“Forebrain steroids fluctuate rapidly during social interactions”

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Supplementary Material

Supplementary Table 1

Dialyzed males were exposed to females in an adjacent cage for a 30-min period. The occurrence of behaviors for the focal males is reported as mean ± standard deviation (range). The experimental groups are as described in the text.

| Group          | Singing | Motifs | Chirping | Drinking | Feeding | Beak wiping | Flights | Preening |
|----------------|---------|--------|----------|----------|---------|-------------|---------|----------|
| Outside NCM (n = 4) | 6.2 ± 1.85 (0-12) | 20.7 ± 7.0 (0-59) | 62.0 ± 23.4 (4-118) | 1.2 ± 1.2 (0-5) | 9.0 ± 5.4 (0-22) | 7.5 ± 4.2 (0-17) | 29.5 ± 9.4 (11-39) | 2.7 ± 1.0 (0-5) |
| Within NCM (n = 9)  | 9.1 ± 2.5 (0-18) | 32.8 ± 8.6 (0-42) | 55.6 ± 11.6 (20-118) | 1.2 ± 0.4 (0-3) | 8.7 ± 2.8 (0-20) | 1.4 ± 0.8 (0-6) | 41.7 ± 11.7 (8-88) | 2.4 ± 0.8 (0-7) |

Supplementary Results

In vitro Probe Recovery

To determine probe recovery, probes were immersed in a microcentrifuge tube containing solutions of $^3$H-estradiol in aCSF and perfused with aCSF, and the concentration of steroids in dialysate was determined in a liquid scintillation counter. Several experiments were conducted to assess the in vitro performance of CMA-7 probes under varying conditions. When the in vitro concentration of $^3$H-estradiol was varied from 36-119 nM the resultant dialysate concentration of $^3$H-estradiol correlated with the in vitro concentration (Fig. 1d; $r^2 = 0.997$; flow rate = 2.0 µl/min). When the perfusion pump flow rate was reduced stepwise at 0.5 µl/min intervals from 2.0 µl/min to 0.5 µl/min the resultant dialysate concentration of $^3$H-estradiol was logarithmically correlated (Fig. 1e; $r^2 = 0.99$). When 1% BSA (Sigma) was added to aCSF it increased $^3$H-estradiol yield by an average of 41% (n = 14 trials), and when the inclusion complex methyl-β-cyclodextrin (5mM, Sigma) was added to BSA-aCSF yield further increased by 18% (n = 4 trials). Adding 4% dextran (Sigma) increased yield by an average of 1.36% (n = 2 trials) and so dextran was not added to aCSF for in vivo experiments (in a pilot experiment (n = 2) with sequential (30 min) ELISA estradiol measures BSA-cyclodextrin aCSF increased estradiol yield in vivo by an average of 20.3% as compared to aCSF
alone). Following all trials of in vitro probe sensitivity, the probe was rinsed internally and externally with methanol to test whether $^3$H-estradiol remained associated with the probe membrane or tubing and would therefore reduce detection and time resolution in vivo. The residual $^3$H-estradiol counts from the rinsed probe was less than 14% of the $^3$H-estradiol counts found in dialysate, indicating that steroids do not adhere strongly to the microdialysis CMA-7 membrane and tubing.

**ZENK activation**

ZENK expression was significantly elevated in dialyzed males exposed to male song vs. silence for 30 min. For the two-way ANOVA there was a significant effect of playback treatment ($F = 13.44; p = 0.001$), no effect of brain region ($F = 0.859; p = 0.36$), and a significant playback*region interaction ($F = 5.13; p = 0.035$). Post-hoc tests revealed that the number of nuclei stained for ZENK was significantly elevated in song playback vs. silence in NCM ($p < 0.0005$), and not significantly different in PoA ($p = 0.41$).

**Behavioral measures**

Behavioral scores (see Supplementary Table 1) for microdialysis males in the Female Presentation Experiment are in accordance with previous observations of male zebra finches responding to female stimuli$^{1,2}$. Factorial ANOVA identified no significant overall differences between the groups ‘within NCM’ vs. ‘outside NCM’ for behavior scores in this experiment ($F = 0.485; p = 0.50$) or for males in the Playback Experiment (data not shown; $F = 1.439$; all $p > 0.10$). These analyses indicate that group differences in the degree of changing neurosteroid levels could not be accounted for by differences in behavioral responses among groups.

