Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird

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Higher cognitive function depends on accurate detection and processing of subtle features of sensory stimuli. Such precise computations require neural circuits to be modulated over rapid timescales, yet this modulation is poorly understood. Brain-derived steroids (neurosteroids) can act as fast signaling molecules in the vertebrate central nervous system and could therefore modulate sensory processing and guide behavior, but there is no empirical evidence for this possibility. Here we report that acute inhibition of estrogen production within a cortical-like region involved in complex auditory processing disrupts a songbird's ability to behaviorally respond to song stimuli. Identical manipulation of local estrogen levels rapidly changes burst firing of single auditory neurons. This acute estrogen-mediated modulation targets song and not other auditory stimuli, possibly enabling discrimination among species-specific signals. Our results demonstrate a crucial role for neuroestrogen synthesis among vertebrates for enhanced sensory encoding. Cognitive impairments associated with estrogen depletion, including verbal memory loss in humans, may therefore stem from compromised moment-by-moment estrogen actions in higher-order cortical circuits.

birdsong | microdialysis | neurotransmission | nongenomic | sex steroid

In vertebrates, steroid hormones can rapidly influence the activity of neurons and neural circuits (1–3), although the functional consequences for higher processing are unclear. The exquisite recognition of species-typical vocalizations (4, 5) and abundant production of neuroestrogens (6, 7) are both especially prominent in songbirds. A cortical-like auditory region (the caudomedial nidopallium, or NCM) of songbirds is a critical locus for song learning as well as auditory processing and song recognition (8–11). NCM exhibits rich expression of the estrogen-synthetic enzyme aromatase in both cell bodies and synaptic terminals (6, 12), and neuroestrogen levels within NCM fluctuate rapidly (<30 min) and independent of peripheral sex-steroid levels during social interactions, song playback, and after neurotransmitter activation (13). In songbirds, therefore, rapid changes in the local production of NCM neuroestrogens could in turn rapidly affect processing of complex auditory stimuli, such as song.

Here, we test this hypothesis in male Australian zebra finches (*Taeniopygia guttata*). Our recent optimization of in vivo microdialysis in this species has enabled the measurement and manipulation of local estrogen production within discrete brain regions in awake, behaving males (13). We have further established that local estrogen levels are dependent on the activity of the enzyme aromatase within NCM, because retrodialysis (reverse delivery using microdialysis) of the aromatase inhibitor fadrozole (FAD) into NCM transiently suppresses local estradiol levels (13).

Results

In Vivo Retrodialysis and Behavior. Adult zebra finches express a robust behavioral preference for acoustic playback of their tutor's song or bird's own song (BOS) compared with conspecific male song (CON) (4, 5, 8), and behavioral scores are highly correlated in both operant and preference tasks (5). We considered whether disruption of aromatase activity with FAD retrodialysis (and resultant acute suppression of estrogen production) into NCM interferes with the preference for BOS vs. CON in a two-choice playback design. Preference for BOS vs. CON was significantly suppressed within 30 min during FAD (100 μM) retrodialysis into the left but not right hemisphere NCM (Fig. 1). Repeated-measures ANOVA showed a FAD treatment effect ($F = 5.626$; $P = 0.016$) and an interaction with left vs. right hemisphere ($F = 4.048$; $P = 0.041$) on preference. Post hoc tests showed a FAD-induced drop in preference ratio for the left NCM (Wilcoxon $P = 0.007$ for artificial cerebrospinal fluid (aCSF) vs. FAD and $P = 0.026$ for FAD vs. wash) but no change for the right NCM (all $P > 0.27$; Mann-Whitney U test $P \leq 0.05$ for left vs. right NCM during FAD). FAD did not significantly alter other behaviors, including singing (Table S1), suggesting a specific effect on auditory processing. This acute FAD effect is consistent with the rapidity of FAD-induced suppression of local estradiol levels in NCM (13), which was observed here in both NCM hemispheres (Table S2). Thus, inhibition of estrogen production within the left-hemisphere NCM acutely suppressed the ability to process and respond appropriately to complex song stimuli.

FAD could have influenced behavior via nonspecific indirect or toxic effects, and thereby produced changes in preference indirectly. However, we observed no significant changes in locomotion, feeding, beak wiping, and singing behaviors during FAD vs. aCSF retrodialysis (Table S1), reflecting observational criteria that the birds were alert and attending to playback stimuli.

