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ORIGINAL ARTICLE

Elevated Aromatase Activity in Forebrain Synaptic Terminals During Song

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The enzyme aromatase, which converts androgens into oestrogens, is expressed throughout the brain in zebra finches. Aromatase is enzymatically active in both cell bodies and synaptic terminals of neurones of the songbird brain, particularly within the forebrain motor and auditory networks. Aromatisation within synaptic terminals could thus provide localised and acute modulatory oestrogens within the forebrain during singing and/or audition. In male zebra finches, we tested the hypothesis that forebrain aromatase activity is elevated during singing behaviour and/or hearing male song. The present study reports that aromatase activity is elevated in males that were singing for 30 min compared to nonsinging males, and that this elevation occurs only within the cellular compartment that contains synaptic terminals. In a separate experiment, males that heard acoustic playback of song for 30 min exhibited no differences in aromatase activity or in aromatase mRNA levels, as revealed by quantitative polymerase chain reaction analysis. Therefore, these results indicate that activation of the motor pathway for song production is linked to local elevations in synaptic aromatase activity within the forebrain of male zebra finches. Future experiments could assess whether elevated synaptic aromatase activity during song is dependent on acute regulation of the aromatase protein.

Key words: estradiol, songbird, rapid, hypothalamus, membrane, nongenomic.

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The enzyme aromatase converts androgens into oestrogens, and aromatase is expressed in endocrine glands and within the central nervous system. In most vertebrates, aromatase is expressed and biologically active within the preoptic area/hypothalamus, a primary locus of reproductive behaviour (1–3). Among individuals, the enzymatic activity of hypothalamic aromatase is related to the intensity of aggressive behaviour in mammals and birds (4–6) and is inversely related to aggression in fish (7, 8). Hypothalamic aromatase activity is also acutely regulated during copulatory behaviour, in birds and mammals (9, 10) and pharmacological inhibition of aromatase suppresses reproductive behaviour in male mice and fish (11, 12). Therefore, the regulation of reproductive behaviour by hypothalamic aromatase activity has emerged as a conserved feature of the vertebrate forebrain (3, 13).

Nonmammalian vertebrates exhibit robust aromatase expression in forebrain regions outside the preoptic area/hypothalamus. Songbirds and teleost fishes exhibit abundant aromatase expression throughout the forebrain (3, 14), although the function of this extra-hypothalamic aromatase is not yet clear. One proposed functional explanation is that forebrain aromatase provides a local source of oestrogen that modulates circuits involved in complex behaviours. In songbirds, the discrete caudal forebrain network of nuclei involved in song production is surrounded by robust aromatase expression (Fig. 1). In zebra finches, injection of oestrogens or aromatisable androgens increases the expression of singing behaviour in males (15) and, in adult white-crowned sparrows, local forebrain aromatisation is responsible for seasonal plasticity of neuronal firing properties within the song network (16). These and other findings raise the possibility that acute, local aromatase activity in the forebrain song network is associated with singing behaviour in songbirds, but this possibility currently remains untested.

In addition to cell bodies, aromatase is expressed "but this possibility is currently untested" in synaptic terminals in the mammalian hypothalamus (17) and hippocampus (18), although the function of this neuronal compartmentalisation is not clear. In zebra finch brain, aromatase has been localised to axonal fibres (14) and synaptic terminals (19) within forebrain nuclei involved in auditory-motor integration [high vocal centre (HVC) and caudomedial nidopallium (NCM)] (Fig. 1). The subcellular compartments enriched with the aromatase enzyme can be separated via differential centrifugation into a microsomal fraction (somal aromatase) and a synaptosomal fraction (synaptic aromatase) for biochemical analysis of enzyme activity (20). Within the purified synaptosomal fraction from the posterior telencephalon of adult zebra finches, aromatase activity is