**No correlation between local steroid changes**

Within NCM, local estradiol levels rapidly increased during exposure to male song, while local testosterone levels rapidly decreased in the same set of animals. This raises the possibility that the decrease in testosterone reflects rapid aromatization (and therefore depletion) of testosterone locally within NCM. To directly address this possibility, we calculated for each bird whether the change in estradiol was correlated with the change in testosterone (a comparison of changes from ‘pre silence’ to ‘playback’ periods). There was no significant correlation between the change in testosterone vs. the change in estradiol ($p = 0.79$; $r^2 = 0.025$), suggesting that either our methods could not resolve the temporal aspects of such changes or that the two steroids could be centrally regulated by separate mechanisms.
Supplementary Methods

Surgery

Adult male zebra finches (at least 120 days of age) were taken from the UCLA Schlinger lab breeding colony on a 14L:10D light cycle. Prior to surgery, males were first housed individually in sound attenuation chambers for minimum 24 hr (ad lib food/water; 14L:10D), and singing behavior was recorded using a Sony microphone connected to a computer with Syrinx software (John Burt, Cornell Laboratory of Ornithology). This recording of song was used for comparison with subsequent postsurgical singing behavior. The forebrain nucleus HVC is a primary center of auditory-vocal integration and is adjacent to NCM, and hence could potentially sustain lesion damage from cannula/probe placement within NCM. Damage to HVC can cause singing deficits, and so the songs of all males were compared pre- and post-surgery for singing deficits using CoolEdit Pro 1.2a software (Syntrillium). In no case did we observe differences between pre- and post-surgery for the elements of song structure (i.e. frequency components; motif sequence) or song complexity (i.e. number of motifs), indicating that the surgical damage to adjacent motor nuclei was minimal. Histological examination also confirmed that probes were targeted to NCM. On the day of surgery, food trays were withdrawn for 1-3 hr prior to surgery to stabilize anesthesia throughout cannula implantation. Following anesthesia birds were placed in a customized stereotaxic device and kept warm with a surgical electric blanket. Lidocaine (4% in ethanol; Sigma) was applied to the scalp and feathers were removed to expose a 4.0 mm² area of scalp, and a small incision was made in the skin along the midline. The position of the head was adjusted to ensure the skull plane dorsal to NCM was perpendicular to the cannula entry point. This plane was leveled by using a flat-tip syringe mounted on a Narshige micromanipulator to match the dorsal-ventral skull depth of the bifurcation of the midsagittal sinus (point-of-origin) with the skull depth 2.0 mm rostral to the point-of-origin. The coordinates of NCM were determined following several pilot studies (following the atlas of Nixdorf-Bergweiler and Bischof) 1.4 mm rostral and 1.1 mm lateral relative to the point-of-origin. A 26-G syringe needle (Beckton Dickinson) was mounted onto a CMA-7 microdialysis clip (CMA Microdialysis) and lowered to create a small incision in the skull above NCM coordinates, and this hole was widened slightly with a 22-G syringe needle. Following CMA-7 cannula implantation, a thin layer of cyanoacrylate (Loctite Corp.) was applied to the exterior cannula/skull boundary and the cannula was immediately sealed to the surrounding skull using dental cement (Perm Reline/Repair Resin; Coltene-Waledent Inc.). The skin incision was sealed with cyanoacrylate and the cannula obdurator remained in place inside the cannula during the period of surgical recovery. This extended post-implantation period was intended to allow stabilization of tissue damage surrounding the cannula and reduction of gliosis during probe implantation.