We next tested for the spatial specificity of aromatase inhibition by orally administering saline or FAD before a similar behavioral experiment. Oral FAD treatment is associated with widespread inhibition of aromatase activity in zebra finch brain (12). There was no significant effect of FAD treatment as compared with saline treatment on song preference (repeated-measures $F = 2.983$; $P = 0.088$ for $n = 7$), although a strong trend was observed (Fig. S1) in the same direction as when FAD was retrodialyzed into the left NCM only. This result suggests that long-term systemic FAD treatment recapitulated some, but not all, of the local and acute effects of aromatase inhibition via FAD retrodialysis within the left hemisphere NCM [FAD retrodialysis is especially effective at acutely suppressing local estradiol levels below detection within NCM (13)]. Together, our behavioral results with FAD are consistent with the hypothesis that depriving the left hemisphere NCM of estrogen production specifically disrupts behavioral responses to natural song stimuli.

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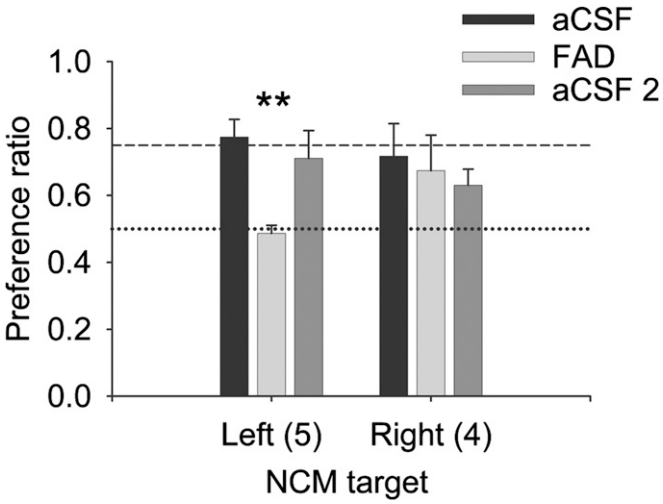

Fig. 1. Song preference depends on estrogen production in the left hemisphere. In freely behaving males, preference for BOS (1.0 = 100% BOS preference vs. 0 CON) is eliminated during retrodialysis of the aromatase inhibitor FAD into the left-hemisphere NCM region (**$P < 0.007$) but not the right. Dashed line represents the species-typical BOS preference ratio (≈75%) (5). Dotted line delineates no preference. Sample sizes in parentheses.

In Vivo Retrodialysis and Electrophysiology. We next tested whether local changes in estradiol (17β-estradiol) modulate auditory-evoked neuronal activity in NCM. All results are reported for the left-hemisphere NCM, the region in which we observed FAD effects on behavior (*Methods*). In anesthetized males multiunit auditory-evoked activity in NCM was significantly altered by local changes in estradiol levels. Retrodialysis of estradiol (30 μg/mL) caused acute (0–30 min) increases in spike rate (spiking activity during song presentation; Fig. 2 *A* and *B*). Repeated-measures ANOVA showed an effect of estradiol treatment ($F = 31.48$; $P < 0.0001$) on spike rate during auditory playback. Wilcoxon signed rank tests (Wilc-SRT) showed that estradiol significantly enhanced spike rate during BOS ($n = 9$; $P = 0.007$), CON1 ($n = 11$; $P = 0.004$), CON2 ($n = 9$; $P = 0.038$), and reverse BOS (REV; $n = 7$; $P = 0.018$), but not during heterospecific song (HET; Bengalese finch song; $n = 4$; $P = 0.999$) or white noise (WN; $n = 4$; $P = 0.465$). Therefore, rapid estradiol-mediated modulation of NCM auditory-evoked activity was species-specific to the type of acoustic stimuli presented. Estradiol boosted auditory-evoked activity only to stimuli that contained elements of zebra finch song and not to other auditory stimuli.

Conversely, inhibition of estrogen production via FAD retrodialysis ($n = 6$) caused no changes in NCM activity during 30 min of song presentation. However, a significant washout effect (increased spike rate in response to song playback) was observed during the 30-min aCSF wash after FAD treatment (Fig. 2*C*; repeated-measures ANOVA $F = 11.88$; $P < 0.001$; Wilc-SRT: BOS, $P = 0.027$; REV, $P = 0.046$; CON1, $P = 0.046$; and CON2, $P = 0.028$). A washout "rebound" effect after FAD treatment is consistent with a disinhibition of local aromatase activity and a modulation of NCM auditory-evoked activity via resurgent local estradiol levels (Table S2) (13). Therefore, we observe that acute elevations in local estradiol levels, during both exogenous estradiol treatment and during "washout" recovery from FAD-mediated aromatase suppression, are each associated with increases in auditory-evoked multiunit activity.

