Published in final edited form as:

Neuroscience. 2015 January 22; 0: 522–535. doi:10.1016/j.neuroscience.2014.10.023.

Neuroestrogen signaling in the songbird auditory cortex propagates into a sensorimotor network via an 'interface' nucleus

Benjamin A. Pawlisch and Luke Ramage-Healey

Neuroscience and Behavior Program, Center for Neuroendocrine Studies, University of Massachusetts Amherst, Amherst, MA 01003

Abstract

Neuromodulators rapidly alter activity of neural circuits and can therefore shape higher-order functions, such as sensorimotor integration. Increasing evidence suggests that brain-derived estrogens, such as 17- β -estradiol, can act rapidly to modulate sensory processing. However, less is known about how rapid estrogen signaling can impact downstream circuits. Past studies have demonstrated that estradiol levels increase within the songbird auditory cortex (the caudomedial nidopallium, NCM) during social interactions. Local estradiol signaling enhances the auditory-evoked firing rate of neurons in NCM to a variety of stimuli, while also enhancing the selectivity of auditory-evoked responses of neurons in a downstream sensorimotor brain region, HVC (proper name). Since these two brain regions are not directly connected, we employed dual extracellular recordings in HVC and the upstream nucleus interfascialis of the nidopallium (Nif) during manipulations of estradiol within NCM to better understand the pathway by which estradiol signaling propagates to downstream circuits. Nif has direct input into HVC, passing auditory information into the vocal motor output pathway, and is a possible source of the neural selectivity within HVC. Here, during acute estradiol administration in NCM, Nif neurons showed increases in baseline firing rates and auditory-evoked firing rates to all stimuli. Furthermore, when estradiol synthesis was blocked in NCM, we observed simultaneous decreases in the selectivity of Nif and HVC neurons. These effects were not due to direct estradiol actions because Nif has little to no capability for local estrogen synthesis or estrogen receptors, and these effects were specific to Nif because other neurons immediately surrounding Nif did not show these changes. Our results demonstrate that transsynaptic, rapid fluctuations in neuroestrogens are transmitted into Nif and subsequently HVC, both regions important for sensorimotor integration. Overall, these findings support the hypothesis that acute neurosteroid actions can propagate within and between neural circuits to modulate their functional connectivity.

© 2014 IBRO. Elsevier Ltd. All rights reserved

Correspondence: Benjamin A. Pawlisch, Neuroscience and Behavior Program, Center for Neuroendocrine Studies, University of Massachusetts Amherst, Amherst, MA 01003 (bpawlisch@cns.umass.edu).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Neuromodulators quickly alter the activity of neural circuits (Bargmann, 2012). For example, neuromodulators, such as norepinephrine and acetylcholine, have been implicated in state-dependent changes in activity, altering sensory processing, motor output, and sensorimotor integration during changes in wakefulness and attention (Wenk, 1997, Berridge and Waterhouse, 2003, Aston-Jones and Cohen, 2005). Recently, estradiol has been implicated as a neuromodulator in sensory circuits in addition to its primary role as a reproductive hormone (Balthazart and Ball, 2006, Cherian et al., 2014). However, the mechanism by which rapid estrogen signaling within sensory processing brain regions is transmitted to other brain regions is unclear.

Like classic neuromodulators, estradiol can rapidly (secs to mins) modulate neural activity (Balthazart and Ball, 2006, Woolley, 2007, Roepke et al., 2011, Meitzen et al., 2012). Rapid, local changes in estradiol occur within brain regions that express the enzyme aromatase, which converts testosterone into estradiol. Aromatase-positive neurons are present in a variety of brain regions in vertebrates, including the human temporal cortex (Cornil et al., 2006, Forlano et al., 2006, Azcoitia et al., 2011, Cohen and Wade, 2011). As in humans, songbirds have populations of aromatase-positive neurons in some pallial regions, including the caudomedial nidopallium (NCM), a higher order sensory processing brain region (Saldanha et al., 2000, Fusani and Gahr, 2006). Microdialysis within the NCM of male and female songbirds has demonstrated that estradiol increases when songbirds hear song and during social interactions (Ramage-Healey et al., 2008, Ramage-Healey et al., 2012). Acute infusions of fadrozole, which blocks aromatase and suppresses estradiol, disrupt both auditory processing and song preference behaviors (Tremere et al., 2009, Ramage-Healey et al., 2010, Tremere and Pinaud, 2011). However, how neural circuits and pathways are modulated by neuroestrogens to support auditory processing and preference behaviors is still relatively unclear.

Because of their discrete, well-characterized pathways involved in auditory processing and vocal motor output and the known connections between these pathways (Figure 1), songbirds have become an excellent model for asking questions regarding how neuromodulators may be involved in sensory processing. Like the auditory system in mammals, there are thalamo-cortical projections from nucleus ovoidalis to a primary cortical region, the Field L complex (Vates et al., 1996, Theunissen et al., 2008). Parts of the Field L complex project to the caudomedial nidopallium (NCM) and the caudal mesopallium, which are distinct, but reciprocally-connected secondary cortical regions (Vates et al., 1996, Gentner, 2008). NCM indirectly connects to the vocal motor pathway through the nucleus interfascialis of the nidopallium (NIf). NIf receives projections from the caudal mesopallium as well as other input from secondary thalamic projections, which are thought to relay information regarding breathing during singing (Bauer et al., 2008, Akutagawa and Konishi, 2010, Lewandowski et al., 2013). NIf provides auditory information directly to HVC (proper name), which is a key nucleus within the vocal motor pathway (Nottebohm et al., 1976, Fortune and Margoliash, 1995, Bottjer et al., 2000). In addition to auditory-evoked activity, NIf and HVC show singing-related (motor) activity (Yu and Margoliash, 1996), indicating that they each have key roles in sensorimotor integration.

