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Forebrain steroid levels fluctuate rapidly during social interactions

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Abstract

Neurosteroids are powerful modulators of brain function and behavior, yet their dynamics within the brain have remained elusive. Using *in vivo* microdialysis in male zebra finches, we show that local estradiol levels increase rapidly within the forebrain during social interactions with females. Further, when males are exposed to other males' song, local estradiol levels also increase and testosterone levels drop within a cortical/pallial auditory region that is analogous to mammalian auditory cortex. We also report that local estradiol and testosterone levels are 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 akin to that of traditional neuromodulators.

Keywords

audition; estrogen; neuromodulation; nongenomic; microdialysis; extranuclear; membrane

The suite of neuromodulators that acutely regulate neural circuit function and behavior has recently expanded to include steroids, oxide gases, and glial amines1,2. The dynamics of storage, release, and action of these novel neuromodulators has proven to be a research challenge. One class, the 'neurosteroids', are synthesized within the brain from cholesterol or from precursors arriving from the periphery to achieve local hormone concentrations independent of the general circulation3. In vertebrates, the enzymes that synthesize neurosteroids are expressed in a region-specific manner3–6. It is possible, therefore, that neurosteroid levels fluctuate locally to modulate circuit function, much in the way of conventional neurotransmitters7, yet there is little direct evidence for this possibility.

Steroids are powerful neuromodulators due to their multiple modes of action. They can act either through 'traditional' nuclear-hormone receptors to affect gene transcription in a protracted timeframe (hours-to-days), or via non-traditional, rapid actions (seconds-to-minutes). In the short-term, steroids influence neuronal excitability via interactions with

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ligand-gated ion channels8,9, and steroids can therefore influence behavior within seconds-to-minutes10,11.

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/mRNA levels differ between experimental groups exposed to stress, social interactions or pharmacological manipulations3,11. Furthermore, *in vitro* synthesis of neurosteroids can shape neural circuit differentiation12 and synaptic plasticity13,14. However, whether local and acute changes in endogenous brain steroid levels occur in the context of natural behavior is currently unknown.

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

We have chosen 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-sensitive15,16. The forebrain expresses the suite of enzymes necessary for steroidogenesis in a region-dependent manner17–19, 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 include a central (brain) source as well15. In contrast, in males estradiol is considered a neurosteroid as it originates exclusively in the brain12,20. In particular, the auditory caudo-medial nidopallium (NCM; analogous to mammalian auditory cortex) exhibits rich expression and activity of the enzyme aromatase21 (which converts androgens into estrogens), and is considered a locus for song memory22,23. Lastly, the most compelling evidence to date for rapid regulation of aromatase enzymatic activity comes from an avian species7,11. We have therefore adapted *in vivo* microdialysis to test whether local steroid levels within the zebra finch auditory forebrain fluctuate during social interactions.

Results

Steroids confirmed in dialysate

Gas chromatography/mass spectrometry (GC/MS) positively confirmed the presence of estradiol in a subset of *in vivo* microdialysate samples (Fig. 1b-c). *In vitro* experiments using ^{3}H-estradiol dissolved in aCSF confirmed the relative recovery (passive diffusion) of steroids across the CMA-7 microdialysis probe membrane (Fig. 1d-e; Supplementary Results).

Aromatase expression

Immunostaining for the aromatase protein showed rich neuronal expression within NCM (Fig. 1a), as reported previously21. In addition, aromatase expression was upregulated surrounding the probe tract (Fig. 1a) and restricted to glial-like cells, consistent with previous observations24. Therefore, glial aromatase upregulation most likely contributes a portion of baseline estradiol as measured here by *in vivo* microdialysis. However, the acute steroid fluctuations observed in this study (see below) are consistent with neuronal rather than glial activation (e.g., microdialysis outside NCM induced glial-like aromatase, but

estradiol levels outside NCM did not fluctuate during social interactions, whereas estradiol changed 'within NCM' where neuronal aromatase was abundant).

ZENK expression

Up regulation of the immediate-early gene ZENK reports neural activity in response to sensory stimulation (see Methods). ZENK was used to determine the auditory activation of two brain regions (auditory NCM and medial preoptic nucleus) by song playback vs. 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 (Fig. 2; see also Supplementary Results). This is consistent with the hypothesis that NCM retains auditory responsiveness during microdialysis. For clarity, the microdialysis probe tract within NCM is not presented in sections in Fig. 2, although the probe tract was histologically verified to be within NCM for all birds in this experiment.