To assess the neurosteroid specificity of estradiol's effects on auditory processing, another group of birds was retrodialyzed with the androgen 5β-dihydrotestosterone (5β-DHT), which is inactive at estrogen or androgen receptors, nonaromatizable, and synthesized naturally in the avian brain (14). The 5β-DHT neurosteroid caused no significant changes in NCM spike rate (Fig. S2). Thus, the infusion of a steroidal molecule was insufficient to replicate the acute effect of estradiol, indicating estradiol's steroid specificity.

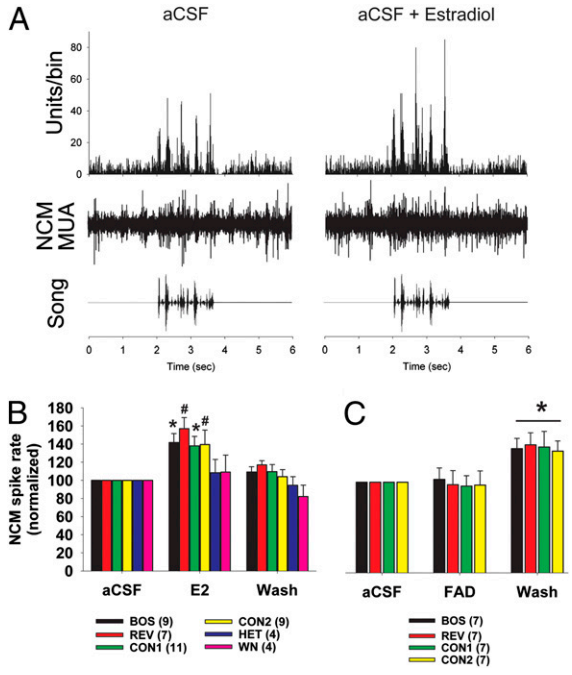

Fig. 2. Acute fluctuations in local estrogens modulate auditory-evoked activity in NCM. (*A*) *Top:* PSTH of 30 presentations of BOS during aCSF (*Left*) vs. aCSF + estradiol treatment (*Right*). *Middle:* Raw multiunit activity (MUA) recording during BOS. *Bottom:* oscillogram of BOS playback stimulus. (*B*) Within 30 min estradiol (E2) retrodialysis increases NCM spike rate during zebra finch song playback (BOS, CON, and REV) but not during HET or WN. (*C*) FAD retrodialysis has no immediate effect, but FAD elevates NCM spike rate during the 30-min washout period for all song stimuli. Spike rates during all treatment periods are standardized to the preceding aCSF period. Sample sizes in parentheses. #$P < 0.007$; *$P < 0.05$.

Multiunit Burst Firing. The majority of auditory responsiveness in NCM occurred in bursts (Fig. S3). Analysis of auditory response strength (*SI Methods*) showed that burst firing accounts for 75–90% of overall auditory responsiveness, whereas isolated activity accounted for <25%, emphasizing the importance of burst-firing in encoding of song within NCM (Fig. S3). Acute estradiol infusion caused a shift from isolated spikes toward elevated burst firing in multiunit NCM activity (Fig. 3*A*). Intermittent bursts occurred during aCSF treatment and increased in number during estradiol treatment, indicated by a leftward shift in interspike interval (ISI) histograms (Fig. 3*A*). Estradiol caused significant changes in multiple burst parameters, including the number of bursts (Fig. 3*B*; repeated-measures ANOVAs: $F = 10.42$; $P = 0.0002$), the average duration of bursts (Fig. S4; $F = 4.59$; $P = 0.015$), the average number of spikes per burst (Fig. S5; $F = 5.80$; $P = 0.005$), and the percentage of all spikes that occurred in bursts (Fig. S6; $F = 4.507$; $P = 0.016$), but not the average ISI within bursts (Fig. S7; $F = 1.98$; $P = 0.148$). Estradiol increased the number of bursts for all zebra finch songs presented (Wilc-SRT: BOS, $P = 0.042$; CON1, $P = 0.05$; and REV, $P = 0.043$) but not for HET or WN ($P > 0.09$; for Wilc-SRT for other parameters, see *SI Results*). Therefore, estradiol increased the occurrence of bursts selectively for playback stimuli that contained elements of zebra finch song and not other auditory stimuli.

