions, such as song memory and retrieval (Gobes and Bolhuis 2007), attentional shifts to song and discrimination among auditory stimuli (Stripling et al. 1997; Terleph et al. 2008; Voss et al. 2007), and/or synaptic plasticity associated with novel individuals and stimuli (Chew et al. 1995; Jarvis et al. 1995). In this context, the salience of incoming auditory stimuli may be thus shaped by preparatory neuroestrogen elevations in response to visual stimuli (for similar findings with the immediate-early gene ZENK, see also Kruse 2004).

Visual input to NCM

Visual stimuli, especially those of conspecifics during social interactions, can exert powerful influences over an animal’s physiology and behavior (Adkins-Regan and Leung 2006; Chen and Fernald 2011; Wingfield and Wada 1989). Although the songbird NCM is considered an auditory region, several previous studies have reported that visual stimuli can evoke or alter responses in NCM, as measured by rapid changes in expression of the immediate early gene ZENK (Avey et al. 2005; see also George et al. 2006; Hara et al. 2009; Kruse 2004). Among vertebrates, multisensory integration is perhaps best understood for the overlap between visual and auditory modalities (e.g., Cohen 2009; Winkowski and Knudsen 2007). Visual input can activate units in
auditory cortex in monkeys (Brosch et al. 2005; Kayser et al. 2010), and the existence of true
'unisensory' cortex (such as the purely auditory NCM) is in question (Stein and Stanford 2008).
To our knowledge, there are no known direct projections from primary visual pathways into
NCM, increasing the likelihood that visual information is integrated in regions afferent to the
NCM, such as the thalamus or midbrain (e.g., George et al. 2011; Winkowski and Knudsen
2007). Although the role of visual input into NCM remains to be fully accounted for, it is
noteworthy that the adjacent caudolateral nidopallium (NCL) receives visual input in pigeons
(Kirsch et al. 2009; Leutgeb et al. 1996), and a region of dorsocaudal nidopallium activated
during courtship receives visual projections in zebra finches (Sadananda et al. 2007; Wild
1994).

Multimodal integration, sensory enhancement, and attentional shifts are proposed to depend
upon neuromodulator systems that can alter or tune neuronal activity to meet different
behavioral demands. The modulators that mediate multisensory integration and attentional
shifts are typically biogenic amines or other neurotransmitters such as thalamocortical
acetylcholine (Ding et al. 2010; Parikh et al. 2007). The current work suggests that
neuroestrogens can participate in multisensory integration in auditory cortex, as either an
independent modulator or in concert with amines or amino acid transmitters associated with
auditory processing in the NCM (Maney et al. 2001; Ribeiro and Mello 2000; Sockman and
Salvante 2008; Vyas et al. 2008). The role of neuroestrogens as modulators of sensory
processing is just emerging (e.g., Jeong et al. 2011; Remage-Healey et al. 2010), and the
interdependence of multimodal processing and brain-derived steroid production and action
provides an exciting new avenue of future research.

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**Figure Legends**

Figure 1. Rapid elevation in NCM neuroestradiol levels in female subjects exposed to song but not white noise stimuli. Each time point represents E$_2$ levels collected over 30-min periods; * p < 0.05  for the period ‘audio’ vs. the period ‘pre 2’ for the group exposed to male song only. Dotted line indicates average background ELISA reading for aCSF alone, prior to microdialysis.

Figure 2. No significant changes in neuroestradiol levels in a control mesopallium region in females exposed to auditory (A) or visual stimuli (B). Each time point represents E$_2$ levels collected over 30-min periods. Dotted line indicates average background ELISA reading for aCSF alone, prior to microdialysis.

Figure 3. (A) Rapid elevation in NCM neuroestradiol levels in male subjects exposed to female, but not male, conspecific visual stimuli. Each time point represents E$_2$ levels collected over 30-min periods; * p < 0.05  for the period ‘video’ vs. the period ‘pre 2’ for the group exposed to male song only. Inset: No significant change in response to heterospecific visual stimulus (Bengalese...
No significant changes in NCM E₂ levels in female subjects exposed to either female or male conspecific visual stimuli. Dotted lines indicate average background ELISA reading for aCSF alone, prior to microdialysis.

Figure 4. Auditory-evoked activity of NCM neurons is rapidly increased in the presence of E₂ in adult females (RS = response strength; see text for derivation). * p < 0.05 for E₂ vs. aCSF comparison for the CON1 and CON2 groups only. Inset: no effect of the abundant neurosteroid 5-beta-DHT on NCM auditory processing.

Figure 5. Local aromatase inhibition causes a rapid decline in RS in NCM to auditory stimuli in adult females. Retrodialysis of the aromatase inhibitor fadrozole (FAD) into NCM rapidly suppresses RS (** p < 0.005 for the pooled WSRT for FAD vs. aCSF).

Figure 6. The neuronal-membrane impermeable estrogen conjugate biotinylestradiol ('E₂-biot') recapitulates the rapid actions of E₂ on auditory-evoked activity of NCM neurons (RS) in adult females. This effect is restricted to the NCM hemisphere ipsilateral to the retrodialysis delivery probe. This is consistent with an acute mode of E₂ acting via a putative membrane-bound receptor (* p < 0.05 for the pooled WSRT for E₂-biot vs. aCSF).
**A. Males**

- Female stimulus (n = 11)
- Male stimulus (n = 7)

**B. Females**

- Female stimulus (n = 7)
- Male stimulus (n = 8)