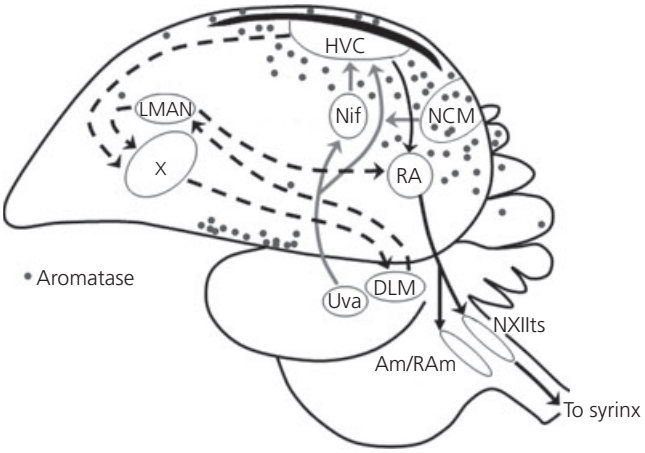

Fig. 1. A saggital view of the zebra finch brain, showing primary motor and auditory circuits, along with aromatase expression (grey dots). The motor network contains two pathways: a posterior forebrain pathway (HVC, RA, nXIIts) involved in song production, and an anterior forebrain pathway (HVC, Area X, DLM, IMAN) involved in song learning. An ascending auditory network contains a series of midbrain (Uva) and forebrain (Nif, NCM) nuclei that project to HVC. The enzyme aromatase (grey dots) is expressed throughout the posterior forebrain, in particular within NCM and surrounding the nucleus HVC. HVC, high vocal centre; RA, robust nucleus of the arcopallium; nXIIts, tracheo-syringeal nucleus of the twelth nerve; DLM, medial dorsolateral nucleus of the thalamus; IMAN, lateral magnocellular nucleus of the anterior nidopallium; Uva, nucleus uvaeformis; NIf, nucleus interface of the nidopallium; NCM, caudomedial nidopallium.

higher in males than in females (21). Because only males sing, this raises the hypothesis that synaptic aromatase is associated with singing behaviour and/or auditory processing in male zebra finches. Furthermore, acute changes in aromatase activity within synaptic terminals could provide a proximate source for local fluctuations in forebrain oestrogen levels during social interactions, as observed recently using *in vivo* microdialysis (22).

In the present study, we tested the hypothesis that forebrain aromatase activity is linked to the expression of singing behaviour and auditory activation in male zebra finches. Aromatase activity could be linked to the production of song, the auditory processing of song, or both. We first examined aromatase activity in forebrain homogenates and then within subcellular compartments by comparing aromatase activity in singing versus nonsinging males. Next, we tested whether playback of conspecific song alters aromatase activity within subcellular compartments and/or the expression of aromatase mRNA in the forebrain of male zebra finches.

Materials and methods

Subjects

All experiments were approved by the UCLA Chancellor's Committee on Animal Care and Use. Animals were adult zebra finches > 120 days of age taken from the Schlinger laboratory breeding colony at UCLA. Males were taken from a mixed-sex aviary room that provided them visual and acoustic contact with females and other males. Males were therefore likely to have been sexually experienced with females. Males were placed individually in acoustic isolation chambers for 24 h prior to each experiment trial. All trials were carried out within 1 h of lights on to minimise circadian variation. Songs were recorded using a Shure-SM57 microphone (Shure, Niles, IL) connected to a computer and digitised with SYRINX software (www.syrinxpc.com; John Burt).