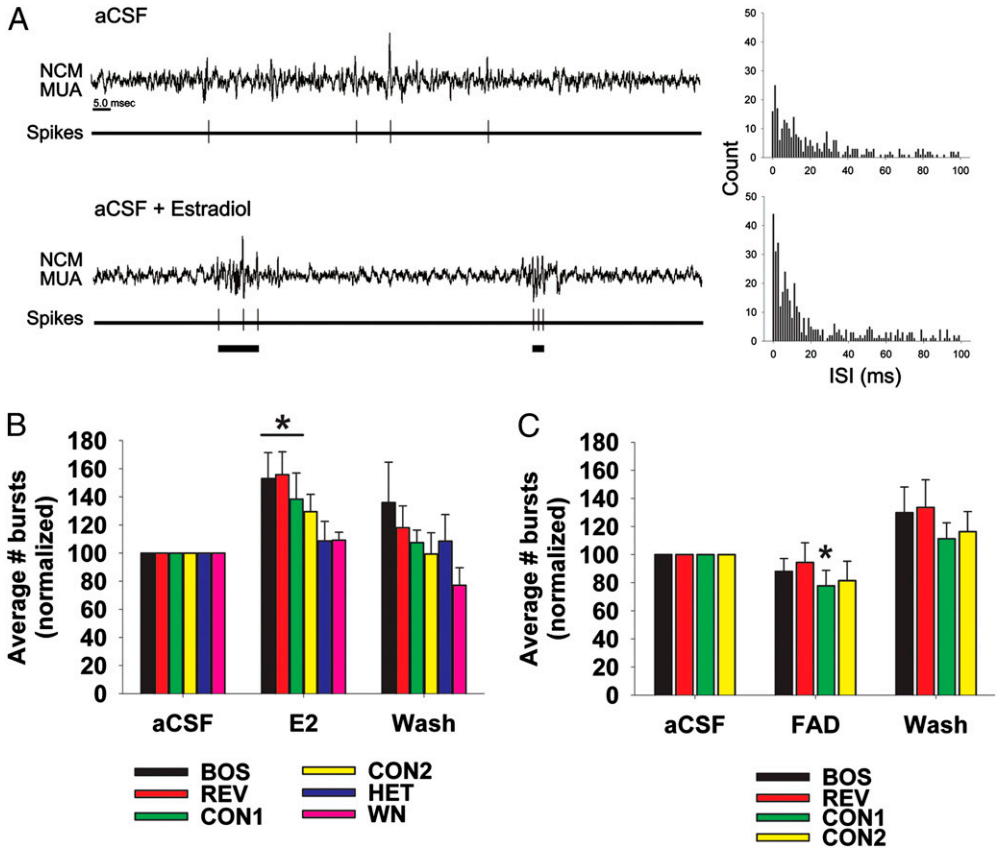

Fig. 3. Estradiol causes acute changes in the occurrence of multiunit bursts. (*A*) Example of NCM spike trains for both aCSF (*Upper*) and estradiol (*Lower*) conditions. Triggered spike plots are depicted below each trace, and two examples of identified burst-periods (black bars) are depicted. *Right:* ISI distribution plots over the entire 30 min treatment periods from the same experiment are shown for aCSF (*Upper*) and estradiol (*Lower*). An increased number of rapid ISI (<5–10 ms) events during estradiol treatment is consistent with a transition from isolated spiking to burst firing. (*B*) As with NCM spike rate activity, estradiol (E2) increases the number of multiunit bursts during playback of zebra finch songs (BOS, CON, REV) but not during HET or WN stimuli. (*C*) FAD significantly decreases the number of multiunit bursts (*$P < 0.05$ for CON1; $P > 0.09$ for all other post hoc tests for aCSF vs. FAD or Wash vs. aCSF).

By contrast, inhibiting local estrogen production with FAD rapidly decreased the occurrence of burst firing in NCM (Fig. 3*C*). Repeated-measures ANOVA showed FAD caused a significant reduction in the number of bursts ($F = 10.49$; $P = 0.0003$; Wilc-SRT: CON1 $P = 0.024$; all other $P > 0.11$) but no significant change in any other burst parameter (range of P values, 0.18–0.93). There were no significant posthoc effects for Wash vs. aCSF for the FAD treatment (all $P > 0.09$). Therefore, experimental increases and decreases in local estradiol levels increased and decreased, respectively, the occurrence of burst firing in NCM multiunit auditory activity.