Tract-tracing studies, delineating the connections between auditory processing and vocal motor pathways, in concert with electrophysiological studies testing the connectivity of these pathways have together begun to shed light on how neuromodulation can alter functional connectivity of the songbird brain. While not the only projection to HVC, NIf has been shown to be the primary source of auditory input into HVC, since inactivation of NIf can greatly reduce auditory-evoked electrophysiological activity HVC (Cardin and Schmidt, 2004a, Coleman and Mooney, 2004, Lewandowski et al., 2013). Furthermore, inactivation of upstream auditory processing regions can reduce auditory-evoked electrophysiological activity in both NIf and HVC (Bauer et al., 2008). Although it has been shown that norepinephrine and acetylcholine act directly in HVC (Dave et al., 1998, Shea and Margoliash, 2003, Shea et al., 2010), upstream regions, such as NIf, are also responsive to varying behavioral states and modulators (Cardin and Schmidt, 2004b). Therefore, neuromodulators in upstream brain regions, including NCM and NIf, are likely key to changes in the activity of HVC neurons.

Aromatase-containing neurons are present within NCM, which is upstream of NIf and HVC, and very few aromatase-containing cells are found in HVC and NIf (Saldanha et al., 2000, Fusani and Gahr, 2006). Changes in estradiol within NCM enhance electrophysiological responses to many types of auditory stimuli within NCM, including a bird's own song (BOS), other male zebra finch's songs, and even white noise (Tremere et al., 2009, Remage-Healey and Joshi, 2012). While it is thought that selective neural responses to BOS gradually emerge along the input pathways into HVC (Janata and Margoliash, 1999, Bauer et al., 2008), local increases in estradiol within NCM result in enhanced neural selectivity downstream in HVC (Remage-Healey and Joshi, 2012). One of the goals of the current study was to identify a possible pathway through which changes in estradiol in NCM can influence selective response properties in HVC. We were interested in examining parallel changes in selectivity and functional connectivity in NIf and HVC to examine estradiol's importance for the emergence of selectivity in HVC. One possible mechanism for estradiol increasing the selectivity in HVC is via altering the flow of auditory information from NIf into HVC. Since activity between NIf and HVC has been shown to be highly correlated, it may be that increasing functional connectivity between NIf and HVC is associated with increasing selectivity. To test these ideas, we retrodialyzed either estradiol or fadrozole into NCM while simultaneously recording extracellularly from NIf and HVC.

Experimental procedures

1.1 Subjects

All experiments were performed with 23 male adult zebra finches (*Taeniopygia guttata*). Zebra finches were colony reared in flight cages and were > 120 d after hatch when used in the experiment. The experiments were conducted in accordance with the University of Massachusetts Amherst Institutional Care and Use Committee and the National Institutes of Health Guidelines.

1.2 Stimuli

Each male ($n=23$) was recorded in a sound attenuation chamber in the presence of a companion female using Sound Analysis Pro (<http://soundanalysispro.com>; version 2A.04). A representative exemplar for every male [bird's own song (BOS)] was selected, which was free of cage noise and calls. Songs were band-pass filtered (0.5– 10 kHz), and the amplitude adjusted to 70 dB using Adobe Audition (Adobe Systems). Each BOS exemplar was also temporally reversed [reverse (REV)], which was used as a control stimulus along with another male zebra finch's song [conspecific (CON)] and white noise [white noise (WN)], all of which were duration-matched to BOS (2.77 ± 0.17 s).

1.3 Surgery

Once songs were recorded, males underwent stereotaxic surgery to prepare them for electrophysiology (approximately 24 or 48 hours before electrophysiological recording). Males were anesthetized with an in-house generic formulation of equithesin (3.33 mL/kg) and were given a topical injection of lidocaine before a midline incision was made to locate the bifurcation of the mid-sagittal sinus. The skin and upper leaflet of skull surrounding the bifurcation of the mid-sagittal sinus was removed, and the bifurcation was used as a reference point for determining and marking the locations of Nif (± 1.70 mm M/L and 2.2 mm rostral from bifurcation of the mid-sagittal sinus), NCM (± 1.10 mm M/L and 1.4 mm rostral), and HVC (± 2.4 mm M/L). A stainless steel head-post and a reference electrode were attached to the head with dental cement.

1.4 Electrophysiology and Retrodialysis

On the day of recording, in order to measure electrophysiological activity in Nif and HVC while manipulating estradiol concentrations in NCM, a microdialysis probe was implanted into the left NCM and electrodes were directed at ipsilateral Nif and HVC in anesthetized male zebra finches (Figure 1). This general methodology has been successfully used in previous experiments (Remage-Healey and Joshi, 2012). Males were anesthetized with three 30 μ L intramuscular injections of 20% urethane over the course of two hours. Thereafter, the head-post was attached to an anchor stage (Herb Adams Engineering), the bird was placed upon a current heating pad (FHC Neurocraft), and a craniotomy surgery and the resection of the dura mater were performed. Once the dura mater was resected, the microdialysis probe and one electrode was carefully lowered into NCM and Nif (respectively) with motorized micromanipulators (Warner Instruments), and another electrode was lowered into HVC with a hydraulic micromanipulator (Narishige).

Estradiol or fadrozole treatment was retrodialyzed through a microdialysis probe (CMA-7 probe membrane length of 1.0 mm; CMA/Microdialysis), as in previous experiments (Remage-Healey et al., 2010, Remage-Healey and Joshi, 2012). Prior to drug treatment, aCSF (199 mM NaCl, 26.2 mM NaHCO₃, 2.5 mM KCl, 1.0 mM MgSO₄, 2.5 mM CaCl₂, 11.0 mM glucose, and 1% bovine serum albumin, pH 7.4) was infused through retrodialysis at a rate of 2.0 μ L/min. Thereafter, estradiol or fadrozole were infused at the same rate, starting 20 minutes prior to the auditory stimuli playback. Estradiol (17 β -estradiol) was administered at a dose of 30 μ g/mL in aCSF (100 μ M; as in Remage-Healey et al., 2010, Remage-Healey and Joshi, 2012), and fadrozole was administered at dose of 30 μ g/mL in

aCSF (110 μ M). Following this playback period, aCSF was infused again, beginning with a 20 minute washout period before the playback of auditory stimuli.