Estradiol Injection

To demonstrate the sensitivity of *in vivo* microdialysis/ELISA methods to artificially-induced changes in steroid levels, five dialyzed birds received intramuscular injections of estradiol, which caused an increase in local estradiol levels within 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 hr following injection ('post 2' vs. '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 within NCM (data not shown, $F = 2.230$; $p = 0.199$).

Fadrozole Retrodialysis

We used Fadrozole (a potent aromatase inhibitor15) to test the hypothesis that Fadrozole retrodialysis reduces local estradiol levels within 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 ($F = 6.772$; $p = 0.002$) as well as testosterone levels ($F = 6.714$; $p = 0.003$) within NCM. Post hoc tests showed that estradiol levels were significantly reduced during Fadrozole retrodialysis ('FAD' vs. 'pre'; $p = 0.017$), and remained significantly reduced during the first period of Fadrozole washout ('post FAD 1' vs. 'pre'; $p = 0.027$). Post hoc tests also showed that testosterone levels were significantly elevated during Fadrozole retrodialysis ('FAD' vs. 'pre'; $p = 0.004$).

A rapid increase in testosterone during Fadrozole treatment could result from a buildup of androgen precursors when the aromatase enzyme is inhibited. We therefore compared for each bird the changes in testosterone vs. estradiol during Fadrozole retrodialysis. There was no significant correlation between the changing testosterone vs. estradiol levels ($n = 5$; $p = 0.74$; $r^2 = 0.068$). Fadrozole is a highly specific inhibitor of aromatase activity15, and this lack of correlation may be due to the limited temporal resolution of our methods. It is also possible that, in addition to aromatase, other enzymes17 regulate forebrain testosterone levels.

Female Presentation Experiment

Thirteen dialyzed males were presented 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 dialyzed males see Supplementary Results and Supplementary Table 1). Histological examination of sections for probe-placement within the caudal forebrain showed that the probe was not sampling from within NCM in four of

these males (i.e., NCM 'misses' were rostral, ventral, and medial). Notably, probes for these misses were sampling from regions that contain few, if any aromatase-positive neurons, though they were adjacent to aromatase-rich regions. The remaining nine birds in this experiment had probes successfully placed within NCM (e.g. Fig. 1a) a region rich in neuronal aromatase21. Therefore, for statistical analyses data were grouped according to whether the probe missed NCM ('outside-NCM' group) vs. successfully sampled from 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- vs. 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 to 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- vs. 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 the aromatase-rich nucleus NCM exhibit 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 (vs. '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 NCM ($n = 3$; probes intentionally targeted to anterior forebrain with reduced aromatase21). 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 (Fig. 5b; probe placement effect: $F = 0.009$, $p = 0.93$; female treatment effect: $F = 7.168$, $p = 0.0017$; placement*treatment interaction: $F = 4.828$, $p = 0.0096$). For the within-NCM group, post hoc tests showed that estradiol levels in the 'females adjacent' period were significantly elevated compared to 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 (Fig. 5c; within vs. outside NCM; $F = 4.293$, $p = 0.107$), female treatment ($F = 2.809$, $p = 0.061$) or a 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 within-NCM or outside-NCM groups. Therefore, social interactions with females caused changes in local estradiol levels within NCM only, and these estradiol changes were not associated with concomitant changes in testosterone levels, either within or outside 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 (i.e. song processing/memory access) within NCM. We therefore tested in a separate set of birds 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 within NCM (too few 'misses' occurred for a separate statistical grouping; results from individual 'outside-NCM' animals are plotted as individual data points in Figs. 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 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 to both the 'pre silence' ($p = 0.011$) and 'post silence' ($p = 0.05$) periods. For the 'colony sounds' group, post hoc analysis identified that estradiol levels were also significantly elevated in the 'playback' period as compared to 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 (vs. '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 to the 'pre silence' period ($p = 0.042$), and that a non-significant decreasing trend existed for the 'playback' vs. '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' vs. '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, at the same time, robust decreases in local testosterone levels and increases in local estradiol levels within NCM.

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: 1) exogenous testosterone treatment leads to detectable changes in local forebrain estradiol and/or testosterone levels; and 2) presentation of social stimuli (adjacent females or male song playbacks) are associated with changes in circulating plasma testosterone and/or estradiol levels.