Single Unit Activity. To investigate whether estradiol's actions on the occurrence of multiunit bursts were due to shifts in the activity of individual neurons, we sorted for clearly identified single units (Fig. 4 *A* and *B*; *Methods*). Estradiol caused significant increases in the number of single-unit bursts during song presentation (Fig. 4*C*; repeated-measures ANOVA $F = 12.78$; $P = 0.0004$; Wilc-SRT: $P = 0.005$) and in the percentage of single-unit spikes that occurred in bursts (Fig. 4*D*; $F = 3.65$; $P = 0.046$; Wilc-SRT: $P = 0.036$). All other single-unit burst parameters were unaffected by estradiol (all $P > 0.39$). By contrast, inhibiting local estrogen production with FAD retrodialysis acutely decreased the number of single-unit bursts during song presentation (Fig. 4*E*; repeated-measures ANOVA $F = 7.56$; $P = 0.007$; Wilc-SRT: $P = 0.018$). All other single-unit burst parameters were unaffected by FAD (all $P > 0.31$). Therefore, experimental increases and decreases in local estradiol levels increased and decreased, respectively, the occurrence of burst firing for NCM single auditory units.

The increase in burst firing could have reflected an epiphenomenon of generalized estradiol-induced increases in tonic activity. However, temporal encoding of bursting NCM single units in response to song stimuli was maintained during estradiol treatment (Fig. 4*B*) consistent with a specific effect on auditory encoding. Moreover, 5β-DHT did not affect single-unit burst firing (*SI Results*) further emphasizing the specificity of estradiol's rapid actions.

Discussion

This study indicates that inhibition of estrogen production in the left-hemisphere NCM suppresses burst firing of auditory neurons and disrupts the ability to process and respond to song stimuli, which is essential to songbird social behavior. Importantly, FAD retrodialysis caused acute suppression of local estradiol levels equally in the left- and right-hemisphere NCM, indicating that hemispheric differences in resultant estradiol levels do not account for the lateralized effect of FAD on song preference. Moreover, local changes in neuroestrogens rapidly influenced neural activity similarly in both hemispheres (*Methods*), indicating that differential neuroestradiol modulation per se does not account for the lateralized effect on song preference. Therefore, our findings are consistent with the hypothesis that the left NCM has a different functional role in song processing than the right, a conclusion that parallels previous observations that song discrimination is biased toward the left forebrain