Before, during, and after estradiol treatment, the same playback stimuli (BOS, CON, REV, and WN) were presented 15 times in a randomized order with an interstimulus interval of 10 ± 2 s. During this time, continuous electrophysiological activity from NIf and HVC was recorded with two carbon fiber electrodes (0.5–1.2 M Ω ; Carbostar-1; Kation Scientific). Electrophysiological activity was amplified, band-pass filtered (0.3–5 kHz, A-M Systems 1700), digitized at 20 kHz (Micro 1401, Cambridge) and stored using Spike 2 software (Cambridge Electronic Design). Both NIf and HVC have characteristic auditory-evoked activity, such that multiunit activity in HVC sites can be correlated with putative NIf activity and activity within NIf slightly precedes activity in HVC (Janata and Margoliash, 1999, Cardin and Schmidt, 2004a). Raw cross-correlograms between NIf and HVC were used to confirm the site of the recording for NIf in every case (e.g., Figure 3B & 5B). After recording, electrolytic lesions were administered to confirm the presence of the electrodes within NIf and HVC. Then, birds were rapidly decapitated, and their brains were fixed in a 30% formalin and sucrose solution. After 24–48 hours, the brains were frozen and sectioned, and the tissue was Nissl stained, dehydrated, and coverslipped. The resulting slides were analyzed for the presence of the lesions in NIf and HVC and probes in NCM (Figure 2).

1.5 Multi-unit and Single-unit Analysis

Multi-unit recordings were analyzed offline by thresholding methods that have been previously described (Remage-Healey and Joshi, 2012). The same threshold was used across all of the different treatments for a given individual. Supra-threshold multi-unit activity was used to obtain a baseline spike rate (measured for the 2 sec prior to stimuli) and auditory-evoked spike rate (measured for the 2 sec during the stimuli). The mean baseline and auditory-evoked firing rates were further used to calculate the response strength (RS) and Z score. RS is a measure of the mean firing rate during the stimulus minus the mean firing rate during the baseline period. We then used the RS to calculate the Z score, which is a measure of the RS divided by the standard deviation of the difference between the stimulus and baseline periods (Coleman and Mooney, 2004). Whereas RS and Z score compare the auditory-evoked activity to the baseline activity, d' calculations can compare the auditory-evoked electrophysiological responsiveness of one stimulus to another stimulus, and d' is an index of stimulus selectivity. For this experiment, d' REV was calculated using the formula below (as in Remage-Healey and Joshi, 2012):

$$d' = \frac{2(RS[STIM] - RS[REV])}{\sqrt{\sigma^2[STIM] + \sigma^2[REV]}}$$

For multi-unit activity, in order to measure how drug treatments changed the connectivity of NIf and HVC, we also measured the average strength of coherency (Shaevitz and Theunissen, 2007). The average strength of coherency was calculated from the cross-correlograms that were used to identify the site of recording for NIf (Figures 3B and 5B). The amplitude and width of the cross-correlograms were computed in Spike 2 to estimate the area of the peak resulting from the cross-correlograms. Assuming a Gaussian distribution

(Shaevitz and Theunissen, 2007), the area under the peak was estimated using the formula below (where dt is a measure of the time interval):

$$\text{Average Coherency Strength} = \sqrt{\frac{\text{Amplitude}^2 \times 2.5 \times \text{width}}{dt}}$$

Multi-unit recordings were sorted to obtain single-units for statistical analysis using Spike 2, since single-unit analyses allow for precise understanding of what is occurring at the level of each neuron. Single-units were identified using principal components analysis based upon the waveform characteristics (see Figures 4D and 5E), using Spike 2 spike-sorting algorithms as previously described (Remage-Healey and Joshi, 2012, Remage-Healey et al., 2010). Only units that met the stringent criterion of a low interspike interval (outside the refractory period duration) were included as single-units. The number of interspike intervals within 1 ms was less than 1 % of the total number of interspike intervals ($0.95\% \pm 0.11$).

1.6 Statistical Analysis

Data were analyzed using OriginPro 8.6 (Origin, Northampton, MA). Two-way repeated measures analyses of variance (ANOVAs) were performed to examine the effects of treatment (before, during, and after estradiol treatment) and song stimuli (including BOS, REV, CON, and WN) on multiple dependent variables, including spikes/sec, response strength, Z score, and d' in NIf and HVC. For the use of two-way repeated measures ANOVAs, the assumptions were tested with Mauchly's Test of Sphericity. If sphericity was violated (Mauchly's Test: $p < 0.05$), multivariate analyses of variance (MANOVAs) were used. Significant main effects or interactions were followed with Fisher's post-hoc analyses. For the fadrozole experiment, MANOVAs could not be used due to insufficient degrees of freedom; therefore, non-parametric Friedman repeated measures ANOVAs were used for each stimulus, which were followed by paired Wilcoxon Signed Rank Tests for significant post-hoc analyses (as in Remage-Healey and Joshi, 2012). In order to test how fadrozole treatment may influence functional connectivity between NIf and HVC, we used Pearson's correlations to compare NIf and HVC multi-unit activity across individuals before, during, and after fadrozole treatment. We also used one-way repeated measures ANOVAs to compare the average strength of coherency before, during, and after fadrozole treatment.

Results

2.1 Rapid Effects of Estradiol in NCM on Downstream NIf

Histology confirmed that all probes were within NCM and that all electrodes were within HVC. Inspection of the NIf recording site showed that 11 male zebra finches had electrodes placed in NIf and HVC, whereas 7 males had lesions in regions surrounding NIf (Figure 2). Previous studies have shown that NIf activity is strongly driven by auditory stimuli (Janata and Margoliash, 1999, Cardin and Schmidt, 2004a, Coleman and Mooney, 2004). Multi-unit analysis of recordings in NIf ($n=11$) showed a significant effect of song stimulus on spike rate (Figure 3C, main effect of song stimulus: $F_{(3,8)}=5.37$, $p=0.025$). Post-hoc tests demonstrated that there were significantly greater auditory-evoked responses to song

stimuli, including REV, than WN in NIf ($p < 0.05$). There was no effect of E2 treatment or interaction between treatment and auditory stimulus presented (main effect of treatment: $F_{(2,9)} = 2.02$, $p = 0.19$, interaction: $F_{(6,5)} = 0.91$, $p = 0.55$) on multi-unit responses.

