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Forebrain steroid levels fluctuate rapidly during social interactions

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Neurosteroids are powerful modulators of brain function and behavior, yet their dynamics in the brain have remained elusive. Using *in vivo* microdialysis in male zebra finches, we found that local estradiol levels increased rapidly in the forebrain during social interactions with females. Furthermore, when males were exposed to other males' songs, local estradiol levels also increased and testosterone levels dropped in a cortical/pallial auditory region that is analogous to mammalian auditory cortex. We also found that local estradiol and testosterone levels were differentially regulated in this same region by the conventional neurotransmitters glutamate and GABA, respectively. This study provides direct evidence that forebrain steroid levels are acutely and differentially regulated during social behavior in a region-specific manner and in a rapid time course similar to that of traditional neuromodulators.

The suite of neuromodulators that acutely regulate neural circuit function and behavior has recently expanded to include steroids, oxide gases and glial amines[1,2]. Understanding the dynamics of the storage, release and action of these neuromodulators has proven to be a research challenge. One class of neuromodulators, the neurosteroids, are synthesized in the brain from cholesterol or from precursors arriving from the periphery to achieve local hormone concentrations that are independent of the general circulation[3]. In vertebrates, the enzymes that synthesize neurosteroids are expressed in a region-specific manner[3–6]. It is possible, therefore, that neurosteroid levels fluctuate locally to modulate circuit function, similar to conventional neurotransmitters[7], but there is little direct evidence for this possibility.

Steroids are powerful neuromodulators because of their multiple modes of action. They can act either through 'traditional' nuclear-hormone receptors to affect gene transcription over a protracted time frame (hours to days) or via nontraditional, rapid actions (seconds to minutes). In the short term, steroids influence neuronal excitability via interactions with ligand-gated ion channels[8,9], and steroids can therefore influence behavior in seconds to minutes[10,11].

Despite the importance of steroids in modulating brain function and behavior, the dynamics of neurosteroids *in vivo* have remained elusive, particularly over the short term. *Ex vivo* analysis of explants or brain region homogenates have revealed that steroid concentrations and steroidogenic enzyme activity or mRNA levels differ between experimental groups that have been exposed to stress, social interactions or pharmacological manipulations[3,11]. Furthermore, *in vitro* synthesis of neurosteroids can shape neural circuit differentiation[12] and synaptic plasticity[13,14]. However, whether local and acute changes in endogenous brain steroid levels occur in the context of natural behavior is currently unknown.

Our study addresses whether steroid levels are rapidly and locally regulated in the forebrain. We optimized an *in vivo* microdialysis system for quantifying 17β-estradiol (a predominant neurosteroid in songbirds) and testosterone levels from the auditory forebrain in actively behaving zebra finches. This approach allowed us to test whether local brain steroids are acutely regulated during social interactions. In addition, retrodialysis experiments (reverse delivery of pharmacological agents during microdialysis) allowed us to address whether brain steroids are regulated by conventional neurotransmitter mechanisms.

We chose zebra finches as our experimental model for several reasons. Steroid binding sites are widely distributed in the zebra finch forebrain and many aspects of zebra finch social behavior are steroid sensitive[15,16]. The forebrain expresses the suite of enzymes that are necessary for steroidogenesis in a region-dependent manner[17–19], although the function of this capacity in adults is essentially unknown. In male zebra finches, the endogenous source for testosterone is largely peripheral (endocrine) but could also include a central (brain) source[15]. In contrast, estradiol is considered to be a neurosteroid in males, as it originates exclusively in the brain[12,20]. In particular, the auditory caudo-medial nidopallium (NCM, analogous to mammalian auditory cortex) shows rich expression and activity of the enzyme aromatase[21] (which converts androgens into estrogens) and is considered to be a locus for song memory[22,23]. Lastly, the most compelling evidence to date for rapid regulation of aromatase enzymatic activity comes from an avian species[7,11]. We therefore adapted *in vivo* microdialysis to test whether local steroid levels in the zebra finch auditory forebrain fluctuate during social interactions.

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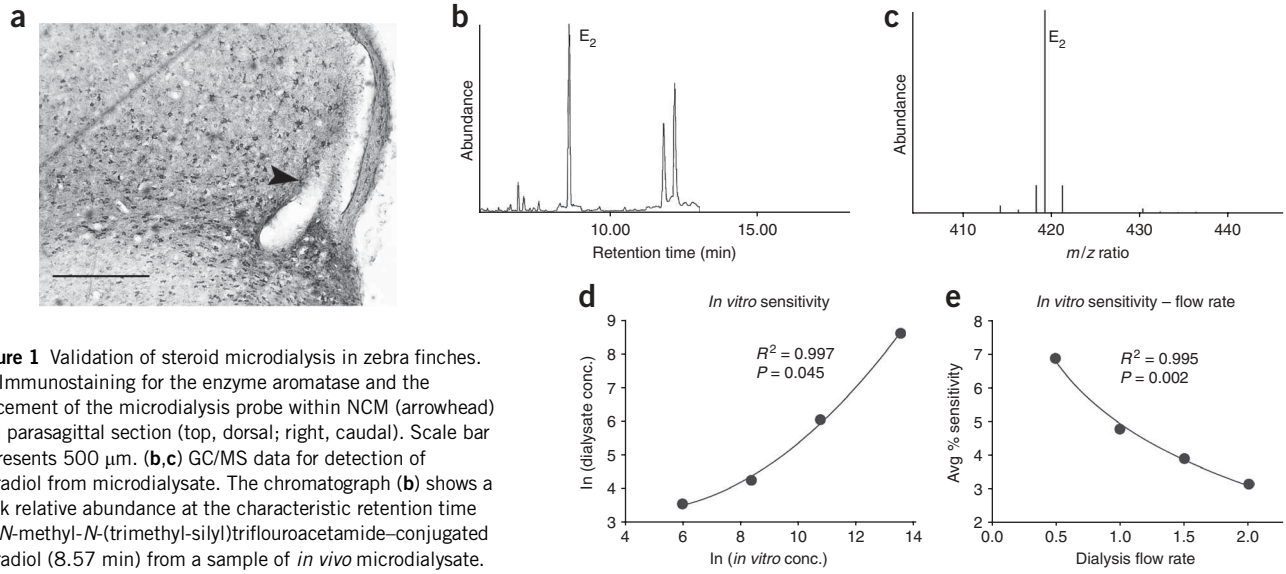