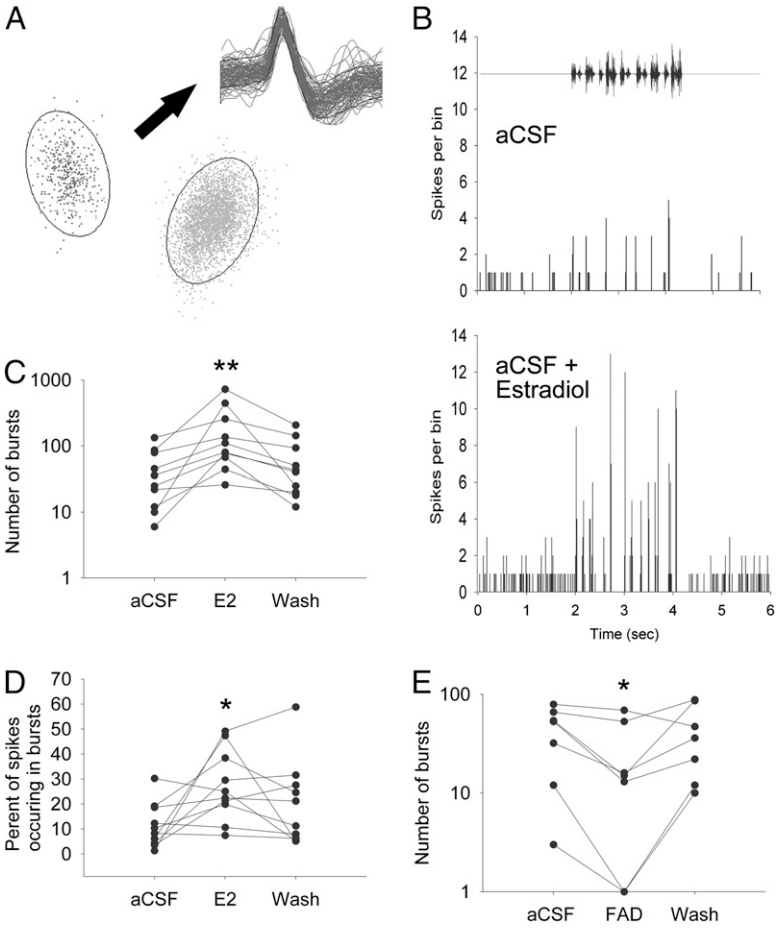

Fig. 4. Local changes in estradiol acutely modulate auditory-evoked activity of single NCM neurons. (*A*) *Upper right:* Overlay of 100 random spike waveforms from a single neuron as indentified by PCA. *Lower left:* PCA plot shows the corresponding unique distribution of spikes (black dots, ellipse = SD) for the same unit (see arrow) during 30 min of playback. (*B*) Single units show auditory-evoked activity that is enhanced during 30-min estradiol treatment. *Inset:* BOS oscillogram. *Lower:* PSTHs for single-unit activity for aCSF and estradiol. (*C*) Estradiol (E2) increases the number of auditory-evoked single unit bursts in NCM. $^{**}P = 0.005$, $n = 10$ units from seven birds. (*D*) Estradiol also increases the percentage of spikes per burst for single NCM units. $^{*}P = 0.036$. (*E*) By contrast, FAD decreases the number of auditory-evoked single unit bursts in NCM. $^{*}P = 0.018$; $n = 7$ units from five birds.

(15, 16). Our study therefore indicates that the left NCM circuit is modulated by neuroestrogens to fine-tune song processing and preference. Circulating estrogens can asymmetrically influence human cortical activity over days to weeks (17, 18), and the present results suggest that acute estrogen actions could be critical for lateralized sensory processing, a widespread feature of the vertebrate forebrain (19). Further, here local estrogen production directly influenced the encoding of species-specific song elements, revealing an exquisite specificity for acute neurosteroid modulation of complex sensory encoding.

Our results indicate that altered burst firing per se—and not simply changes in neuronal activity—is linked to (*i*) fluctuations in estradiol levels in NCM, (*ii*) acute changes (gain/loss) in species-specific song encoding, and (*iii*) resultant modulation of downstream song processing and preference behavior. Burst firing can be dissociated from accompanying increases in firing frequency, because some bursting states depend on membrane hyperpolarization, leading to constant or even decreases in overall firing frequency (20). Our results suggest that fluctuating local estradiol levels cause NCM multiunit activity to shift from isolated spiking to bursting. At a population-wide level, multiunit auditory-evoked activity does not decrease below a "baseline" during FAD-induced suppression of estradiol levels, whereas rising estradiol levels (both exogenous or endogenous during FAD washout) acutely boost auditory-evoked isolated activity above this baseline. Further analysis revealed that the occurrence of multiunit bursts is acutely and significantly suppressed by FAD and enhanced by estradiol. These findings at the multiunit level led us to focus on the modulation of auditory-evoked activity of single units during up- and down-regulation of neuroestradiol levels. Isolation of single units allowed us to precisely monitor the firing patterns of auditory neurons over periods of neuroestrogen manipulation, revealing that FAD causes a transient drop in single-unit burst firing, whereas estradiol causes bursting to increase specifically to stimuli that contain zebra-finch song. Such bidirectional, estrogen-dependent, and species-specific modulation of burst firing is particularly important for song encoding, because bursts account for more than 75% of total song responsiveness in NCM (Fig. S3). Our electrophysiology results with FAD and estradiol strongly suggest that the bursting activity of auditory units in NCM is integral to the encoding of song and the expression of song preference. Future intracellular approaches will shed light on how estrogens acutely impact firing modes via possible neuronal membrane effects in NCM. (During manuscript preparation a study was published reporting that estrogen manipulation in NCM changes auditory multiunit activity and gene expression [21].)

A transition to neuronal burst firing has emerged as a unique mode of sensorimotor information transfer in the forebrain of songbirds and other vertebrates (20, 22–26). Burst firing has been associated with increased attention/wakefulness (23, 27, 28), synaptic efficacy (29, 30), and discrimination among sensory stimuli (20, 24, 28) and is thought to depend on the activity of projections from the basal forebrain (e.g., cholinergic projections; ref. 31). This study introduces neuroestrogens as a class of local modulators for the transition to burst firing, a processing mode that may be fundamental for sensory discrimination in the numerous estrogen-producing circuits that occur in the CNS of vertebrates (1, 7). Although the cellular mechanisms for these effects are unknown, burst-mode firing is enabled by changing

membrane ionic conductances (20, 32), which can in turn be acutely modulated by estrogens (1). Long-term estrogen blockade in humans has been associated with cognitive impairments, including verbal memory deficits (33–35), and this study emphasizes that pharmacologic estrogen depletion could have as yet unknown consequences for moment-by-moment cortical function. In conclusion, songbirds reveal how a highly adapted suite of behaviors to discriminate, learn, and replicate complex song features is linked to the evolutionary innovation of neuroestrogen synthesis and the control of neuronal burst firing.