By contrast, we were able to isolate more than one single-unit from many of our recording sites, such that isolating single-units from these same recordings increased the sample size ($n = 21$ units) and a significant effect of E2 treatment became apparent. Retrodialysis of $100\mu\text{M}$ estradiol (E2) increased auditory-evoked spike rate in NIf neurons (Figure 4A, main effect of treatment: $F_{(2,19)} = 5.30$, $p = 0.015$, song stimulus: $F_{(3,18)} = 3.10$, $p = 0.053$, interaction: $F_{(6,15)} = 0.63$, $p = 0.707$). Post-hoc tests demonstrated significant increases in spike rate during E2 treatment (E2 relative to pre: $p = 0.033$ and E2 to post: $p = 0.002$). This increase in spiking was due to both an increase in auditory-evoked responses as measured by Z score (Figure 4B, main effect of treatment: $F_{(2,19)} = 7.16$, $p = 0.005$, main effect of stimulus: $F_{(3,18)} = 6.88$, $p = 0.003$, interaction: $F_{(6,15)} = 0.36$, $p = 0.893$; E2 relative to pre: $p = 0.067$ and E2 to post: $p = 0.025$), and also because of an increase in baseline spike rate (Figure 4C, main effect of treatment: $F_{(2,19)} = 5.04$, $p = 0.018$, main effect of stimulus: $F_{(3,18)} = 2.87$, $p = 0.067$, interaction: $F_{(6,15)} = 0.73$, $p = 0.637$), which post-hoc tests demonstrate increases during treatment (E2 relative to pre: $p = 0.003$ and E2 to post: $p = 6.4 \times 10^{-4}$). There was no effect of treatment or interaction on d' in NIf neurons (main effect of treatment: $F_{(2,19)} = 0.70$, $p = 0.509$, song stimulus: $F_{(3,18)} = 8.81$, $p = 8.2 \times 10^{-4}$, interaction: $F_{(6,15)} = 0.63$, $p = 0.707$). Therefore, estradiol in NCM caused an acute elevation in the activity of single NIf neurons, both at rest and when they were driven by auditory inputs.

The recordings from neurons within the nidopallium immediately surrounding NIf ('NIf-surround') served as a comparison to determine the spatial specificity of the E2-dependent modulation in NIf. The same analysis of estradiol on auditory-evoked electrophysiological responses did not show significant effects of treatment. Multi-unit analysis of recordings in NIf-surround ($n = 5$) did not show a significant effect treatment on spike rate (Figure 5C, main effect of treatment: $F_{(2,5)} = 2.58$, $p = 0.137$, main effect of song stimulus: $F_{(3,4)} = 0.74$, $p = 0.55$, interaction: $F_{(6,1)} = 0.92$, $p = 0.498$). Similarly, single-unit analysis of recordings in NIf-surround ($n = 7$) did not show a significant effect of treatment (main effect of treatment: $F_{(2,5)} = 3.55$, $p = 0.110$, main effect of stimulus: $F_{(3,4)} = 1.60$, $p = 0.324$, interaction: $F_{(6,1)} = 7.13$, $p = 0.279$). Furthermore, there was no increase in baseline spike rate, despite a significant effect of treatment (Figure 5D, main effect of treatment: $F_{(2,5)} = 10.66$, $p = 0.016$, main effect of stimulus: $F_{(3,4)} = 2.00$, $p = 0.257$, interaction: $F_{(6,1)} = 77.65$, $p = 0.087$). Post-hoc tests demonstrate this effect is the result of a significant decrease in baseline spike rate after the treatment (post relative to pre: $p = 3.1 \times 10^{-4}$ and post relative to E2: $p = 0.003$). Therefore, single units in the immediate surround of NIf showed a different pattern of activity as a result of E2 modulation in NCM compared to single units within NIf. Overall, estradiol in NCM did not cause an acute elevation in the activity of NIf-surround neurons in contrast to NIf neurons (i.e., those identified to have strong synaptic inputs to HVC).

2.2 Rapid Effects of Estradiol in NCM on downstream HVC

Previous studies demonstrated that elevating estradiol concentrations in NCM specifically increased the Z score and neuronal selectivity (d' for the BOS stimulus) in HVC (Remage-

Healey and Joshi, 2012). In contrast to the previous study, multi-unit electrophysiological activity in HVC did not show a significant effect of treatment on firing rate (Figure 6A, main effect of treatment: $F_{(2,9)}=0.60$, $p=0.568$, main effect of stimulus: $F_{(3,8)}=5.20$, $p=0.028$, interaction: $F_{(6,5)}=1.17$, $p=0.439$), and single-unit analysis did not show a significant effect of treatment on firing rate either (Figure 6B, main effect of treatment: $p=0.496$, main effect of stimulus: $p=0.002$, interaction: $p=0.187$). Furthermore, there was not a significant effect of treatment on d' (Figure 6C, main effect of treatment: $F_{(2,9)}=0.64$, $p=0.667$, main effect of stimulus: $F_{(3,8)}=54.53$, $p=1.7 \times 10^{-15}$, interaction: $F_{(6,5)}=1.46$, $p=0.200$). However, a subset of single HVC neurons (~ 53 %) did show a marked increase in selectivity to BOS as measured by d' , although this was not as systematic across the population of HVC neurons in this study as in the previous study.