Figure 1 Validation of steroid microdialysis in zebra finches. (**a**) Immunostaining for the enzyme aromatase and the placement of the microdialysis probe within NCM (arrowhead) in a parasagittal section (top, dorsal; right, caudal). Scale bar represents 500 μm. (**b**,**c**) GC/MS data for detection of estradiol from microdialysate. The chromatograph (**b**) shows a peak relative abundance at the characteristic retention time for *N*-methyl-*N*-(trimethyl-silyl)triflouroacetamide–conjugated estradiol (8.57 min) from a sample of *in vivo* microdialysate. The mass spectrum (**c**) shows a peak at the characteristic mass/charge (*m*/*z*) ratio for MSTFA-conjugated estradiol (419) from a sample of *in vivo* microdialysate. (**d**,**e**) Microdialysis probes readily absorbed ^{3}H-estradiol from an *in vitro* ^{3}H-estradiol aCSF solution. The concentration of ^{3}H-estradiol in collected microdialysate increased as the *in vitro* concentration of ^{3}H-estradiol was increased (flow rate = 2.0 μl/min, **d**). The concentration of ^{3}H-estradiol in collected microdialysate (percentage sensitivity of *in vitro* concentration) increased logarithmically as the microdialysis flow-rate decreased to a theoretical maximum at zero-flow (**e**). For this experiment, the *in vitro* concentration of ^{3}H-estradiol was held constant.

RESULTS

Validation of microdialysis

We carried out gas chromatography/mass spectrometry (GC/MS) to positively confirm the presence of estradiol in a subset of *in vivo* microdialysate samples (**Fig. 1**). Complimentary *in vitro* experiments using ^{3}H-estradiol dissolved in artificial cerebrospinal fluid (aCSF) confirmed the relative recovery (passive diffusion) of steroids across the CMA-7 microdialysis probe membrane (**Fig. 1** and **Supplementary Results** online).

Immunostaining for the aromatase protein showed rich neuronal expression in NCM (**Fig. 1**), as has been reported previously[21]. In addition, aromatase expression was upregulated surrounding the probe tract (**Fig. 1**) and was restricted to glial-like cells, consistent with previous observations[24]. Therefore, glial aromatase upregulation probably contributes a portion of baseline estradiol, as measured here by *in vivo* microdialysis. However, the acute steroid fluctuations that we observed here (see below) are consistent with neuronal, rather than glial, activation. For example, microdialysis outside of the NCM induced glial-like aromatase, but estradiol levels outside of the NCM did not fluctuate during social interactions, whereas estradiol levels changed in NCM, where neuronal aromatase was abundant.

Upregulation of the immediate-early gene *EGR1* (also known as *ZENK*) indicates neural activity in response to sensory stimulation (see Methods). We used *ZENK* to determine the auditory activation of two brain regions (auditory NCM and medial preoptic nucleus) by song playback versus silence in a subset of males with microdialysis probes directed at NCM. Song induced significant *ZENK* upregulation in NCM, despite the presence of microdialysis probes ($P < 0.0005$; **Fig. 2** and **Supplementary Results**). This is consistent with the hypothesis that NCM retains auditory responsiveness during microdialysis.

To demonstrate the sensitivity of *in vivo* microdialysis and ELISA methods to artificially induced changes in steroid levels, we gave five dialyzed birds intramuscular injections of estradiol, which caused an increase in local estradiol levels in NCM (**Fig. 3**). Repeated-measures ANOVA revealed a significant effect of time after injection ($F = 3.316$, $P = 0.037$) on local estradiol levels. *Post hoc* tests showed that estradiol was significantly elevated by 1 h following injection ('post 2' versus 'pre-injection', $P = 0.048$) and had reached a peak during this sampling period. There were no significant effects of estradiol injection on testosterone levels in NCM (data not shown, $F = 2.230$, $P = 0.199$).

Fadrozole retrodialysis

We used fadrozole (a potent aromatase inhibitor[15]) to test the hypothesis that fadrozole retrodialysis reduces local estradiol levels in NCM. Fadrozole (100 μM) caused a robust decrease in estradiol levels, as well as an increase in testosterone levels within NCM (**Fig. 4**). Repeated-measures ANOVA revealed a significant effect of time after retrodialysis on local estradiol levels ($F = 6.772$, $P = 0.002$) and on testosterone levels ($F = 6.714$, $P = 0.003$) in NCM. *Post hoc* tests showed that estradiol levels were significantly reduced during fadrozole retrodialysis ('FAD' versus 'pre', $P = 0.017$) and remained significantly reduced during the first period of fadrozole washout ('post FAD 1' versus 'pre', $P = 0.027$). *Post hoc* tests also showed that testosterone levels were significantly elevated during fadrozole retrodialysis ('FAD' versus 'pre', $P = 0.004$).

A rapid increase in testosterone during fadrozole treatment could be the result of a buildup of androgen precursors when the aromatase enzyme is inhibited. We therefore compared the changes in testosterone versus estradiol during fadrozole retrodialysis for each bird. There was no significant correlation between the changing testosterone versus estradiol levels ($n = 5$, $P = 0.74$, $r^2 = 0.068$). Fadrozole is a highly specific inhibitor of aromatase activity[15] and this lack of correlation might result from the limited temporal resolution of our methods. It is also possible that, in addition to aromatase, other enzymes[17] regulate forebrain testosterone levels.