Six birds received intramuscular injections of testosterone ($n = 3$ each for within NCM and outside NCM). Testosterone injection caused a significant increase in local testosterone levels within NCM (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 (mean ± SEM pg/ml); post-injection: 41.06 ± 1.37; p = 0.05) and an increase in estradiol outside 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, detectable increases in estradiol outside NCM are due to 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 ± SEM pg/ml); 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 minutes. Testosterone and estradiol levels were within the low but physiological range for male zebra finches; unlike some bird species15, zebra finches may not exhibit socially-induced acute changes in gonadal steroid secretion.

Neurotransmitter Retrodialysis

Because steroidogenic enzymes are found in neurons8, including presynaptic terminals in NCM19, local steroid levels could be regulated by neurotransmitter activation. We tested in a separate set of birds whether estradiol and testosterone levels within NCM were altered in response to infusion of either NMDA, glutamate or GABA. Glutamate retrodialysis caused a significant decrease in local estradiol levels within NCM (Fig. 7a; Repeated measures ANOVA F = 2.977; p = 0.044). Post hoc tests showed that estradiol was significantly reduced during the period of glutamate retrodialysis (vs. ‘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 activity11 and *in vitro* hippocampal estradiol production (GM Rune, personal communication).

By contrast, GABA retrodialysis caused a significant increase in local testosterone levels within NCM (Fig. 7b; Repeated measures ANOVA F = 7.400; p = 0.015). Post hoc tests showed that testosterone was significantly elevated during the period of GABA retrodialysis (vs. ‘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 impacting testosterone levels, while GABAergic activation elevated testosterone levels without significantly impacting estradiol levels.

Discussion

This study provides direct evidence that acute modulation of local steroid levels occurs within the forebrain during social interactions. In actively behaving zebra finches, steroids within 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 the rapid regulation of their activity[7,11] have direct consequences for local and acute changes in neurosteroid concentrations. These findings are consistent with the hypothesis that local brain steroid levels are regulated in a timescale similar to that of traditional neuromodulators.

We performed several validations for *in vivo* neurosteroid microdialysis in zebra finches. First, the presence of estradiol in microdialysate was confirmed 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 significant increases in local estradiol levels, and pharmacological inhibition of aromatase caused significant changes in local estradiol and testosterone levels. Together, these 'proof of principle' results confirm predictions for neurosteroid microdialysis in zebra finches. Importantly, results with the song-responsive immediate-early gene ZENK indicate that the auditory region NCM retains auditory responsiveness during microdialysis. Lastly, estradiol levels changed in response to social interactions only 'within NCM' and not 'outside NCM', emphasizing that steroid levels are locally regulated within the forebrain.

A prevailing view holds that increased testosterone production from the gonads leads to parallel increases in central estradiol levels[25,26]. Indeed, in this study exogenous testosterone injection caused significant elevations in forebrain testosterone as well as 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, yet these same treatments led to robust changes in forebrain estradiol levels in the same timeframe. Second, local forebrain estradiol and testosterone levels change in opposite directions during audition, as well as following aromatase inhibition. Third, only local forebrain estradiol levels (and not testosterone levels) within NCM change during social interactions with females. Lastly, retrodialysis of neurotransmitters into NCM differentially altered forebrain steroid levels, presumably via local, forebrain-specific mechanisms. Therefore, in contrast to previous reports that steroidal substrates and products change concomitantly within the brain[13,27] the current findings illustrate that changing forebrain steroid levels are not always accompanied by parallel changes in precursor concentrations. However, these findings 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 timecourse 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]. While 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 within discrete brain nuclei could reflect their divergent (e.g. excitatory vs. inhibitory) downstream modulatory actions on neural circuit function[30]. For example, in songbirds, estrogens prolong plasticity during development and song learning, while 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 vs. androgens occur on an acute timescale within the forebrain of adult zebra finches.

GABAergic stimulation caused an increase in testosterone levels within NCM, which could be due to fast downregulation of testosterone-metabolizing enzyme activity, or upregulation of testosterone-synthesizing enzyme activity. Aromatase activity is unlikely to be responsible for our testosterone result with GABA, primarily because we observe no concomitant changes in estradiol levels following GABA infusion. Other androgen metabolizing enzymes (e.g. 5α- and 5β-reductase) are expressed in forebrain GABAergic and glutamatergic neurons32 and are abundant in the songbird telencephalon15,17, 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 hippocampus14, and is expressed33 and active6 in avian brain tissue. In addition, GABAergic, steroidogenic, and calbindin-positive neurons appear to overlap within NCM34, 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 within the songbird forebrain. Although steroids influence auditory encoding in the peripheral nervous system35,36, there are few studies that address a neuromodulatory role for steroids on central mechanisms of audition. Steroids exert long-term, genomic actions on auditory processing within the inferior colliculus in rats37, and within NCM in songbirds38. This study now predicts that brain-derived steroids acutely modulate auditory processing within pallial/cortical regions, which could provide clues about the function of neuronal aromatase expression within auditory cortex of other vertebrates, including humans39.