2.3 Blocking estradiol synthesis in NCM Affects Nlf and HVC

In a separate set of birds, we retrodialyzed the compound fadrozole (FAD) into NCM, which inhibits aromatase activity to block endogenous synthesis of estradiol in NCM. Only 3 of 5 of FAD-infused individuals had event correlations that showed characteristic correlated activity in Nlf and HVC. Therefore, single-unit analysis was performed in both Nlf ($n=6$ units) and HVC ($n=7$ units), and Friedman repeated measures ANOVAs were used to measure the effects of FAD treatment for each stimulus. There was not a significant effect of FAD treatment on baseline spike rate ($X^2=1.33$, $p=0.513$), auditory-evoked spike rate during BOS ($X^2=0.33$, $p=0.847$), and Z score for BOS (Figure 7A, $X^2=3.00$, $p=0.223$) in Nlf. However, there was a significant effect of FAD treatment on d' for BOS in Nlf (Figure 7B, $X^2=6.33$, $p=0.042$). Wilcoxon signed rank post-hoc tests showed decreases in d' for BOS during FAD treatment (FAD relative to pre: $Z=2.10$, $p=0.031$ and post to pre: $Z=0.63$, $p=0.529$). There was no effect of FAD treatment on d' for CON ($X^2=0.333$, $p=0.846$) or d' for WN ($X^2=1.00$, $p=0.606$) in Nlf. Simultaneous HVC recordings showed a concurrent decrease in Z score during FAD treatment in HVC (Figure 8, main effect of treatment: $p=0.122$, main effect of stimuli: $p=1.6 \times 10^{-4}$, interaction: $p=0.012$). Post-hoc tests showed that there were significant decreases in Z score for BOS during and after FAD (FAD relative to pre: $p=0.008$ and post to pre: $p=0.007$). There was a trend for a decrease in d' in HVC (main effect of treatment: $p=0.072$, main effect of stimulus: $p=1.7 \times 10^{-4}$, interaction: $p=0.060$). As in Nlf, the auditory-evoked spike rate did not change in HVC (main effect of treatment: $p=0.348$, main effect of stimulus: $p=0.101$, interaction: $p=0.850$).

Since we observed that fadrozole retrodialysis in NCM resulted in concurrent changes in the response properties of neurons in Nlf and HVC, we next directly compared simultaneous changes in Nlf and HVC. We used two types of analyses to address this question. First, we performed correlations across individuals to more directly compare the relationship between multi-unit, auditory-evoked activity in Nlf alongside HVC. Prior to fadrozole treatment, there was a significant correlation between auditory-evoked activity in Nlf and HVC, as measured by Z score (Figure 9, $r=0.366$, $p=0.0145$). By contrast, during and immediately after fadrozole treatment, this correlation was lost for Z score between Nlf and HVC (Figure 9, FAD: $r=0.214$, $p=0.163$; Post: $r=-.102$, $p=0.510$). Second, we performed one-way repeated measures ANOVAs on the average strength of coherency before, during, and after fadrozole treatment. The average strength of coherency did not change following fadrozole

treatment (effect of treatment: $F_{(2,6)}=0.787$, $p=0.497$; pre = 59.5 ± 18.0 (mean $\pm$ sem); FAD = 38.5 ± 19.6 ; post = 44.2 ± 12.8). These results indicate that changes in selectivity could be the result of altered functional connectivity between NIf and HVC, although this interpretation warrants caution since these changes do not appear to depend on changes in the strength of coherency.

Discussion

There is increasing evidence indicating that estradiol can rapidly modulate the electrophysiological activity of neural circuits, including sensory processing circuits, in addition to initiating long-term effects on gene expression (Maney and Pinaud, 2011, Cherian et al., 2014). A recent study reported that estrogens acting rapidly in the auditory processing region, NCM, exert simultaneous downstream effects in the sensorimotor HVC (Remage-Healey and Joshi, 2012). NIf became an interesting candidate for downstream effects because it connects auditory and vocal motor pathways and therefore is an important region for sensorimotor integration like HVC. In this study, increasing estradiol concentrations in NCM increased both the baseline and the auditory-evoked electrophysiological activity in NIf. Furthermore, we observed that blocking endogenous estrogen production decreased the selectivity of NIf neurons in parallel with decreased selectivity in HVC neurons. These findings are consistent with the hypothesis that local estrogen production exerts rapid, transsynaptic effects in the songbird brain.

3.1 Estradiol Modulation Propagates to the Nucleus Interfacialis of Nidopallium (NIf), a Downstream Region Involved in Sensorimotor Integration

Retrodialysis of estradiol in NCM increased the firing rate evoked by all stimuli in NIf, suggesting a generalized (and not stimulus selective) increase in auditory-evoked electrophysiology responses in NIf. The generalized increase in auditory-evoked electrophysiology in NIf partially can be explained by the surprising result that estradiol in NCM increased the baseline firing rate in NIf. Changes in the baseline firing rate are surprising because previous studies showed that changes in electrophysiology in upstream NCM and downstream HVC occurred in the auditory-evoked firing rates only, and baseline firing rates remained unchanged in both regions (Remage-Healey and Joshi, 2012). Also, the direction of the result is surprising since estradiol increases the baseline firing rate in NIf in contrast to what has been seen with some other neuromodulators, in which the signal-to-noise ratio is altered by either increasing the responsiveness to specific auditory stimulation or by decreasing the baseline levels of activity (Berridge and Waterhouse, 2003, Cardin and Schmidt, 2004b, Aston-Jones and Cohen, 2005). Baseline activity in NIf may be a particularly important parameter for sensorimotor integration in the songbird brain. The change in baseline activity in NIf observed here is consistent with past studies that have demonstrated a large variability in NIf baseline firing rate across behavioral states, with large increases in baseline activity in awake birds (Cardin and Schmidt, 2004a). This suggests that estradiol, as well as norepinephrine and acetylcholine, may have a role in state-dependent changes in activity in sensory processing regions. However, the functional significance of this finding is more difficult to interpret at present, since we did not observe

simultaneous changes in the activity or selectivity of HVC neurons during increases in baseline firing rates in NIf.

Retrodialysis of estradiol in NCM also increased Z scores to all stimuli in NIf, which are above and beyond changes in baseline activity. Therefore, the changes in baseline activity do not fully explain the generalized (and not stimulus selective) increases in NIf after estradiol infusions. In contrast to estradiol, fadrozole treatment in NCM lead to decreases in auditory-evoked activity for the BOS only, indicating that fadrozole selectively decreased the responsiveness of NIf neurons. These patterns may reflect NIf's role as an interface between auditory and sensorimotor functions. General changes in electrophysiology in NIf are similar to what has been seen in NCM for estradiol actions, and the selective changes in electrophysiology in NIf for fadrozole actions are similar to what has been seen in HVC (Remage-Healey and Joshi, 2012). NIf may therefore act as an intermediary for these actions in the sensorimotor pathway. The differences may also be due to differences in the selectivity between the populations of neurons that we recorded from during the estradiol experiment versus the fadrozole experiment. Prior to the drug treatment, the neurons in NIf were more selective on average in the fadrozole experiment than in the estradiol experiment. Past studies have shown variability in the selectivity of NIf neurons, and the range of selectivity in the present results overlap with previous results. Furthermore, it is not clear whether specific neuron sub-types might have differences in selectivity in NIf (Janata and Margoliash, 1999, Coleman and Mooney, 2004, Bauer et al., 2008). As was mentioned above, we feel that it is most instructive to consider the changes in electrophysiological activity in NIf in conjunction with the concurrent changes in electrophysiological activity in HVC.