Female presentation experiment

We presented 13 dialyzed males with females adjacent to their own cage to determine whether social interactions are associated with local changes in forebrain estradiol levels (for behavioral measures of

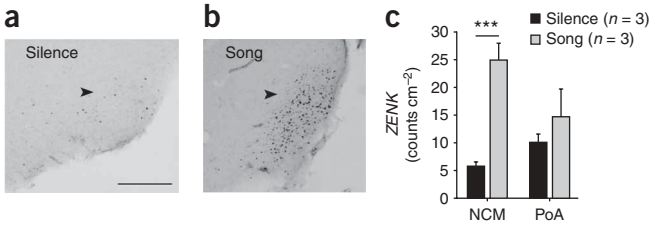

Figure 2 Expression of the immediate-early gene *ZENK* is upregulated in NCM during auditory stimulation, despite the presence of a microdialysis cannula and probe within NCM. Three microdialyzed birds received playback of male song, whereas three other birds received silence. (**a**,**b**) Representative parasagittal sections (top, dorsal; right, caudal) through NCM showing the NCM border (arrowheads) in a male that received silence (**a**) and in a male that heard song (**b**). Scale bar represents 500 μm. For clarity, the microdialysis probe tract in NCM is not presented in sections, although the probe tract was histologically verified to be within NCM for all birds in this experiment. (**c**) *ZENK* expression (mean stained nuclei per cm^2 ± s.e.m.) was significantly upregulated (***$P < 0.0005$) in the song versus silence group only in NCM, whereas expression in the preoptic area (PoA, nonauditory) was no different between groups.

dialyzed males, see **Supplementary Results** and **Supplementary Table 1** online). Histological examination of sections for probe placement in the caudal forebrain showed that the probe was not sampling from within NCM in four of these males (that is, NCM 'misses' were rostral, ventral and medial). Notably, probes for these misses were sampling from regions that contain few, if any, aromatase-positive neurons, although they were adjacent to aromatase-rich regions. The remaining nine birds in this experiment had probes that were successfully placed within NCM (for example, **Fig. 1a**), a region that is rich in neuronal aromatase[21]. Therefore, for statistical analyses, data were grouped according to whether the probe missed the NCM ('outside NCM' group) or successfully sampled from the NCM ('within NCM' group). Local estradiol levels increased during female presentation trials for the within NCM group and not for the outside NCM group (**Fig. 5**). Repeated-measures ANOVA revealed an overall effect of probe placement (within versus outside NCM, $F = 5.155$, $P = 0.044$) and a significant effect of time ($F = 4.257$, $P = 0.005$) but no significant time × probe placement interaction ($F = 1.967$, $P = 0.116$) on local estradiol levels. For the within NCM group, *post hoc* tests showed that estradiol levels in the 'females adjacent' period were significantly elevated compared with the 'lights on' period ($P = 0.006$) and the 'post-females' period ($P = 0.046$). For the outside NCM group, *post hoc* tests identified no significant differences among sampling periods. For between group differences, *post hoc* tests revealed that estradiol levels were significantly elevated in the within versus outside NCM group for the 'overnight' ($P = 0.018$) and 'pre–lights on' ($P = 0.023$) sampling periods but not for the 'lights on' period ($P = 0.146$). This suggests that forebrain regions outside of the aromatase-rich nucleus NCM have circadian changes in steroid levels that could be peripheral or central in origin.

The local increases in estradiol within NCM were unrelated to the production of song in female-exposed males. For each of the within NCM birds (including 8 singers and 1 non-singer), local estradiol levels were elevated during the female adjacent period (versus 'lights on'). Moreover, there was no significant correlation between the number of songs each male sang and the degree of increase in dialysate estradiol for each male ($R = 0.13$, $P = 0.73$). Together, these results suggest that fast increases in local estradiol within NCM are not strictly related to activation of the motor pathway(s) for song production. However, as above, limits on microdialysis temporal resolution may restrict the power of such correlative analysis.

We reasoned that fast changes in local estradiol levels within NCM in response to females could depend, in part, on changing androgen levels (centrally or peripherally derived), which are then aromatized into estrogens. In a separate set of birds ($n = 6$), we measured fluctuations in local estradiol and testosterone levels simultaneously in response to female presentation, both within NCM ($n = 3$) and outside of the NCM ($n = 3$, probes intentionally targeted to anterior forebrain with reduced aromatase[21]). Consistent with our previous observations, local estradiol levels within NCM increased significantly during social interactions with females, whereas estradiol levels did not change significantly outside NCM (probe placement effect: $F = 0.009$, $P = 0.93$; female treatment effect: $F = 7.168$, $P = 0.0017$; placement × treatment interaction: $F = 4.828$, $P = 0.0096$; **Fig. 5b**). For the within NCM group, *post hoc* tests showed that estradiol levels in the females adjacent period were significantly elevated compared with the pre-females period ($P = 0.035$) and the post-females period ($P = 0.0087$). For the outside NCM group, *post hoc* tests identified no significant differences in estradiol levels among sampling periods.

In contrast, for testosterone levels, there were no significant effects of probe placement (within versus outside NCM, $F = 4.293$, $P = 0.107$; **Fig. 5c**) or female treatment ($F = 2.809$, $P = 0.061$), nor was there a significant placement × treatment interaction ($F = 0.419$, $P = 0.79$). *Post hoc* tests showed no significant changes in testosterone levels in response to females for either the within NCM or outside NCM groups. Therefore, social interactions with females caused changes in local estradiol levels only within NCM and these estradiol changes were not associated with concomitant changes in testosterone levels, either inside of or outside of the NCM.