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 mechanisms22,23,40. There is evidence that endogenous/exogenous estrogens can improve spatial memory in mammals41,42 as well as songbirds43. 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 play a significant role in song learning15,44 and recent observations indicate that steroids can act via long-term mechanisms within the forebrain in a localized, nucleus-specific manner31,45. One question that arises from this work is whether local pharmacological inhibition of steroidogenesis within forebrain auditory regions affects acute auditory memory function. Furthermore, ontogenetic applications of microdialysis technology could shed light on how the developing brain, as a source as well as target of steroids8,12,25,33, is acutely modulated by steroids during critical learning and acquisition phases.

These findings emphasize the importance of monitoring real-time changes in steroids within neural circuits in order 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 excitability8–10 with the rapid regulation of steroidogenic enzyme activity in the context of behavior7,11. It is now evident that steroids are part of a network of local and rapid modulatory mechanisms intrinsic to the vertebrate forebrain.

Methods

Subjects

Animal care and use protocols were approved by the UCLA Chancellor's Committee on Animal Care and Use. Microdialysis cannula/probe implantation procedures were adapted from techniques routinely used to analyze neurotransmitters in rodents46 and recently in zebra finches47. Birds were anesthetized with Equithesin (dose = 3.2 mL/kg) injected into the pectoralis muscle. Using stereotaxic coordinates, a CMA-7 microdialysis guide cannula with obdurator (CMA Microdialysis) were inserted 1.2 mm from the surface of the dura mater to target NCM. Dental cement and cyanoacrylate were applied to stabilize the cannula, and birds were returned to individual sound attenuation chambers and monitored for at least 6 days prior to microdialysis probe implantation (Supplementary Methods).

Microdialysis

At the start of microdialysis, the obdurator was removed from the guide cannula and replaced by a continuously-perfused (2.0 μl/min aCSF, Harvard Apparatus 22 infusion pump) CMA-7 microdialysis probe (CMA-7; OD 0.24 mm; shaft length 7.0 mm; membrane length 1.0 mm; CMA Microdialysis) under isoflurane anesthesia (Hospira). Sample collection did not begin until 8-12 hours after probe implantation to allow implantation-induced neurochemical changes to subside. Following the completion of all experiments, birds were perfused, 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, a subset of brain tissue from dialyzed birds was processed for immunocytochemical expression of the aromatase protein using established methods19,21 (Supplementary Methods). Upregulation of the immediate-early gene ZENK (a transcription factor also known as egr-1, ngfi-a, krox-24 and zif-268) reflects neuronal activity in response to auditory stimulation48. Therefore, in a separate set of males we also determined whether probe/cannula placement within NCM reduces or eliminates 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 gas chromatography/mass spectrometry (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 ± SEM = 6.53 ± 0.57 pg/ml). The intra-assay CV was 3.32%, and inter-assay CV 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 ± SEM = 11.58 ± 3.25 pg/ml). The intra-assay CV was 7.86 %, and inter-assay CV 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

Estradiol Injection

In vivo, we predicted that local estradiol levels within NCM are 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; Supplementary Methods).