Methods

Animals. Animal care and use protocols were approved by the University of California, Los Angeles Chancellor's Committee on Animal Care and Use and by the Institutional Care and Use Committee at the Joint Science Department of the Claremont Colleges. Surgery for microdialysis and electrophysiology procedures followed existing methods (*SI Methods*) optimized previously for this species (13). After the completion of all experiments, birds were processed for histology, and brain sections were examined under light microscopy to determine probe placement (Fig. S8).

Song Preference Trials. Male zebra finches express a reliable behavioral preference for acoustic playback of their tutor's song or their own song (BOS) when compared with a conspecific song (CON). This preference is expressed in two-choice phonotaxis in both males and females (4, 5, 36, 37). Here, a test microdialysis cage was divided into three zones, containing a perch at each end (preference perches) and a perch in the middle (neutral). Food and water were available ad libitum. Two speakers were placed adjacent to the preference perches, and a digital sound level meter calibrated the amplitude of songs played back to ≈70 dB. Microdialyzed males readily responded to song playback and were observed hopping and searching at each preference perch.

Song stimuli consisted of four repetitions of song (≈5 s length) with 3–6 s silence between each song repetition, followed by 30 s of silence [resembling natural singing rates for male zebra finches (38)]. For each 30-min trial, BOS was broadcast from one side of the cage (1 min) and CON from the other side (1 min), for a total of 2 min of continuous alternation (looped 15×). After a 1-h rest period, the side for each stimulus was reversed in a balanced design to account for any side bias. The total time spent on the perch next to the BOS speaker vs. the CON speaker was summed for both trials and expressed as a preference ratio (preference for BOS/total time spent choosing). To meet motivation criteria (e.g., ref. 5) males were required to have visited each perch at least once during the trial and had to spend at least 10% of the total test duration at either stimulus perch (all microdialyzed males fulfilled these criteria; mean = 31.5%). All trials were videotaped and sounds recorded onto a hard drive, and behaviors scored offline with J-Watcher software (http://www.jwatcher.ucla.edu).

FAD Retrodialysis. The retrodialysis and song preference experiments were completed over 3 successive days for each microdialysis subject. Song preference trials were carried out during continuous retrodialysis of aCSF on day 1 and day 3. On day 2, the same set of trials was carried out during FAD retrodialysis as outlined below. The water-soluble aromatase inhibitor FAD (gift of Novartis) acutely inhibits aromatase activity in zebra finch brain (13, 39). FAD was dissolved in aCSF and retrodialyzed at 100 μM for $n = 9$ birds (left-hemisphere NCM, 5; right-hemisphere NCM, 4). After a baseline period of retrodialysis of aCSF for 30 min, aCSF plus FAD was retrodialyzed for 30 min. During the subsequent 30-min period the first song preference trial was carried out during FAD retrodialysis. A 1-h rest interval followed. A second song preference trial was then carried out for 30 min (side/stimuli reversed as above) during FAD retrodialysis. Subsequently, the solution was switched to aCSF for two successive 30-min periods of washout. All trials were videotaped and scored as above.

In Vivo Retrodialysis and Extracellular Recordings. To assess whether acute changes in local forebrain steroids modulate auditory-evoked activity in NCM, in vivo microdialysis was combined with multiunit electrophysiology recordings. Data are presented from the left-hemisphere NCM (the region where retrodialyzed FAD modulated behavior), and electrophysiology experiments with the right NCM did not reveal substantial differences from the patterns in the left. Playback stimuli were a combination of BOS, REV, CON, HET, and WN (generated using CoolEdit Pro software; Adobe). All stimuli were presented at an interstimulus interval of 10 ± 2 s at ≈70 dB sound pressure level (root mean square, A-weighted) via an audio amplifier connected to a speaker directed at a custom-designed headpost stage (Herb Adams Engineering). Each experiment presented only a subset of the above stimuli (typically four; e.g., BOS, REV, CON, and HET), and stimuli were 2 to 3 s in duration. Each experiment presented the same set of song stimuli for three successive 30-min periods, with a total of 30 presentations of each stimulus per period. Dorsocaudal NCM neurons are known to habituate to repeated presentation of song stimuli, but we did not observe substantial habituation in our recordings in ventral NCM (as in ref. 40; see also *SI Results*). Three recording/playback periods consisted of a baseline aCSF retrodialysis (30 min), followed by 30 min of either estradiol (30 μg/mL) or FAD (100 μM) retrodialysis, and then followed by 30 min of washout with aCSF. To test for the neurosteroid specificity of estradiol effects on auditory processing, a separate group of birds ($n = 3$) was tested with retrodialysis of the nonaromatizable androgen 5β-DHT, with experimental procedures as above for aCSF pretreatment and stimuli playback. Similar to aromatase, the enzyme that synthesizes 5β-DHT from testosterone (5β-reductase) is expressed throughout zebra finch telencephalon (14, 41).