Playback experiment

Because local estradiol levels within NCM changed during social interactions, we reasoned that these changes could be the result of auditory activation (that is, song processing/memory access) within NCM. In a separate set of birds, we tested whether playback of auditory stimuli led to rapid changes in local steroid levels within NCM. Birds in this experiment were histologically confirmed to have probes in the NCM (too few misses occurred for a separate statistical grouping; results from individuals from the outside NCM group are plotted as individual data points in **Fig. 6a–c**). Playback treatments containing male song caused an acute increase in local estradiol levels within NCM (**Fig. 6a**,**b**). Repeated-measures ANOVA revealed an overall effect of sampling time ($F = 5.711$, $P = 0.007$), no significant effect of playback

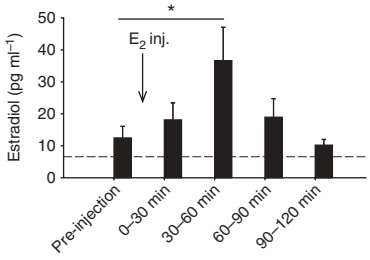

Figure 3 Estradiol injection causes a substantial and detectable increase in estradiol levels within NCM. Each histogram depicts a 30-min microdialysis sample in series (mean ± s.e.m.). Intramuscular injection of estradiol (6.0 μg, arrow) caused a significant increase in dialysate estradiol levels in the second 30-min period following injection (*$P = 0.048$; $n = 5$). The dashed line indicates the average background estradiol concentration as reported by ELISA for aCSF alone.

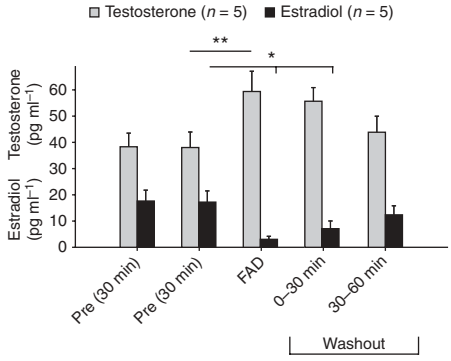

Figure 4 Fadrozole (100 μM, delivered via retrodialysis) significantly alters local steroid levels within NCM. Each histogram depicts a 30-min microdialysis sample in series (mean ± s.e.m.). When compared with pre-treatment baseline (pre (30 min)), local estradiol levels were reduced during fadrozole (FAD) retrodialysis and during the first 30 min of washout (0–30 min; $*P < 0.03$). When compared with pre-treatment baseline, local testosterone levels were significantly increased during FAD retrodialysis ($**P = 0.004$).

treatment ($F = 1.834$, $P = 0.177$) and a significant playback treatment × time interaction ($F = 7.695$, $P = 0.004$) on local estradiol levels within NCM. For the 'male song' group, *post hoc* analysis showed that estradiol levels were significantly elevated in the 'playback' period as compared with both the 'pre-silence' ($P = 0.011$) and 'post-silence' ($P = 0.05$) periods. For the 'colony sounds' group, *post hoc* analysis indicated that estradiol levels were also significantly elevated in the playback period as compared with both the pre-silence ($P = 0.010$) and post-silence ($P = 0.009$) periods. For both the 'white noise' and 'female chirps' playback groups, *post hoc* analysis showed no significant within-subject changes in estradiol levels among sampling periods (all $P > 0.47$). Lastly, between-subject *post hoc* analysis showed that estradiol levels were significantly elevated during the playback period in both the male song ($P = 0.023$) and colony sounds ($P = 0.039$) groups (versus white noise). Therefore, local estradiol levels within NCM were only elevated in response to playback stimuli that contained male song.

Testosterone was measured in a subset of dialysate samples in the playback experiment (the male song and white noise groups). Male song playback treatment caused decreases in local testosterone levels within NCM (**Fig. 6c**). Repeated-measures ANOVA revealed a significant overall effect of sampling time ($F = 3.842$, $P = 0.047$), a significant effect of playback treatment ($F = 7.978$, $P = 0.026$) and no significant playback treatment × time interaction ($F = 2.978$, $P = 0.084$) on local testosterone levels within NCM. For the male song group, *post hoc* tests showed that testosterone levels were significantly reduced in the playback period as compared with the pre-silence period ($P = 0.042$) and that a nonsignificant decreasing trend existed for the playback versus post-silence periods ($P = 0.064$). For the white noise group, *post hoc* tests identified no significant within-subject changes in testosterone levels among sampling periods (all $P > 0.34$). Lastly, between-subject *post hoc* tests revealed that testosterone levels were significantly reduced in the male song versus white noise group during the playback period ($P = 0.029$; for correlations between changing testosterone and estradiol levels, see **Supplementary Results**). Therefore, male song stimulation caused robust decreases in local testosterone levels and increases in local estradiol levels within NCM at the same time.

Influence of circulating steroids

Changes in local forebrain estradiol levels could depend, in part, on changing circulating testosterone, which is then converted to estradiol via brain aromatase. We conducted several experiments to determine whether exogenous testosterone treatment leads to detectable changes in local forebrain estradiol and/or testosterone levels, and whether presentation of social stimuli (adjacent females or male song playbacks) are associated with changes in circulating plasma testosterone and/or estradiol levels.

We gave six birds intramuscular injections of testosterone ($n = 3$ each for the within NCM and outside NCM groups). Testosterone injection caused a significant increase in local testosterone levels within NCM (mean ± s.e.m., pg ml^{-1}; pre-injection, 19.65 ± 2.51; post-injection, 58.33 ± 14.33; $P = 0.03$) and outside NCM (pre-injection, 24.48 ± 2.86; post-injection, 49.36 ± 16.29; $P = 0.04$). Likewise, testosterone injection caused a significant increase in local estradiol levels within NCM (pre-injection, 17.87 ± 8.42; post-injection, 41.06 ± 1.37; $P = 0.05$) and an increase in estradiol outside of the NCM that approached significance (pre-injection, 15.69 ± 4.57; post-injection, 31.22 ± 8.33; $P = 0.06$). Therefore, surges in plasma testosterone levels can lead to detectable, local increases in forebrain levels of estradiol and testosterone. Presumably,