Singing experiment 1

We investigated whether an acute period of singing behaviour (30 min in duration, consisting of intermittent song bouts characteristic of male zebra finches) is linked to forebrain aromatase activity. The 30-min sampling time was chosen, in part, to match the rapidity of observed changes in forebrain immediate–early gene expression associated with the production of song in male zebra finches (23, 24). For each of 12 males (one male only per trial), two female zebra finches were added to a cage adjacent to the focal male's cage inside an acoustic isolation chamber for a 30-min period. Two females were used because pilot testing showed that some males preferentially sang to one female of a pair only. During each trial, the focal male was monitored and scored by one or two observers through a one-way glass partition for the occurrences of courtship songs, chirps, beak wipes, flights, preening, feeding and drinking. According to their within-trial behaviours, males were separated into two groups for comparison: (i) males that sang at least one song in the presence of females ('singers'; n = 8; song bouts: mean $\pm$ SD = 18.75 $\pm$ 10.66) and (ii) males that did not sing at all in the presence of females ('nonsingers'; n = 4). The proportion of nonsingers observed here is similar to previous studies in which 20–30% of male zebra finches do not sing courtship song when individually presented with females for brief trials (25). As also shown previously, these nonsinger males are considered capable of song, but did not exhibit song when presented with our stimulus females. At the end of trials, males were caught and immediately euthanised by rapid decapitation (< 1 min after initial disturbance). Trunk blood was collected, centrifuged and plasma stored at −80 °C. Brains were immediately dissected on ice into major brain regions following established protocols (21, 26): anterior hypothalamus-preoptic area (AH-POA; separated from optic tecta and posterior hypothalamus), ventral forebrain containing nucleus taeniae (NT), anterior telencephalon (AT; separated from NT and hippocampus, containing song nuclei Area X and lateral magnocellular nucleus of the anterior nidopallium) and posterior telecephalon (PT; separated from NT and hippocampus, containing song nuclei HVC, robust nucleus of the arcopallium, and auditory nuclei NCM, Field L, CMM and Nif; see also Fig. 1). Aromatase mRNA and protein within the ventral forebrain region that contains nucleus taeniae is restricted to the bounds of nucleus taeniae (14, 27) and our measure of biochemical activity of aromatase from this region is therefore presented as 'NT'. Dissected brain regions were immediately frozen on dry ice and stored at −80°C until the aromatase activity assay.

Singing experiment 2

In Experiment 2, we sought to determine the subcellular compartment (microsomes versus synaptosomes) that accounts for differences in aromatase activity between singers and nonsingers. All experimental procedures were as outlined above for the singing Experiment 1 for a new set of 12 birds (eight singers, four nonsingers). As in Experiment 1, all singers sang at least one song in the presence of females (song bouts: mean $\pm$ SD = 10.50 $\pm$ 10.18), whereas nonsingers did not produce a single bout of song when presented with females. Immediately following brain region dissection, AT and PT samples were processed for cellular subfractionation. We focused on telencephalic tissue because singing was associated with differences in aromatase activity only in the posterior telencephalon in

Experiment 1 (see Results). Brain subfractions were prepared according to established protocols with some modifications (20, 21, 28). Briefly, AT and PT samples were homogenised in 2 ml of ice-cold sucrose phosphate buffer (pH = 7.2–7.6; on ice) in 3 × 5 s intervals with an electric homogeniser (Tissue Tearor; Biospec Products, Bartelsville, OK, USA). Homogenates were then centrifuged for 15 min at 1046 *g* at 4 °C to separate whole cell debris, nuclei and dense organelles (P1 pellet), from mitochondria, synaptosomes, cytosol and microsomes (S1 supernatant). The resulting S1 supernatant was decanted and centrifuged for 30 min at 10 900 *g* at 4 °C to separate synaptosomes and mitochondria (P2 pellet) from microsomes and cytosol (S2 supernatant). The S2 supernatant was collected and frozen at −80 °C until the aromatase assay ('S2 microsomes', see below). The P2 pellet was resuspended in 2 ml of fresh sucrose phosphate buffer and gently shaken before being centrifuged again for 30 min at 10 900 *g* at 4 °C to purify the synaptosomal/mitochondria sample. The resulting purified P2 pellet was collected and frozen at −80 °C until the aromatase activity assay ('synaptosomal fraction') and the supernatant was discarded. We report on aromatase activity in the synaptosomal fraction (P2), which contains both purified synaptosomes and mitochondria, and in the microsomal fraction (S2), which contains both microsomes and cytosol. Aromatase activity is not found in cytosol or mitochondria (20); thus, the present study reports P2 aromatase activity as 'synaptosomal' and S2 aromatase activity as 'S2 microsomal'.