Surgery and extracellular recording procedures were as described previously (42–44), with some modifications (*SI Methods*) with a new set of subjects. All electrophysiologic recordings were analyzed offline with Spike2 software. The threshold for detecting multiunit activity was determined by the user at a level crossed by only high-amplitude events and excluded small-amplitude events (e.g., 42–44).

Multiunit activity. Multiunit activity in response to auditory playback was calculated by averaging spike rate over the 2 s of each playback stimulus for all 30 repetitions of each stimulus (e.g., BOS, HET, etc.). Average spike rates for treatment periods were then standardized to spike rates during the aCSF control period for each bird (e.g., vs. estradiol) and analyzed via repeated-measures ANOVA (for analyses with song motifs, see *SI Methods*).

Spike rate. Typically, treatment effects on zebra finch auditory-evoked activity examine the parameter response strength, which calculates spike rate activity for song playback relative to preceding baseline spontaneous activity. However, we observed qualitative changes in NCM spontaneous activity at baseline (i.e., no song playback) in response to local estradiol retrodialysis [spike rate over 10-s period: 221.33 ± 35.01 (spikes, mean ± SEM) for aCSF; 313.33 ± 105.91 (mean ± SEM) for aCSF + estradiol], which causes shifts in underlying response strength measurement. Therefore for each experiment spike rates were standardized according to responsiveness during aCSF treatment (re 100%). Important for this analytical approach, there was no statistical difference in spontaneous activity among treatment periods ($F = 1.414$; $P = 0.31$), and the calculated response strengths for all playback stimuli at baseline (aCSF period) were not significantly different from each other ($F = 1.945$; $P = 0.107$). Although the average response strength was slightly negative for white noise (−0.635 ± 1.013; mean ± SE), the response strength for the remaining stimuli ranged from 2.904 ± 1.156 (HET) to 5.080 ± 1.148 spikes/s (BOS). Treatment effects were very similar when computed according to either response strength or aCSF standardization, and between-experiment variability was reduced when standardizing to aCSF.

Multiunit burst analysis. The ISI distributions for spike train data revealed a substantial increase in short ISI (<10 ms) during estradiol as compared with aCSF treatment (Fig. 3*A*). To evaluate this elevation in burst firing, a custom software script for burst identification and analysis (CED) was used to process spike trains for bursts consisting of three or more action potentials separated by ≤10 ms (for similar burst parameters, see ref. 25). Burst parameters were quantified in response to each playback stimulus (30 iterations) for both estradiol and FAD experiments as described for spike rate data above. Burst analysis was restricted to the time during auditory presentation, and for each treatment period bursts were quantified for the following: average number of bursts; average burst duration; average spikes per burst; average ISI within bursts; and the percentage of overall spikes that occurred in bursts. As with spike rate data, burst parameters were then standardized to the aCSF control period for all treatment conditions for each bird (e.g., vs. estradiol).

Spike sorting and single unit analysis. Retrodialysis of estradiol and FAD each caused changes in NCM burst parameters, as revealed by multiunit analysis. To investigate whether changes in bursting were due to a shift to burst-firing of individual neurons, spike train data were sorted for single-unit analysis. Large-amplitude single units (high signal/noise ratio; 1 to 2 units per bird) were identified using spike-sorting methods in Spike2. Single units identified by the offline spike-sorting algorithm were verified by inspecting waveform characteristics and by PCA (Fig. 4*A*). In all cases, single units were verified to be auditory-responsive by inspecting playback PSTHs (Fig. 4*B*). Spike-sorted waveform templates were maintained across treatment periods, and individual spike parameters (PCA plots) were well segregated across treatment periods. Isolated unit spike trains were then analyzed as described above

using identical burst parameters. To achieve normality, the numbers of bursts from single units were log-transformed before ANOVA.

Statistical Analysis. Behavioral, recording, and hormone data were analyzed with repeated-measures ANOVA, with all relevant factors included in each model. Post hoc tests were Wilc-SRTs for within-subject factors, and Mann-Whitney *U* tests for between-subject factors, both of which are robust to sample sizes below $n = 10$. Post hoc tests were Bonferroni corrected for multiple comparisons.

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