Microdialysis

Prior to microdialysis probe implantation, the probe, swivel, and FEP tubing were all rinsed with 70% ethanol and subsequently filled with aCSF (199 mM NaCl; 26.2 mM NaHCO₃; 12.0 mM KH₂PO₄; 2.0 mM MgCl₂; 2.0 mM CaCl₂; 25.0 mM glucose).
NaHCO₃; 2.5 mM KCl; 1 mM NaH₂PO₄; 1.3 mM MgSO₄; 2.5 mM CaCl; 11 mM glucose; bovine serum albumin cyclodextrin, pH = 7.4) previously filtered through 0.2 µm HT syringe filters (Acrodisc 25 mm filters, Pall Life Sciences). The analyte cutoff of CMA-7 probe membranes is 6000 Daltons which permits the passive diffusion of all compounds in this study, including steroids (MW estradiol = 272.38; testosterone = 288.42) and pharmacological agents (GABA = 103.12; glutamate = 147.13; NMDA = 147.13; Fadrozole = 268.75). Once implanted, the top of the probe housing was sealed to the guide cannula using cyanoacrylate and protected with tape. The probe in-flow and out-flow tubes were connected to polyethylene FEP tubing (CMA Microdialysis) which was in turn connected a low-torque quartz-lined dual-channel swivel (375/D/22QM; Instech Laboratories). Outflow tubing was connected to microcentrifuge tubes for sample collection. The length of all inflow and outflow connection tubing was measured to calculate “dead volume”, which causes a delay between the beginning of a behavioral or pharmacological manipulation and the resultant collection time. All sampling times reported in this study after behavioral or pharmacological manipulations account for this ‘dead-volume’ delay. At the end of each sampling period samples were immediately sealed and stored at –80 °C.

Birds were observed to adapt quickly to this microdialysis system. The liquid microdialysis swivel (375/D/22QM; Instech Laboratories) is designed specifically to account for the rotational torque generated by small animals, and we observed that our adapted tether system allowed birds 360-degree range of motion including flight. Birds were observed perch hopping, feeding, drinking, preening, chirping and singing within hours after probe implantation (see Supplementary Table 1 for behavioral scores). The duration of microdialysis sampling did not exceed four days for any animal. To maximize data gathered from each animal and to minimize animal use, a subset of multiple experiment categories (i.e. pharmacological vs. behavioral) were performed on each animal. However, to preserve statistical independence, in no case was a behavioral experiment performed more than once per animal, but in some cases more than one pharmacological manipulation (e.g. fadrozole vs. glutamate treatment) were performed on the same animal.

Probe Placement

Birds were euthanized via anesthetic overdose and perfused through the heart with 0.9% avian phosphate-buffered saline followed by ice-cold 4% formalin. The cannula and probe were left in place during perfusion to minimize tissue damage. Cannulae were recovered during craniotomy and probes were discarded. Brains were removed, sunk overnight in 30 % sucrose in 4 % formalin, sectioned at 40 µm thickness on a cryostat, and either stored for immediate immunocytochemical processing (see below) in 0.1 M phosphate buffer, or mounted onto gel-subbed superfrost plus microscope slides (Fisher). Mounted, air-dried sections were then stained with cresyl violet and viewed and photographed under a light microscope for probe placement.
Aromatase expression

Injury to brain tissue of zebra finches and other vertebrates is known to cause localized upregulation of the expression of the enzyme aromatase (the enzyme that converts androgens into estrogens). This study focused on local, in-vivo measurement of estradiol and testosterone surrounding the microdialysis probe, and it was therefore important to evaluate the degree of local, reactive aromatase caused by microdialysis cannula/probe placement. Aromatase expression was evaluated in sections from birds sacrificed 3-4 days after probe implantation. Briefly, sections were washed 3x15 min in 0.1 M PBT (1 mL Triton X in 0.1 M phosphate buffer), and then treated in series with: 1) 0.6 % hydrogen peroxide, 2) 3x15 min in 0.1 M PBT, 3) 60 min in 10 % normal goat serum (Vector Laboratories), 4) 72 hr in 1:2000 rabbit anti-aromatase in 0.3 % PBT, 5) 3x15 min in 0.1 % PBT, 6) 60 min in 1:200 biotinylated goat anti-rabbit IgG in 0.3 % PBT (Vector), 7) 3x15 min in 0.1 % PBT, 8) 90 min in 1:200 avidin-biotin complex in 0.3 % PBT (Vector), 9) 3x15 min 0.1 % PBT, and then visualized with 0.03 % diaminobenzidene activated with 0.01 % hydrogen peroxide. Sections were then washed with 0.1 M PB, mounted onto gel-subbed superfrost slides, air-dried, processed through alcohol dehydration, cleared for lipids with CitriSolv (Fisher), and coverslipped with Permount (Fisher).