Playback experiment

We reasoned that the differences in posterior telencephalon aromatase activity we observed between singers versus nonsingers (see Results) could be due to activation of the auditory pathway (i.e. self-stimulation) and not singing behaviour *per se*. To test this hypothesis for aromatase activity, a separate set of 19 males were individually housed in acoustic isolation chambers for 24 h prior to experimental trials. For 13 males (one male per trial only), conspecific male song (1-min recording of a single song motif sequence from each of three individual males, looped 30 times) was broadcast inside the acoustic chamber for 30 min. For six other males, pulsed white noise (1-min intermittent white noise looped 30 times) was broadcast inside the acoustic chamber for 30 min. All sound stimuli were band-pass filtered (1–12 kHz; CoolEdit Pro Syntrillium, Phoenix AZ) and standardised for peak amplitude < 70 dB (after 29). At the end of trials, males were caught and immediately euthanised by rapid decapitation (mean = 39 ± 11 s after initial disturbance). Trunk blood was collected and brains dissected as above. The left hemisphere AT and PT samples were homogenised and processed for subfractionation as above. The right hemisphere AT and PT samples were immediately frozen on dry ice and processed for quantitative polymerase chain reaction (PCR) for aromatase mRNA expression (see below). We focused once again on telencephalic tissue because singing was associated with differences in aromatase activity only in the posterior telencephalon in Experiments 1 and 2 (see Results). Three males in the 'male song' group responded to song playback by singing at least one song bout. Samples from these three singing birds were not included in the biochemistry assay with the 16 nonsinging birds (resultant n = 10 'male song' birds, n = 6 'white noise' birds). None of the birds in the 'white noise' treatment group sang during the 30-min white noise playback period. Samples analysed for the quantitative PCR of aromatase mRNA included a subset of six males from the 'male song' group, six males from the 'white noise' group and three males from the 'male song' group who sang during the playback experiment ('MS Singers').

Biochemistry assay

Aromatase activity was measured using standard biochemical techniques, as reported previously for zebra finches and quail (20, 21). Tissue homogenates (either whole homogenate in 180 μl of ice-cold sucrose-phosphate buffer, a synaptosomal pellet homogenised in 180 μl of ice-cold sucrose-phosphate buffer, or 180 μl of ice-cold S2 fraction) were incubated with 100 nM [1,2,6,7-^{3}H] AE (androstenedione; New England Nuclear, Waltham, MA, USA) and cofactor mix (20 μl) in a final volume of 200 μl. We did not isolate a purified P3 (microsomal) pellet (20, 21), but assayed aromatase activity in the S2 supernatant, which contains microsomes and cytosol. To confirm that microsomes within the S2 cytosolic fraction exhibited aromatase activity, we conducted pilot time course experiments on a separate set of S2 samples in which the concentration of [1,2,6,7-^{3}H] AE was varied from 20, 50 and 100 nM, and the time course was in the range 5–30 min. Based on this validation, we chose a 10-min incubation time and 100 nM [1,2,6,7-^{3}H] AE starting concentration for all S2 samples containing microsomes. We assessed the validity of this approach by confirming that an excess of radio-isotopic substrate ([1,2,6,7-^{3}H] AE) was present during and after the aromatase reaction (data not shown) because high activity of cytosolic enzymes (such as 5β-reductase) in the S2 supernatant could shunt substrate away from the aromatase reaction. The regenerating cofactor mix for all experiments consisted of 22.2 mM NAD-P, 22.2 mM βNAD, 103.8 mM glucose-6-phosphate, 22.2 mM ATP and 20 IU/ml glucose-6-phosphate dehydrogenase. Whole brain homogenate and synaptosomal samples were incubated for 5 min, whereas S2 microsomal samples were incubated for 10 min at 40 °C, each tube was then snap frozen in a dry ice/methanol bath to stop the reaction. Steroids were extracted with diethyl ether (× 3), and the aqueous phase stored at −20 °C for a Bradford protein assay. The organic phase was dried in a warm water bath and resuspended with CH_2Cl_2/methanol. Samples were air-dried and oestrogens were separated from androgens by phenolic partition using carbon tetrachloride and 0.1 M sodium hydroxide (20). All synaptosomal samples were processed in parallel for activity of aromatase and the androgen-metabolic enzymes 5α-reductase, 5β-reductase and 17β-hydroxysteroid dehydrogenase (20). Radioinert steroids were added to each tube as markers for visualisation (E_1 and E_2 for aromatase; 5α-androstanedione, 5β-androstanedione, androstenedione and testosterone for other androgen-metabolic pathways) and samples were separated using thin-layer chromatography with ether : hexane (3 : 1) as a mobile phase. Bands containing oestrogens were visualised using iodine and bands containing androgens were visualised under ultraviolet-radiation after primulin spray (Sigma, St Louis, MO, USA). All bands were scraped, and steroids were eluted from silica using methanol. Radioisotope decay counts were measured on a liquid scintillation counter (E_1 and E_2 counts were combined for total oestrogen content to reflect aromatase activity). Protein content was estimated for each sample using the Bradford method. To account for procedural losses (recovery), separate tubes containing known quantities of [1,2,6,7-^{3}H] AE and [2,4,6,7-^{3}H] E_1 were processed in parallel through the entire assay.