Fadrozole Retrodialysis

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 7 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). Since male zebra finches sing robustly in the morning, all female presentation trials were carried out immediately after lights-on. Dialysate sampling periods were: 1) 8-10 hr overnight (‘overnight’); 2) 30 min prior to lights-on (‘pre lights on’); 3) 30 min immediately following lights-on (‘lights on’); 4) 30 min with females adjacent (‘females adj’; three unfamiliar females presented to the microdialyzed male in an adjacent cage inside the acoustic attenuation chamber); and 5) 30 min following removal of the female cage (‘post females’). 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 as well as testosterone levels within and outside 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 (‘playback’): 1) 5-min recording of female chirping behavior (‘female chirps’; looped 6 times; n = 6); 2) 5-min recording of the zebra finch colony (‘colony sounds’; looped 6 times; n = 5); 3) 1-min recording of male song from three individual males (‘male song’; looped 30 times; n = 6); 4) 1-min intermittent white noise stimulus (‘white noise’; looped 30 times,; n = 5). After playback, a 30-min silent-period dialysate sample was collected to examine post-treatment effects (‘post silence’). 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 effects on local forebrain estradiol and testosterone levels within and outside 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 neurons8 and synaptic terminals19, which raises the possibility that neurosteroid levels are regulated by neurotransmitter activation. We therefore tested whether neurosteroids in NCM are altered in response to local infusion of either: 1) the predominant excitatory neurotransmitter glutamate (L-glutamatic acid; n = 6); 2) the predominant inhibitory neurotransmitter gamma-aminobutyric-acid (GABA; n = 6): or 3) the selective glutamatergic agonist N-methyl-daspartic acid (NMDA; n = 5). Baseline dialysate was 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 ANOVA) 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 as well as behavior occurrences), and unpaired t-tests (between-group comparisons of plasma steroid levels).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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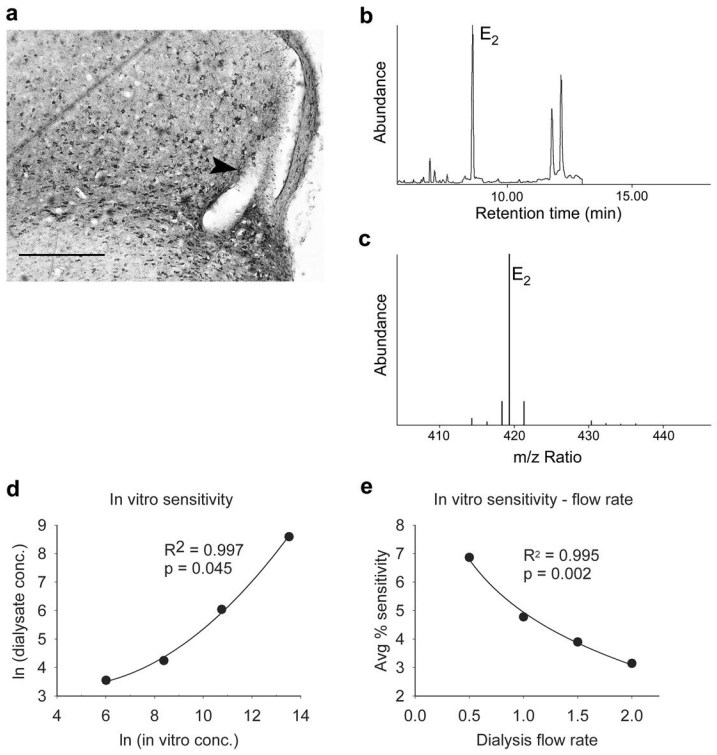

Figure 1.

Validation of steroid microdialysis in zebra finches. Immunostain for the enzyme aromatase **(a)** showing the placement of the microdialysis probe within NCM (arrowhead) in a parasaggital section (top = dorsal; right = caudal). Scale bar = 500 μm. **(b-c)** Gas chromatogaphy/ mass spectrometry data for detection of estradiol from microdialysate. The chromatograph **(b)** shows a peak relative abundance at the characteristic retention time for MSTFA-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 absorb ^{3}H-estradiol from an *in vitro* ^{3}H-estradiol aCSF solution. **(d)** The concentration of ^{3}H-estradiol in collected microdialysate increases as the *in vitro* concentration of ^{3}H-estradiol is increased (flow rate = 2.0 μl/min) **(e)** The concentration of ^{3}H-estradiol in collected microdialysate (% sensitivity of *in vitro* concentration) increases logarithmically as the microdialysis flow-rate decreases, to a theoretical maximum at zero-flow. For this experiment the *in vitro* concentration of ^{3}H-estradiol was held constant.