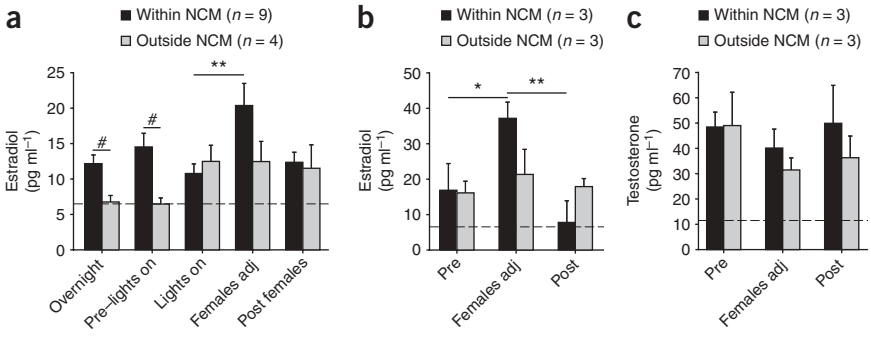

Figure 5 Local forebrain estradiol levels change rapidly within NCM during social interactions with females, while testosterone levels remain unchanged. Each histogram depicts a 30-min microdialysis sample in series (mean ± s.e.m., overnight is >8 h). Within NCM animals were a group with microdialysis probes successfully directed at the NCM and outside NCM were a group with probes in forebrain surrounding the NCM. (**a**) For the 30-min period when females were adjacent to dialyzed males (females adj), estradiol levels within NCM were significantly elevated as compared with the 30-min lights on and post-females periods ($**P < 0.006$ for within-group comparison of lights on versus females adj), indicating fast regulation of estradiol levels locally within NCM. Local estradiol levels were significantly higher in within NCM birds during the lights off periods (# $P < 0.02$ for between group comparisons for within versus outside NCM groups). (**b**,**c**) In a separate group of birds, estradiol levels within NCM were again elevated during social interactions with females. For the females adj period, estradiol levels (**b**) were significantly elevated within NCM (versus pre, $*P < 0.035$; versus post, $**P < 0.009$), but not outside the NCM. Testosterone levels (**c**) in these same males did not change within or outside the NCM when females were adjacent. The dashed line indicates the average background estradiol (**a**,**b**) or testosterone (**c**) concentration as reported by ELISA for aCSF alone.

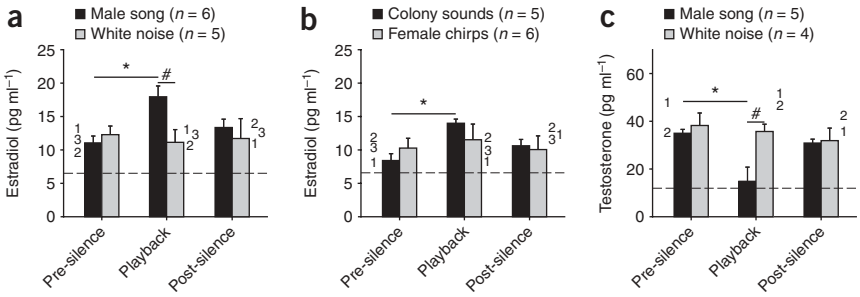

Figure 6 Local steroid levels within NCM change rapidly in response to acoustic playbacks. Each histogram depicts a 30-min microdialysis sample in series (mean ± s.e.m.). (**a**) Estradiol levels within NCM were rapidly elevated during playback of male song versus pulsed white noise (*$P = 0.011$ for playback versus pre-silence in the male song group; # $P = 0.023$ for male song versus white noise during playback). Bars are group means for the within NCM group, whereas the numbers indicate individual results for the outside NCM group (1 and 2 represent male song, 3 represents white noise). (**b**) Local estradiol levels within NCM were rapidly elevated during playback of zebra finch colony sounds and not female chirps (colony sounds group, *$P = 0.01$ for playback versus pre-silence and *$P = 0.009$ for playback versus post-silence). Bars are group means for the within NCM group, whereas the numbers indicate individual results for the outside NCM group (1 represents colony sound, 2 and 3 represent female chirps). (**c**) Local testosterone levels within NCM were rapidly reduced during playback of male song and not pulsed white noise (*$P = 0.042$ for playback versus pre-silence in the male song group; # $P = 0.039$ for male song versus white noise during playback). Bars are group means for the within NCM group, whereas the numbers indicate individual results for the outside NCM group (1 represents male song, 2 represents white noise). Testosterone levels were from the same subjects as in **a** (one sample was lost from each group during extraction). The dashed line indicates the average ELISA background estradiol or testosterone concentration for aCSF alone.

detectable increases in estradiol outside NCM are the result of aromatization in adjacent regions or from reactive glia.

We next sought to determine whether social stimuli that cause changes in local forebrain steroid levels (see above) also lead to changes in plasma steroid levels. In a female presentation experiment, a separate set of 12 males were exposed to either adjacent females ($n = 6$) or control manipulations ($n = 6$) for 30 min. There were no group differences for either plasma testosterone (female exposed, 158.83 ± 9.52 (mean ± s.e.m., pg ml^{-1}); control, 160.58 ± 11.34; $P = 0.64$) or plasma estradiol (female exposed, 58.18 ± 26.48; control, 42.88 ± 17.80; $P = 0.66$). In an acoustic playback experiment, a separate set of 16 males were exposed to either male song ($n = 10$) or white noise ($n = 6$) for 30 min. There were no group differences for either plasma testosterone (male song, 180.71 ± 66.28; white noise, 137.32 ± 62.52; $P = 0.68$) or plasma estradiol (male song, 41.96 ± 10.16; white noise, 51.24 ± 24.65; $P = 0.70$). Together, these results indicate that the same social stimuli that cause rapid changes in local forebrain steroid levels do not appear to be accompanied by changes in plasma steroid levels at 30 min. Testosterone and estradiol levels were in the low, but physiological, range for male zebra finches; unlike some bird species[15], zebra finches may not show socially induced acute changes in gonadal steroid secretion.