3.2 Blocking Estrogen Production in NCM Propagates from the Nucleus Interfacialis of Nidopallium (NIf) into HVC

Retrodialysis of fadrozole strongly influenced the stimulus selectivity of NIf neurons as well as the selectivity of HVC neurons. In fact, our observation of concurrent changes in selectivity of NIf neurons, and HVC is consistent with other studies that have shown a strong relationship between activity in NIf and HVC (Cardin and Schmidt, 2004a, Coleman and Mooney, 2004). In a previous study, similar doses of estradiol in NCM have increased the selectivity of HVC neurons. Although the present study did not detect changes in selectivity of HVC neurons in response to estradiol retrodialysis, a subset of single neurons did show a strong increase in selectivity (Figure 6C). One possible explanation is the presence of a ceiling effect, where high concentrations of estradiol were already present within NCM and the selectivity of HVC remained consistently high after the retrodialysis of estradiol. This possibility may explain why blocking endogenous synthesis of estradiol in NCM altered stimulus selectivity in both NIf and HVC. Little is known about the dose response relationships for neuroestrogen signaling and, for that matter, the concentrations of estradiol in specific regions of the brain. Future work may elucidate a mechanism that explains the importance of dose in estradiol signaling.

3.3 Evidence for changes in functional connectivity between NIf and HVC

Our results with the aromatase inhibitor fadrozole in NCM led us to test whether increases in selectivity were the result of changes in the functional connectivity between NIf and HVC. While the retrodialysis of fadrozole led to concurrent increases in the activity of NIf and HVC, repeated measures ANOVAs of each region only allow us to indirectly infer changes in the connectivity of NIf and HVC. To better address this specific idea, we used correlations across individuals for evoked NIf and HVC activity. As expected, the activity in NIf and HVC was highly correlated, as has been previously shown (Cardin and Schmidt, 2004a). However, the correlation was weakened after the treatment of fadrozole in NCM, largely due differences in the selective responses to BOS stimuli. This is consistent with a change in the connectivity between NIf and HVC as a result of neuromodulation. However, the average strength of coherency did not significant change as a result of fadrozole treatment. So, our conclusions with respect to functional connectivity are preliminary.

3.4 The Function of Neuromodulation of Sensory Processing Regions

Despite the complexity of transsynaptic and distributed effects, the functional implications of estrogen-dependent neuromodulation in behavioral circuits are becoming clearer. One role for acute estradiol elevation in sensory circuits may be to alter sensorimotor integration, via gating information flow through the auditory processing and vocal motor pathways. Changing the functional connectivity between NIf and HVC may have important behavioral consequences for adult males because changes in functional connectivity could regulate auditory feedback into the vocal motor pathway. It may be that rapid, local changes in estradiol within the NCM are an important mechanism by which auditory feedback shapes future singing behavior. Future work using awake, singing songbirds are needed to further understand how neuromodulation by estradiol can influence singing behavior. Furthermore, an exploration of the transsynaptic effects of neuroestrogen signaling in the female zebra finch brain may help demonstrate with the importance of neuroestrogens in song recognition.

Acknowledgements

This work was supported by NIH grants R00NS066179 and R01NS082179 and the University of Massachusetts. The authors thank Vanessa Lee, Joseph Starrett, and Clemens Probst for their technical assistance and Dr. Melissa Coleman and two anonymous reviewers for valuable input on the manuscript.

References

- Akutagawa E, Konishi M. New brain pathways found in the vocal control system of a songbird. *J Comp Neurol.* 2010; 518:3086–3100. [PubMed: 20533361]
- Aston-Jones G, Cohen JD. Adaptive gain and the role of the locus coeruleus-norepinephrine system in optimal performance. *J Comp Neurol.* 2005; 493:99–110. [PubMed: 16254995]
- Azcoitia I, Yague JG, Garcia-Segura LM. Estradiol synthesis within the human brain. *Neuroscience.* 2011; 191:139–147. [PubMed: 21320576]
- Balthazart J, Ball GF. Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 2006; 29:241–249. [PubMed: 16580076]
- Bargmann CI. Beyond the connectome: how neuromodulators shape neural circuits. *Bioessays.* 2012; 34:458–465. [PubMed: 22396302]