Quantitative PCR

Differences in the activity of the aromatase protein between individuals could arise from constitutive or acute differences in expression of aromatase mRNA levels, or from post-transcriptional modifications. To test the former hypothesis, we isolated RNA from the right hemisphere from birds in the playback experiment using quantitative real-time PCR. Total RNA was extracted from brain tissue using the TRIzol method, following the manufacturer's instructions (Invitrogen, Carlsbad, NM, USA). Tissue was suspended in TRIzol and homogenised with an electric homogeniser for 20 s on ice. Following homogenisation, tubes were manually shaken for 45 s, and samples were separated with choloroform. The aqueous phase was pipetted into a new set of tubes and washed with isopropanol. Following 10 min of centrifugation (12 *g* at 4 °C), pelleted RNA was washed with 75% ethanol, redissolved to final stock volume with DEPC water and incubated for 10 min at 60 °C. Isolated RNA was verified via gel electrophoresis and the final

concentration was determined via spectrophotometer at 260 nm. Isolated RNA was stored at −80 °C until PCR.

RNA was treated for contamination using DNase (Promega, Madison, WI, USA) and 1 μg of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) on a thermal cycler (MJ Research, Waltham, MA, USA) for 50 min at 42 °C followed by 15 min at 70 °C. The resulting cDNA was analysed by quantitative PCR for aromatase mRNA using specific zebra finch aromatase primers: forward: 5′-GGATGAGCACATGGATTTTGC-3′; reverse 5′-GCAGTCAGATCCCCTCTGTTC-3′. GAPDH was used as internal control, primers: forward 5′-CCATCAGCAGCAGCCTTCA-3′; reverse 5′-GACCTGCCGTCTGGAAAA-3′. Reactions were carried out an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with Sybr Green. Dissociation curves of PCR products were checked to verify the absence of DNA contamination, and all samples were run in duplicate. The delta C_t method was used for quantification.

Hormone levels

Plasma oestradiol was quantified via enzyme immunoassay (Cayman Chemical, Ann Arbor MI), according to previously established protocols (22). Assay sensitivity was 5.40 pg/ml.

Statistical analysis

Enzyme activity results (fmol/mg protein/minute) for each experiment were analysed using STATVIEW 4.5 (Abacus, Berkeley, CA, USA) and tested for normality using JMP 5.0.1 (SAS Institute, Cary, NC, USA). For all experiments, biochemistry results did not fit normal distributions, and common transformations did not improve normality (see Results), so Mann–Whitney nonparametric U-tests were performed on all biochemistry data with two-tailed probability distributions. When multiple comparisons were performed for a given experimental run (i.e. anterior versus posterior telencephalon), Bonferroni correction was used to correct P-values. For quantitative PCR, delta C_t values were log-transformed to achieve normality and analysed via two-way ANOVA. Plasma steroid levels were analysed by unpaired t-tests.