ZENK experiment

Zebra finches and other songbirds exhibit rapid upregulation of the immediate-early gene ZENK during auditory processing in the nucleus NCM, among other auditory nuclei. Six birds that had undergone successful microdialysis within NCM were divided into two groups. Three birds served as controls and received no sound playback during a 1 hr silent period, while three other birds received 30 min of looped male song playback, followed by 30 min of silence. All birds were immediately killed via anesthetic overdose and perfused with 4% formalin. Brains were removed and sectioned as above, and processed for immunocytochemical staining for ZENK (procedures identical to that for aromatase as above, with the exception that the primary antibody step was 72 hr in 1:10,000 rabbit anti-ZENK (Santa Cruz Biotechnology) in 0.3 % PBT). To strengthen comparisons of silent vs. song treatments, brain sections were processed in pairs (1 silent-treatment, 1 song-treatment) in each of 3 immunocytochemistry runs. Positively-stained ZENK nuclei within NCM and the medial preoptic nucleus were counted manually by two observers blind to the treatment group (correlation coefficient between observer scores was 0.93).

Steroid Analysis

To confirm the presence of estradiol in dialysate, gas chromatography/mass spectrometry was performed on dialysate after methanol/solid phase extraction (with PrepSep 12-well vacuum manifold (Fisher) equipped with 3 ml Empore high performance extraction disk cartridges C18 SD (3M Corp)). Extracted dialysate samples were derivatized with TMS-Keto reagent (MSTFA) and analyzed in SIM mode following
established methods on an Agilent 5075 GC/MS system (Agilent Technologies, with EI/CI package, equipped with an HP1 16.2 m x 0.2 mm x 11 µm column).

ELISA

The estradiol ELISA is highly-specific for estradiol (e.g. cross-reactivity: 14% for estradiol-3-glucuronide; 12% for estrone; 1% for estradiol-17-glucuronide; < 0.10% for all other major steroids including testosterone). When analyzing steroid samples such as plasma or culture media, organic extraction and subsequent dilution is often required to separate steroids from lipids and proteins which normally interfere with ELISA. However, the low-molecular weight cutoff of the CMA-7 probe membrane (6 kDa) normally prevents large molecules (such as steroid binding proteins) from diffusing into the collected dialysate, and pilot experiments demonstrated that neither ether- nor methanol-extraction of dialysate substantially aided detection of estradiol. Pilot experiments with varying dilutions of dialysate in triplicate (1:4, 1:1, no dilution) revealed that ‘interference’ from undiluted substances in dialysate was not a significant factor for the measurement of estradiol using ELISA. Therefore, to maximize detection, samples pipetted directly on the 96-well plates consisted of whole, undiluted dialysate. Dialysate estradiol concentrations were distributed throughout the linear portion of the ELISA standard curve, and samples maintained linearity when spiked with known concentrations of estradiol (all levels reported in this study are unspiked microdialysate).

The testosterone ELISA is also highly-specific for testosterone (e.g. cross-reactivity: 14.6% for 19-hydroxytestosterone; 7.2% for androstenedione; < 1.0% for all other major steroids including estradiol). Following the incubation step of the first (estradiol) ELISA plate with primary antibody and estradiol tracer, all samples were pipetted from the plate (unbound fraction; this fraction is normally discarded) and stored individually in labeled microcentrifuge tubes at –20 °C until the day of extraction. The estradiol assay (fraction bound to the 96-well plate) continued according to the manufacturer’s instructions with the proscribed wash steps prior to plate development. Prior to the testosterone ELISA, thawed samples were first ether-extracted to eliminate proteins from the estradiol ELISA (antibodies, etc.) which were shown in pilot experiments to interfere with the testosterone ELISA. Briefly, samples were thawed and 1 ml ether was added to each sample tube and vortexed for 30 sec. Samples were then flash-frozen in a methanol/dry ice bath for 7-9 sec, and the ether fraction was poured into a new set of labeled tubes. This extraction was completed in triplicate for each sample, and the ether fractions were combined, dried down in a warm water bath and resuspended in assay buffer provided with the testosterone ELISA (Assay Designs). The testosterone ELISA was then performed on these extracted samples. A set of spiked testosterone samples were processed and extracted from the beginning of the estradiol assays, and the average testosterone recovery for these samples was 68.1% when compared with an identical set of unprocessed and unextracted samples. Dialysate testosterone concentrations were distributed throughout the linear portion of the ELISA standard curve, and samples maintained linearity when spiked with known concentrations of testosterone (all levels reported in this study are unspiked microdialysate accounting for losses due to extraction). ELISA plates for both estradiol and testosterone assays were measured on
a Thermo Multiskan EX plate reader (Thermo Corp) with 415 and 405 nm filters (respectively) using Ascent Software 2.6.