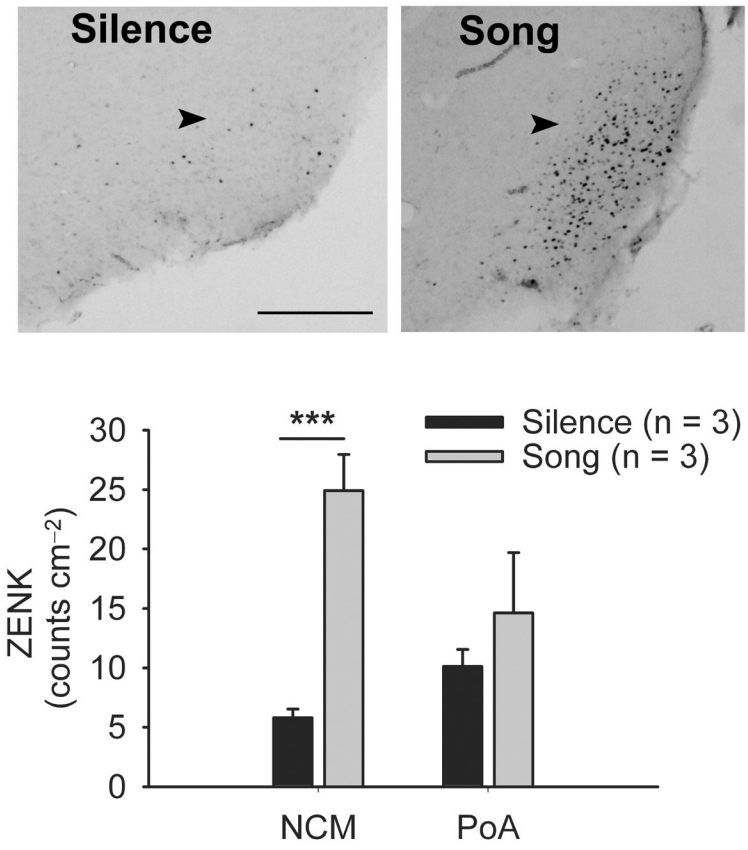

Figure 2.

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, while three other birds received silence. **(a-b)** Representative parasaggital 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 = 500 μm. **(c)** ZENK expression (mean stained nuclei per cm^2 ± s.e.m.) is significantly upregulated (*** $p < 0.0005$) in the song vs. silence group only in NCM, while expression in the preoptic area (PoA; non-auditory) is no different between groups.

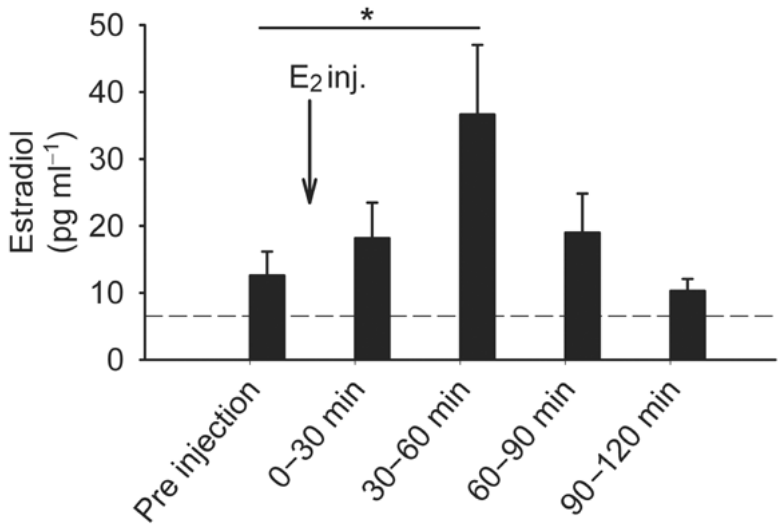

Figure 3.

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 (* $p = 0.048$; $n = 5$). Dashed line indicates average background estradiol concentration as reported by ELISA for aCSF alone.

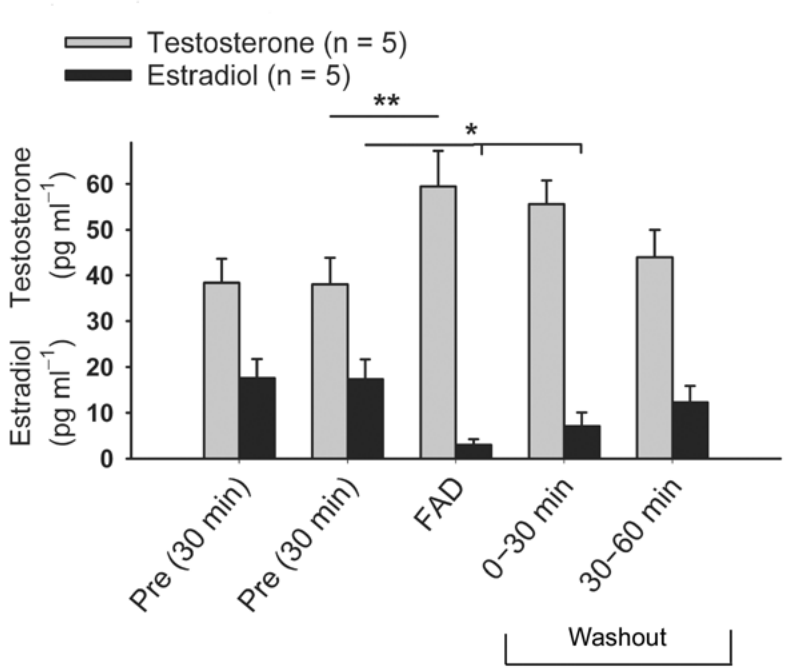

Figure 4.