Neurotransmitter retrodialysis

Because steroidogenic enzymes are found in neurons[8], including presynaptic terminals in the NCM[19], local steroid levels could be regulated by neurotransmitter activation. In a separate set of birds, we tested whether estradiol and testosterone levels within NCM were altered in response to infusions of NMDA, glutamate or GABA. Glutamate retrodialysis caused a significant decrease in local estradiol levels within NCM (repeated-measures ANOVA, $F = 2.977$, $P = 0.044$; **Fig. 7a**). *Post hoc* tests showed that estradiol was significantly reduced during the period of glutamate retrodialysis (versus 'pre', $P = 0.008$). Repeated-measures ANOVA identified no significant effects of NMDA ($F = 0.319$, $P = 0.86$) or GABA ($F = 1.038$, $P = 0.41$) on local estradiol levels within NCM. Glutamate-mediated estradiol suppression is consistent with recent reports of glutamatergic suppression of *in vitro* hypothalamic aromatase activity[11] and *in vitro* hippocampal estradiol production (G.M. Rune, personal communication).

In contrast, GABA retrodialysis caused a significant increase in local testosterone levels within NCM (repeated-measures ANOVA, $F = 7.400$, $P = 0.015$; **Fig. 7b**). *Post hoc* tests showed that testosterone was significantly elevated during the period of GABA retrodialysis (versus 'pre', $P = 0.038$). Repeated-measures ANOVA identified no significant effects of glutamate ($F = 0.699$, $P = 0.52$) or NMDA ($F = 0.424$, $P = 0.70$) on local testosterone levels within NCM. Taken together, these results indicate that glutamatergic activation suppressed estradiol levels without significantly affecting testosterone levels, whereas GABAergic activation elevated testosterone levels without significantly affecting estradiol levels.

DISCUSSION

This study provides direct evidence that acute modulation of local steroid levels occurs in the forebrain during social interactions. In actively behaving zebra finches, steroids in the auditory forebrain fluctuate during singing, audition and in response to neurotransmitter activation. These findings indicate that the region-specific expression of steroidogenic enzymes throughout the forebrain of vertebrates[4–6] and

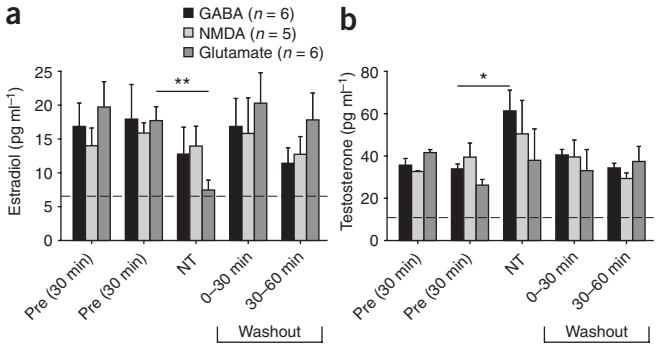

Figure 7 Neurotransmitter activation causes acute changes in steroid levels within NCM. (**a**,**b**) Infusion of glutamate into the NCM via retrodialysis caused a rapid decrease in local estradiol levels (**a**), whereas infusion of GABA into the NCM caused a rapid increase in local testosterone levels (**b**). Each histogram depicts a 30-min microdialysis sample in series (mean ± s.e.m.). When compared with pre-treatment baseline, estradiol levels were significantly reduced during the period of glutamate retrodialysis only (10 mM; **$P = 0.008$). When compared with pre-treatment baseline, testosterone levels were significantly increased during the period of GABA retrodialysis only (100 μM; *$P = 0.038$). NT, 30-min period of neurotransmitter retrodialysis. The dashed line indicates the average background estradiol (**a**) and testosterone (**b**) concentrations as reported by ELISA for aCSF alone.

the rapid regulation of their activity[7,11] have direct consequences for local and acute changes in neurosteroid concentrations. These results are consistent with the hypothesis that local brain steroid levels are regulated on a time scale that is similar to that of traditional neuromodulators.

We carried out several validations for *in vivo* neurosteroid microdialysis in zebra finches. First, we confirmed the presence of estradiol in microdialysate using three independent methods: estradiol radioisotope detection *in vitro*, and *in vivo* detection of estradiol using both ELISA and GC/MS (see Methods). Second, systemic estradiol injection resulted in increases in local estradiol levels and pharmacological inhibition of aromatase caused substantial changes in local estradiol and testosterone levels. Together, these proof of principle results confirm predictions for neurosteroid microdialysis in zebra finches. Notably, the results of our experiments with the song-responsive immediate-early gene *ZENK* indicate that the auditory region of the NCM retains auditory responsiveness during microdialysis. Lastly, estradiol levels changed in response to social interactions only in the within NCM group and not in the outside NCM group, emphasizing that steroid levels are locally regulated in the forebrain.

A prevailing view holds that increases in testosterone production from the gonads lead to parallel increases in central estradiol levels[25,26]. Indeed, in our study, exogenous testosterone injection caused elevations in forebrain testosterone and estradiol levels. However, although birds in this study were not castrated or adrenalectomized, we observed fluctuations in forebrain steroid levels that did not appear to be accompanied by changing circulating levels. First, social stimuli (interactions with females and hearing male song) did not alter circulating steroid levels, but these same treatments led to robust changes in forebrain estradiol levels in the same time frame. Second, local forebrain estradiol and testosterone levels changed in opposite directions during audition and following aromatase inhibition. Third, only local forebrain estradiol levels (and not testosterone levels) within NCM changed during social interactions with females. Lastly, retrodialysis of neurotransmitters differentially altered forebrain steroid levels, presumably via local, forebrain-specific mechanisms. Therefore, in contrast with previous reports that steroidal substrates and products change concomitantly in the brain[13,27], our findings illustrate that changing forebrain steroid levels are not always accompanied by parallel changes in precursor concentrations. However, these results do not rule out an interaction between circulating and central levels of steroids. Future tests of this hypothesis will be aided by improvements in microdialysis technology and assay sensitivity to allow greater time course resolution of changing forebrain steroids vis-à-vis peripheral steroid concentrations.