In vivo microdialysis

In all cases, in vivo microdialysis experiments were conducted in series, so that no more than one male was exposed to each stimulus or pharmacological treatment at a time. This was intended to eliminate any stimulatory effects of the focal male’s behavior (e.g. song) on the behavior and/or physiology of males in adjacent acoustic attenuation chambers.

Estradiol Injection

The estradiol injection dose was as used previously in zebra finches\(^5\). Estradiol was injected into the pectoralis muscle. Dialysate was collected for 30 min prior to injection, and then for four sequential 30-min periods immediately following the injection (total experiment duration = 2.5 hrs).

Fadrozole Retrodialysis

The water-soluble aromatase inhibitor Fadrozole (gift of Novartis) was confirmed previously to inhibit aromatase activity in zebra finch brain tissue explants\(^6\). Fadrozole was dissolved in aCSF and retrodialyzed at two doses: 10 \(\mu\)M (n = 2) and 100 \(\mu\)M (n = 5). The low dose of Fadrozole (10 \(\mu\)M) retrodialyzed into NCM produced no detectable changes in estradiol levels in a pilot experiment (data not shown), and the dose was consequently increased to 100 \(\mu\)M in a subsequent set of five animals. For this experiment aCSF alone was dialyzed for two 30 min periods for baseline measurement, followed by aCSF + 100 \(\mu\)M Fadrozole retrodialysis for 30 min, and then followed by two 30 min periods of aCSF alone for post-treatment washout.

Female Presentation Experiments

In the second female presentation experiment, microdialysis probes were directed within NCM (n = 3) and outside NCM (n = 3; probes intentionally targeted to a region near Area X) of microdialyzed males (n = 6 birds total). All of these trials were carried out in the morning (1 hr after lights on), and dialysate sampling periods were: 1) 30 min before insertion of females (‘pre’); 2) 30 min with females adjacent as above (‘females adj’); and 3) 30 min following removal of the female cage (‘post’). Male behaviors were scored during the trials as above.

Acoustic Playback Experiment

Experiments were completed within 11:00-16:00 to minimize possible circadian variation. All stimuli were band-pass filtered (Chebychev ramped pass filter, cutoffs: 1 kHz and 12 kHz, CoolEdit Pro) to remove cage noises and low-frequency ambient
sounds. A calibrated speaker inside the chamber was used for all playbacks, and stimuli were standardized so that peak amplitude did not exceed 70 dB.

Influence of Circulating Steroids

Testosterone Injection

Six birds (n = 3 for each within and outside NCM group) received intramuscular injections of testosterone (20 µl of 300 µg/ml) into the pectoralis muscle. Dialysate was collected for 30 min prior to injection and then for a two-hour period immediately following the injection (total experiment duration = 2.5 hrs).

Social Stimulation

In a female presentation experiment, a separate set of 12 males were exposed individually (‘one-at-a-time’) in acoustic isolation chambers to either novel females in an adjacent cage for 30 min (n = 6) or to equivalent cage manipulations without inserting females (housed alone for 30 min; n = 6). At the end of the trial males were captured and blood was immediately withdrawn (> 2 min upon disturbance) from the alar wing vein, centrifuged and plasma frozen for analysis. In an acoustic playback experiment, a separate set of 16 males were exposed individually in acoustic isolation chambers to playback of either male song (n = 10) or white noise (n = 6; as above) for 30 min. At the end of the trial males were captured, rapidly decapitated, and trunk blood was immediately collected (< 30 sec upon disturbance), centrifuged, and plasma frozen for analysis.

Neurotransmitter Retrodialysis

All neurotransmitter drugs were purchased from Sigma and dissolved in microdialysis aCSF. The dose for NMDA treatments (1.0 mM in aCSF) was based on previous work showing NMDA-dependent estradiol release in hippocampal culture. The glutamate dose (10 mM) and GABA dose (100 µM) were based on studies using similar methods of infusion. Experiments were completed within 11:00-16:00 to minimize possible circadian variation.

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