- Bauer EE, Coleman MJ, Roberts TF, Roy A, Prather JF, Mooney R. A synaptic basis for auditory-vocal integration in the songbird. *J Neurosci*. 2008; 28:1509–1522. [PubMed: 18256272]
- Berridge CW, Waterhouse BD. The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev*. 2003; 42:33–84. [PubMed: 12668290]
- Bottjer SW, Brady JD, Cribbs B. Connections of a motor cortical region in zebra finches: relation to pathways for vocal learning. *J Comp Neurol*. 2000; 420:244–260. [PubMed: 10753310]
- Cardin JA, Schmidt MF. Auditory responses in multiple sensorimotor song system nuclei are co-modulated by behavioral state. *J Neurophysiol*. 2004a; 91:2148–2163. [PubMed: 14724261]
- Cardin JA, Schmidt MF. Noradrenergic inputs mediate state dependence of auditory responses in the avian song system. *J Neurosci*. 2004b; 24:7745–7753. [PubMed: 15342742]
- Cherian S, Wai Lam Y, McDaniels I, Struziak M, Delay RJ. Estradiol rapidly modulates odor responses in mouse vomeronasal sensory neurons. *Neuroscience*. 2014; 269:43–58. [PubMed: 24680884]
- Cohen RE, Wade J. Aromatase mRNA in the brain of adult green anole lizards: effects of sex and season. *J Neuroendocrinol*. 2011; 23:254–260. [PubMed: 21121975]
- Coleman MJ, Mooney R. Synaptic transformations underlying highly selective auditory representations of learned birdsong. *J Neurosci*. 2004; 24:7251–7265. [PubMed: 15317851]
- Cornil CA, Taziaux M, Baillien M, Ball GF, Balthazart J. Rapid effects of aromatase inhibition on male reproductive behaviors in Japanese quail. *Horm Behav*. 2006; 49:45–67. [PubMed: 15963995]
- Dave AS, Yu AC, Margoliash D. Behavioral state modulation of auditory activity in a vocal motor system. *Science*. 1998; 282:2250–2254. [PubMed: 9856946]
- Forlano PM, Schlinger BA, Bass AH. Brain aromatase: new lessons from non-mammalian model systems. *Front Neuroendocrinol*. 2006; 27:247–274. [PubMed: 16828853]
- Fortune ES, Margoliash D. Parallel pathways and convergence onto HVC and adjacent neostriatum of adult zebra finches (*Taeniopygia guttata*). *J Comp Neurol*. 1995; 360:413–441. [PubMed: 8543649]
- Fusani L, Gahr M. Hormonal influence on song structure and organization: the role of estrogen. *Neuroscience*. 2006; 138:939–946. [PubMed: 16310314]
- Gentner, TQ. Temporal auditory pattern recognition in songbirds. In: Zeigler, HP.; Marler, P., editors. *Neuroscience of Birdsong*. Cambridge University Press; Cambridge; New York: 2008. p. 187-198.
- Janata P, Margoliash D. Gradual emergence of song selectivity in sensorimotor structures of the male zebra finch song system. *J Neurosci*. 1999; 19:5108–5118. [PubMed: 10366643]
- Lewandowski B, Vyssotski A, Hahnloser RH, Schmidt M. At the interface of the auditory and vocal motor systems: NIf and its role in vocal processing, production and learning. *J Physiol Paris*. 2013; 107:178–192. [PubMed: 23603062]
- Maney D, Pinaud R. Estradiol-dependent modulation of auditory processing and selectivity in songbirds. *Front Neuroendocrinol*. 2011; 32:287–302. [PubMed: 21146556]
- Meitzen J, Grove DD, Mermelstein PG. The organizational and aromatization hypotheses apply to rapid, nonclassical hormone action: neonatal masculinization eliminates rapid estradiol action in female hippocampal neurons. *Endocrinology*. 2012; 153:4616–4621. [PubMed: 22865367]
- Nottebohm F, Stokes TM, Leonard CM. Central control of song in the canary, *Serinus canarius*. *J Comp Neurol*. 1976; 165:457–486. [PubMed: 1262540]
- Remage-Healey L, Coleman MJ, Oyama RK, Schlinger BA. Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. *Proc Natl Acad Sci U S A*. 2010; 107:3852–3857. [PubMed: 20133597]
- Remage-Healey L, Dong SM, Chao A, Schlinger BA. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *J Neurophysiol*. 2012; 107:1621–1631. [PubMed: 22190616]
- Remage-Healey L, Joshi NR. Changing neuroestrogens within the auditory forebrain rapidly transform stimulus selectivity in a downstream sensorimotor nucleus. *J Neurosci*. 2012; 32:8231–8241. [PubMed: 22699904]

- Remage-Healey L, Maidment NT, Schlinger BA. Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci.* 2008; 11:1327–1334. [PubMed: 18820691]
- Roepke TA, Ronnekleiv OK, Kelly MJ. Physiological consequences of membrane-initiated estrogen signaling in the brain. *Front Biosci (Landmark Ed).* 2011; 16:1560–1573. [PubMed: 21196248]
- Saldanha CJ, Tuerk MJ, Kim YH, Fernandes AO, Arnold AP, Schlinger BA. Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J Comp Neurol.* 2000; 423:619–630. [PubMed: 10880992]
- Shaevitz SS, Theunissen FE. Functional connectivity between auditory areas field L and CLM and song system nucleus HVC in anesthetized zebra finches. *J Neurophysiol.* 2007; 98:2747–2764. [PubMed: 17898149]
- Shea SD, Koch H, Baleckaitis D, Ramirez JM, Margoliash D. Neuron-specific cholinergic modulation of a forebrain song control nucleus. *J Neurophysiol.* 2010; 103:733–745. [PubMed: 19939956]
- Shea SD, Margoliash D. Basal forebrain cholinergic modulation of auditory activity in the zebra finch song system. *Neuron.* 2003; 40:1213–1226. [PubMed: 14687554]
- Theunissen, FE.; Amin, N.; Shaevitz, S.; Woolley, SMN.; Fremouw, T.; Hauber, ME. Song selectivity and the songbird brain. In: Zeigler, HP.; Marler, P., editors. *Neuroscience of Birdsong*. Cambridge University Press; Cambridge; New York: 2008. p. 157-173.
- Tremere LA, Jeong JK, Pinaud R. Estradiol shapes auditory processing in the adult brain by regulating inhibitory transmission and plasticity-associated gene expression. *J Neurosci.* 2009; 29:5949–5963. [PubMed: 19420261]
- Tremere LA, Pinaud R. Brain-generated estradiol drives long-term optimization of auditory coding to enhance the discrimination of communication signals. *J Neurosci.* 2011; 31:3271–3289. [PubMed: 21368039]
- Vates GE, Broome BM, Mello CV, Nottebohm F. Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches. *J Comp Neurol.* 1996; 366:613–642. [PubMed: 8833113]
- Wenk GL. The nucleus basalis magnocellularis cholinergic system: one hundred years of progress. *Neurobiol Learn Mem.* 1997; 67:85–95. [PubMed: 9075237]
- Woolley CS. Acute effects of estrogen on neuronal physiology. *Annu Rev Pharmacol Toxicol.* 2007; 47:657–680. [PubMed: 16918306]
- Yu AC, Margoliash D. Temporal hierarchical control of singing in birds. *Science.* 1996; 273:1871–1875. [PubMed: 8791594]

Aromatase-containing neurons in the songbird cortex locally synthesize estradiol.
Estradiol modulated electrophysiological activity in the downstream region NIf.
Estradiol treatment increased baseline and auditory-evoked activity in NIf.
Blocking aromatase simultaneously decreased selectivity in NIf and HVC.
Estradiol signaling in the songbird cortex alters downstream sensorimotor regions.

Figure 1.