Our results indicate that local forebrain estradiol and testosterone levels are differentially regulated by the neurotransmitters glutamate and GABA, respectively. Mechanisms to uncouple brain estrogens from androgens could be widespread among vertebrates, as estradiol and testosterone concentrations in post-mortem human brain–region homogenates are not directly correlated[28]. Although estrogens primarily exert rapid excitatory effects on neurons[8], 5α-reduced metabolites of testosterone generally reduce neuronal excitability[29]. Therefore, acute uncoupling of neurosteroid levels in discrete brain nuclei could reflect their divergent (for example, excitatory versus inhibitory) downstream modulatory actions on neural circuit function[30]. In songbirds, estrogens prolong plasticity during development and song learning, whereas surging androgen levels are thought to be responsible for the 'crystallization' of learned song[15,16] via synaptic development and maturation[31]. Our findings predict that similarly divergent roles for estrogens versus androgens occur on an acute time scale in the forebrain of adult zebra finches.

GABAergic stimulation caused an increase in testosterone levels within NCM, which could be the result of fast downregulation of testosterone-metabolizing enzyme activity or upregulation of testosterone-synthesizing enzyme activity. Aromatase activity is probably not responsible for our testosterone results with GABA, primarily because we observed no concomitant changes in estradiol levels following GABA infusion. Other androgen metabolizing enzymes (for example, 5α- and 5β-reductase) are expressed in forebrain GABAergic and glutamatergic neurons[32] and are abundant in the songbird telencephalon[15,17], although the neurotransmitter-dependent regulation of these enzymes is poorly understood for any vertebrate. Testosterone levels could change locally in the forebrain due to the actions of testosterone-synthesis enzymes, such as 17α-hydroxylase/17,20-lyase, which is expressed in synaptic terminals in mammalian hippocampus[14] and is expressed[33] and active[6] in avian brain tissue. In addition, GABAergic, steroidogenic and calbindin-positive neurons appear to overlap within NCM[34], indicating that local inhibitory circuits involved in zebra finch audition are steroidogenic. Together, these observations raise the possibility that testosterone is locally synthesized as a neurosteroid independent of the circulation in some physiological contexts (including auditory processing in songbirds), although a direct demonstration of this possibility is not yet evident.

The localized nature of these phenomena suggests that neurosteroids modulate acute auditory processing in the songbird forebrain. Although steroids influence auditory encoding in the peripheral nervous system[35,36], there are few studies that address the neuromodulatory role of steroids on central mechanisms of audition. Steroids exert long-term, genomic actions on auditory processing in the inferior colliculus in rats[37] and in the NCM in songbirds[38]. Our study predicts that brain-derived steroids acutely modulate auditory processing in pallial/cortical regions, which could provide clues about the function of neuronal aromatase expression in the auditory cortex of other vertebrates, including humans[39].

Local changes in NCM steroids could modulate the adjacent forebrain song motor pathway or they could be important for the regulation of activity within NCM itself, including auditory memory mechanisms[22,23,40]. There is evidence that endogenous/exogenous estrogens can improve spatial memory in mammals[41,42] and songbirds[43]. One potential function for rapid fluctuations in neurosteroids within NCM could therefore be to modulate short-term auditory memory processing and/or vocal learning. Brain-derived steroids are hypothesized to be important in song learning[15,44], and recent observations indicate that steroids can act via long-term mechanisms in the forebrain in a localized, nucleus-specific manner[31,45]. One question that arises from our results is whether local pharmacological inhibition of steroidogenesis in forebrain auditory regions affects acute auditory memory function. Ontogenetic applications of microdialysis technology could shed light on how the developing brain, as both a source and target of steroids[8,12,25,33], is acutely modulated by steroids during critical learning and acquisition phases.

Our findings emphasize the importance of monitoring real-time changes in steroids in neural circuits to reveal how those circuits are modulated *in vivo* during social behavior. They provide an essential logical link between the well-established rapid effects of steroids on neuronal excitability[8–10] and the rapid regulation of steroidogenic enzyme activity in the context of behavior[7,11]. It is now evident that steroids are part of a network of local and rapid modulatory mechanisms that are intrinsic to the vertebrate forebrain.

METHODS

Subjects. Animal care and use protocols were approved by the University of California Los Angeles Chancellor's Committee on Animal Care and Use. Microdialysis cannula/probe implantation procedures were adapted from techniques that are routinely used to analyze neurotransmitters in rodents[46] and more recently in zebra finches[47]. Birds were anesthetized with equithesin (3.2 ml per kg of body weight), which was injected into the pectoralis muscle. Using stereotaxic coordinates, a CMA-7 microdialysis guide cannula with obdurator (CMA Microdialysis) was inserted 1.2 mm from the surface of the dura mater to the target NCM. Dental cement and cyanoacrylate were applied to stabilize the cannula and the birds were returned to individual sound-attenuation chambers and monitored for at least 6 d before microdialysis probe implantation (**Supplementary Methods** online).

Microdialysis. At the start of microdialysis, the obdurator was removed from the guide cannula and replaced by a continuously perfused (2.0 μl min^{-1} aCSF, Harvard Apparatus 22 infusion pump) CMA-7 microdialysis probe (CMA-7, outer diameter of 0.24 mm, shaft length of 7.0 mm, membrane length of 1.0 mm, CMA Microdialysis) under isoflurane anesthesia (Hospira). Sample collection did not begin until 8–12 h after probe implantation to allow implantation-induced neurochemical changes to subside. Following the completion of all experiments, the birds were killed by perfusion and brain sections were examined under light microscope to determine probe placement (**Supplementary Methods**).