The aromatase inhibitor 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 to pre-treatment baseline ('pre (30 min)'), local estradiol levels are reduced during fadrozole (FAD) retrodialysis, and during the first 30 min of washout (0-30 min; * $p < 0.03$). Also when compared to pre-treatment baseline, local testosterone levels are significantly increased during FAD retrodialysis (** $p = 0.004$).

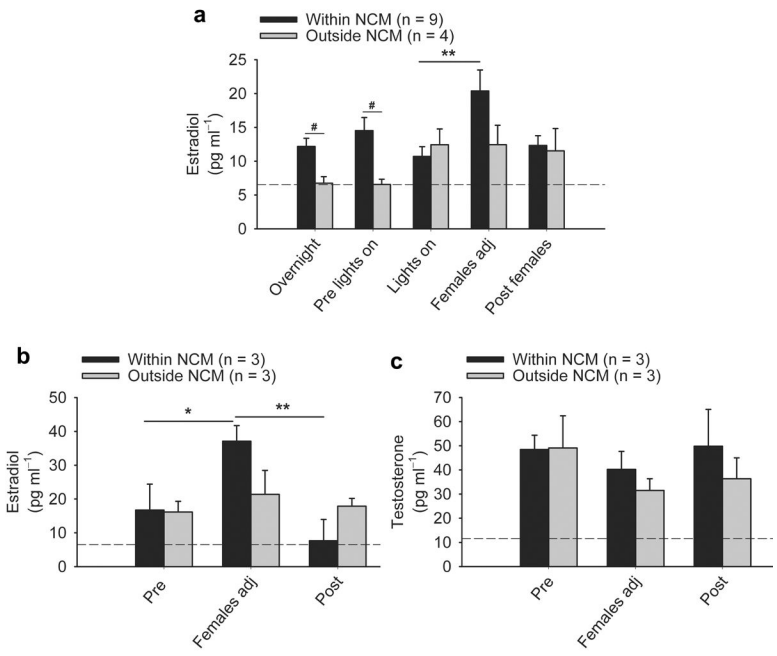

Figure 5.

Local forebrain estradiol levels change rapidly in 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 hr. 'Within-NCM' animals were a group with microdialysis probes successfully directed at NCM; 'outside-NCM' were a group with probes in forebrain surrounding 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 to 30-min 'lights on' and 30-min 'post-females' periods (** $p < 0.006$ for within-group comparison of lights-on vs. females adjacent), indicating fast regulation of estradiol levels locally within NCM. Local estradiol levels were significantly higher in within-NCM birds during lights off periods (# $p < 0.02$ for between group comparisons for within- vs. 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 30-min period when females were adjacent to dialyzed males ('females adj'), estradiol levels **(b)** were significantly elevated within NCM (vs. 'pre' $p < 0.035$; vs. 'post' $p < 0.009$) but not outside NCM. Testosterone levels **(c)** in these same males did not change within NCM or outside NCM when females were adjacent. Dashed line indicates average background estradiol **(a-b)** or testosterone **(c)** concentration as reported by ELISA for aCSF alone.

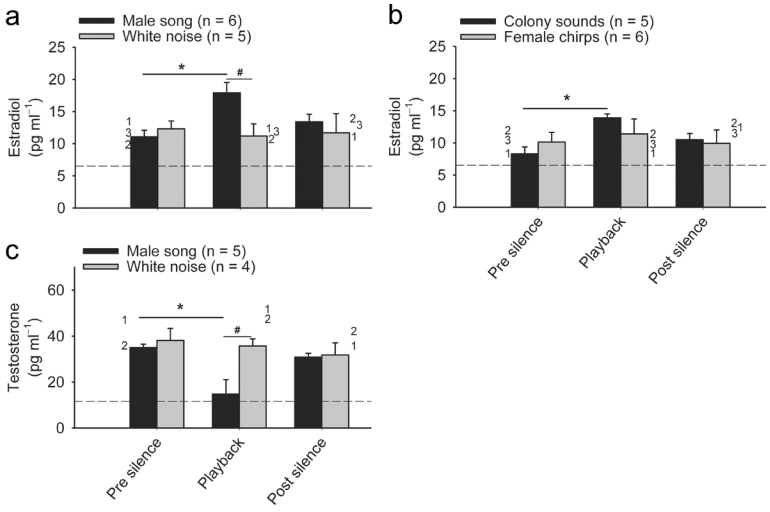

Figure 6.