A. Schematic of a sagittal view of the auditory and vocal motor pathways of the male zebra finch brain, showing the placements of a retrodialysis probe and electrodes during retrodialysis and dual electrophysiological recording. The green triangle depicts the presence of a retrodialysis probe within the caudomedial nidopallium (NCM), which is a higher order auditory region that contains aromatase-positive neurons. The blue triangle represents the presence of an extracellular electrode into the nucleus interfascicularis of the nidopallium (Nif), a region connecting auditory and vocal motor regions, and the red triangle represents the presence of an extracellular electrode in HVC (proper name), a vocal motor region critical for singing behavior in male songbirds. Other abbreviations: the nucleus ovoidalis (Ov), Field L complex (Field L), caudal mesopallium (CM), and robust nucleus of the arcopallium (RA).

B. Exemplar of simultaneous electrophysiological activity in Nif and HVC. Supra-threshold activity is shown in raster form above the multi-unit electrophysiological activity in HVC and Nif. Supra-threshold activity was used to measure firing rate, Z score, and d' REV (for more detail see the methods). Song, as shown as a waveform, elicits auditory-evoked activity above baseline activity simultaneously in Nif and HVC.

Figure 2.

Schematic of the location of lesions from electrodes in NIf and immediately surrounding NIf. NIf is found ventral to the mesopallial lamina (LaM) and just dorsal to the pallial-subpallial lamina (PSL). The Field L complex (Field L) extends caudo-dorsally along the PSL. NIf is shaded and contains placement lesions showing that many of the lesions are inside of NIf ($n=11$), and some were present outside of the NIf ($n=7$). Two were not depicted that were too lateral to be in NIf and thus were outside of the plane of section of the diagram. Some lesions are not depicted due to tissue degradation/loss.

Figure 3.

Multi-unit electrophysiological activity in NIf. A. Photomicrograph of the lesion inside NIf (black arrow points to lesion). B. Exemplar of raw cross-correlograms highlighting the relationship between NIf and HVC activity. The time of the peak denotes the difference in timing between NIf and HVC activity; the size of the peak denotes the strength of the correlation in activity between NIf and HVC. The large peak before zero shows that activity in NIf occurs slightly before activity in HVC. C. Mean (+ SEM) spike rate during auditory stimuli before (pre), during (E2), and after (post) estradiol treatment. For each treatment, the same stimuli were presented: a male zebra finch's own song or bird's own song (BOS), that bird's own song reversed (REV), another male zebra finch's song (CON), and white noise (WN) in a randomized order (see methods for more details). The asterisks denote significant post-hoc tests of the main effect of song stimuli between BOS, CON, REV, and WN.

Figure 4.

Single-unit electrophysiological activity in NIf. A. Mean (+ SEM) spike rate during auditory stimuli before, during, and after estradiol treatment. The asterisk denotes significant post-hoc tests of the main effect of treatment between pre, E2, and post. B. Mean (+ SEM) Z score during auditory stimuli before, during, and after estradiol treatment. The same statistical tests show similar results for Z score. The asterisk denotes significant post-hoc test of the main effect of treatment between pre, E2, and post. C. Mean (+ SEM) baseline firing rate of NIf neurons before, during, and after estradiol treatment. As in the evoked responses in A–B, the same statistical tests show similar result for baseline firing rate. The asterisk denotes significant post-hoc tests of the main effect of treatment between pre, E2, and post. D. Top, Principle component analysis plot of multi-unit electrophysiological recording, showing three auditory units in NIf. Below, Overlay of the 50 random spike waveforms from the same single units.

Figure 5.

Electrophysiological activity in NIF-surround. A. Photomicrograph of the lesion outside of Nif (black arrow points to lesion). B. Exemplar of raw cross-correlograms highlighting in the relationship between Nif-surround and HVC activity showing no clear 'leading' relationship between the activity of neurons in the immediate surround of Nif and the activity of HVC neurons. C. Multi-unit electrophysiological activity in Nif-surround. Mean (+ SEM) firing rate during auditory stimuli before, during, and after estradiol treatment. D. Mean (+ SEM) baseline firing rate during auditory stimuli before, during, and after estradiol treatment. The asterisk denotes significant post-hoc tests of the main effect of treatment between pre, E2, and post. E. Top, Principle component analysis plot of multi-unit electrophysiological recording, showing two auditory units in HVC. Below, Overlay of the 50 random spike waveforms from the same single units.

Figure 6.

Electrophysiological activity in HVC. A. Multi-unit electrophysiological activity in HVC. Mean (+ SEM) firing rate during auditory stimuli before, during, and after estradiol treatment. The asterisk denotes significant post-hoc tests of the main effect of song stimuli between BOS, CON, REV, and WN. B. Single-unit electrophysiological activity in HVC. After single-unit sorting, the same statistical tests show similar results for firing rate during auditory stimuli. C. Single-unit electrophysiological activity in HVC. Mean (+ SEM) d' REV during auditory stimuli before, during, and after estradiol treatment. Inset shows individual data for all single units for d' REV for the BOS stimulus.

Figure 7.

Single-unit electrophysiological activity in NIf shows that selectivity for the BOS is reduced during fadrozole treatment in NCM. A. Mean (+ SEM) Z score during auditory stimuli before, during, and after fadrozole treatment. B. Mean (+ SEM) d' REV during auditory stimuli before, during, and after fadrozole treatment. The asterisk denotes significant post-hoc test of the main effect of treatment between pre, FAD, and post.

Figure 8.

Single-unit electrophysiological activity in HVC shows that selectivity for the BOS is reduced during fadrozole treatment in NCM. A. Mean (+ SEM) Z score during auditory stimuli before, during, and after fadrozole treatment. The asterisk denotes significant post-hoc tests of the interaction of treatment and song stimulus. B. Mean (+ SEM) d' REV during auditory stimuli before, during, and after fadrozole treatment.

Figure 9.

Concurrent electrophysiological activity in NIf and HVC shows a direct influence of fadrozole in NCM on the correlated activity between NIf and HVC. A. Prior to fadrozole treatment, a trend line shows there is a significant correlation between NIf and HVC. B. During fadrozole treatment, there is no longer a significant correlation between NIf and HVC activity. C. After fadrozole treatment, there is also no longer a significant correlation between NIf and HVC activity.