Immunocytochemistry. To determine the extent of reactive aromatase expression in response to cannula/probe implantation, we processed a subset of brain tissue from dialyzed birds for immunocytochemical expression of the aromatase protein using established methods[19,21] (**Supplementary Methods**). Upregulation of the immediate-early gene *ZENK* (a transcription factor that is also known as egr-1, ngfi-a, krox-24 and zif-268) reflects neuronal activity in response to auditory stimulation[48]. Therefore, in a separate set of males, we also determined whether probe/cannula placement in the NCM reduced or eliminated the well-established *ZENK* response to auditory stimulation (**Supplementary Methods**).

Steroid analysis. Analysis of microdialysate steroid concentrations was determined using enzyme immunoassays (ELISA, see below). The presence of estradiol in dialysate was unequivocally confirmed with GC/MS (**Supplementary Methods**). ELISA was used exclusively for quantification of steroid levels after optimization (**Supplementary Methods**). For each estradiol ELISA (Cayman Chemical), unmanipulated aCSF was included with dialysate samples in the plate for comparison as a background/baseline estradiol concentration (mean ± s.e.m. = 6.53 ± 0.57 pg ml^{-1}). The intra-assay coefficient of variation was 3.32%, and the inter-assay coefficient of variation was 12.16% (n = 17 assays).

For a subset of the estradiol ELISAs, samples were processed for subsequent testosterone analysis using a second commercial ELISA (Assay Designs; **Supplementary Methods**). For each testosterone ELISA, unmanipulated aCSF was included with dialysate samples in each ELISA plate for comparison as a background/baseline testosterone concentration (mean ± s.e.m. = 11.58 ± 3.25 pg ml^{-1}). The intra-assay coefficient of variation was 7.86%, and inter-assay coefficient of variation was 18.76% (n = 10 assays). This serial assay design provided quantification of both estradiol and testosterone from the same set of microdialysate samples; this procedure was only performed on a subset of microdialysate samples (see below).

***In vivo* microdialysis.** For estradiol injection *in vivo*, we predicted that local estradiol levels within NCM would be elevated following peripheral estradiol injection. Five actively behaving male zebra finches with microdialysis probes directed at NCM received intramuscular injections of estradiol (20 μl of 300 μg ml^{-1}; **Supplementary Methods**).

To test whether local pharmacological inhibition of aromatase alters local estradiol or testosterone levels within NCM, we used the aromatase inhibitor fadrozole. Fadrozole was dissolved in aCSF and perfused via retrodialysis into the NCM of seven actively behaving males (**Supplementary Methods**).

Female presentation experiments. We tested whether social interactions with females were accompanied by changes in estradiol levels within NCM of microdialyzed males (n = 13 males). Because male zebra finches sing robustly in the morning, all female presentation trials were carried out immediately after lights on. The sequential dialysate sampling periods were 8–10 h overnight, 30 min before lights on, 30 min immediately following lights on, 30 min with females adjacent (three unfamiliar females presented to the microdialyzed male in an adjacent cage inside the acoustic attenuation chamber) and 30 min following removal of the female cage. The male's singing behavior was recorded using Syrinx software (**Supplementary Methods**). The occurrence of other behaviors (drinking, feeding, beak wiping, flights and preening) were monitored and scored by an observer hidden behind a one-way glass partition during the trial.

In a second female presentation experiment, we again tested whether the presence of females would be accompanied by changes in local estradiol and testosterone levels inside and outside of the NCM (**Supplementary Methods**).

Acoustic playback experiment. We next tested whether local estradiol and testoterone levels within NCM change in response to acoustic playback of auditory stimuli. Dialysate was first collected from males housed in sound-attenuation chambers in silence for 30 min to establish baseline ('pre-silence'). Then, one of the following four playback stimuli were broadcast (**Supplementary Methods**) in loop mode for 30 min during dialysis: 5-min recording of female chirping behavior (looped 6 times, n = 6), 5-min recording of the zebra finch colony (looped 6 times, n = 5), 1-min recording of male song from three individual males (looped 30 times, n = 6) or 1-min intermittent white noise stimulus (looped 30 times, n = 5). After playback, dialysate was collected during a 30-min silent period so that we could examine the post-treatment effects. During playback trials, the focal male's vocalizations were recorded using Syrinx and other behaviors were scored as above.

Influence of circulating steroids. To test whether peripheral steroids contribute to fluctuating forebrain levels of testosterone and estradiol, we injected testosterone peripherally and examined the effects on local forebrain estradiol and testosterone levels inside and outside of NCM (**Supplementary Methods**). Next, we examined whether social stimuli that caused changes in local forebrain estradiol and testosterone levels also lead to changes in plasma estradiol and/or testosterone levels (**Supplementary Methods**).

Neurotransmitter retrodialysis experiment. Neurosteroidogenic enzymes are expressed in neurons[8] and synaptic terminals[19], which raises the possibility that neurosteroid levels are regulated by neurotransmitter activation. We therefore tested whether neurosteroids in the NCM are altered in response to local infusion of either the predominant excitatory neurotransmitter glutamate (L-glutamatic acid, n = 6), the predominant inhibitory neurotransmitter GABA (n = 6) or NMDA (n = 5). Baseline dialysates were collected for two 30-min periods, followed by 30 min of GABA/glutamate/NMDA retrodialysis, followed by two 30-min post-treatment sampling periods (**Supplementary Methods**).

Analysis. Statistical tests (one-way, multi-way and repeated-measures ANOVAs) were performed using Statview 4.57 (Abacus Concepts). *Post hoc* tests included paired t tests (within-subject comparisons of dialysate steroid levels from adjacent time periods), Tukey's *post hoc* tests (between-subject *post hoc* comparisons of hormone levels and behavior occurrences) and unpaired t tests (between-group comparisons of plasma steroid levels).

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

L.R.-H. conducted the experiments and wrote the manuscript. N.T.M. provided methodological expertise and contributed to manuscript preparation. B.A.S. supervised the project and wrote the manuscript.

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