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 are rapidly elevated during playback of male song vs. pulsed white noise (* $p = 0.011$ for 'playback' vs. 'pre silence' in the male song group; # $p = 0.023$ for male song vs. white noise during 'playback'). Bars are group means for 'within-NCM', while numbers show individual results for 'outside-NCM' (1, 2 = male song; 3 = white noise). **(b)** Local estradiol levels within NCM are rapidly elevated during playback of zebra finch colony sounds and not female chirps (within the colony sounds group * $p = 0.01$ for 'playback' vs. 'pre-silence' and * $p = 0.009$ for 'playback vs. post-silence'). Bars are group means for 'within-NCM', while numbers indicate individual results for 'outside-NCM' (1 = colony sounds; 2, 3 = female chirps). **(c)** Local testosterone levels within NCM are rapidly reduced during playback of male song and not pulsed white noise (* $p = 0.042$ for 'playback' vs. 'pre-silence' within the male song group; # $p = 0.039$ for male song vs. white noise during 'playback'). Bars are group means for 'within-NCM', while numbers indicate individual results for 'outside-NCM' (1 = male song; 2 = white noise). Testosterone levels are from the same subjects in **(a)** (one sample was lost from each group during extraction). Dashed line indicates average ELISA background estradiol or testosterone concentration for aCSF alone.

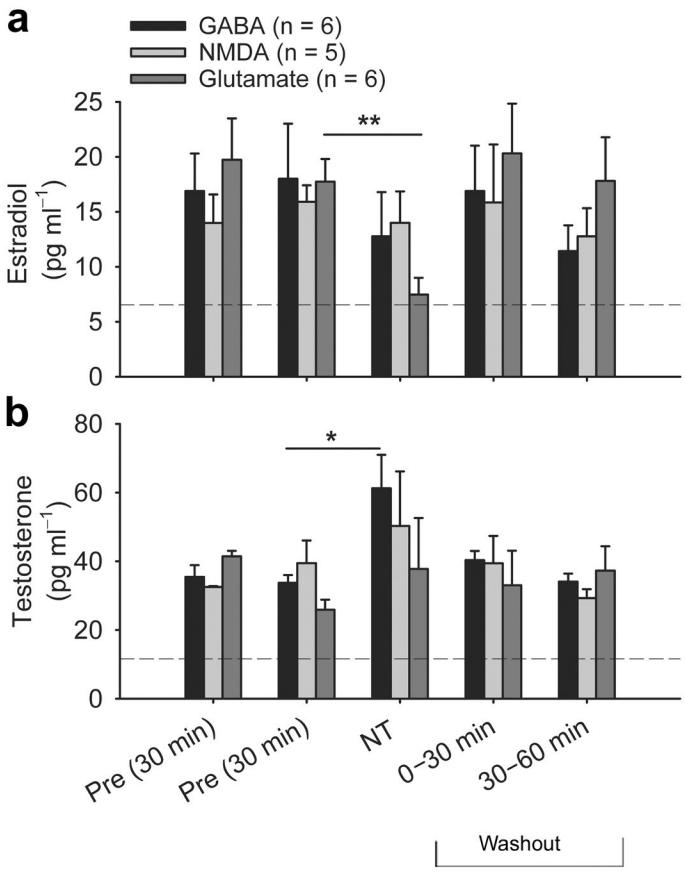

Figure 7.

Infusion of glutamate into NCM via retrodialysis causes a rapid decrease in local estradiol levels **(a)** while infusion of GABA into NCM causes 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 are significantly reduced during the period of glutamate retrodialysis only (10 mM; ** $p = 0.008$). When compared with pre-treatment baseline, testosterone levels are significantly increased during the period of GABA retrodialysis only (100 μM; * $p = 0.038$). NT = 30 min period of neurotransmitter retrodialysis. Dashed line indicates average background estradiol **(a)** and testosterone **(b)** concentrations as reported by ELISA for aCSF alone.