Results

Singing experiment 1

We investigated whether a brief (30 min) bout of singing behaviour was linked to aromatase activity in the forebrain of male zebra finches. The results obtained (including log-transformed data) did not fit normal distributions (Shapiro–Wilk, $P < 0.0001$), so the results were analysed using nonparametric statistics. Aromatase activity was significantly higher in singers than nonsingers in the posterior telencephalon, but not the anterior telencephalon. Mann–Whitney U-tests showed that aromatase activity (Fig. 2A–B) was significantly higher in singers versus nonsingers in the posterior telencephalon ($U = 30$; Bonferroni corrected $P = 0.032$) but not in the anterior telencephalon ($P = 0.49$). Mann–Whitney U-tests also showed there were no differences between singers and nonsingers in AH-POA ($P = 0.39$) or nucleus taeniae ($P = 0.73$). Despite higher mean PT aromatase activity levels in singers versus nonsingers, there was no significant correlation between the number of songs sung by each male during the trial and PT aromatase activity in singers ($R^2 = 0.28$; Spearman rank, $z = 1.705$; $P = 0.088$). Other than singing behaviour (see Materials and methods), all other scored behaviours were not significantly different between singers versus nonsingers (data not shown; chirps, $P = 0.80$; beak wipes, $P = 0.44$; flights, $P = 0.29$; preening, $P = 0.71$; drinking, $P = 0.23$; feeding, $P = 0.33$). There was no significant difference in plasma levels of oestradiol between singers (58.18 ± 23.23 pg/ml) and nonsingers (27.47 ± 11.58; $P = 0.29$). There was also no significant correlation between plasma oestradiol and the number of songs each male sang ($R^2 = 0.104$; $F = 0.581$; $P = 0.48$), or between plasma oestradiol and either PT aromatase activity or AT aromatase activity (all $P > 0.54$).

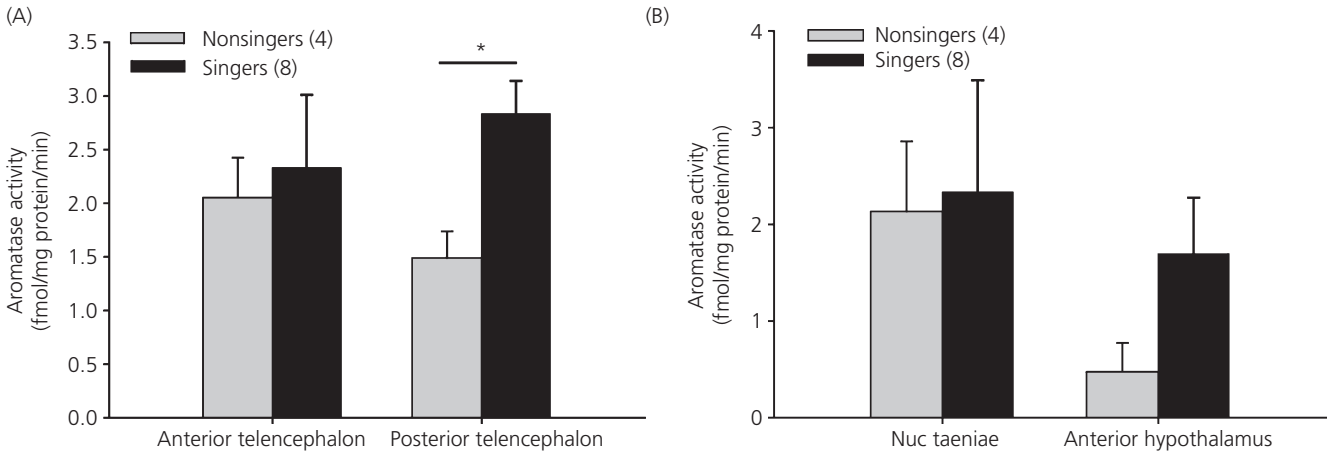

Fig. 2. Aromatase activity is elevated in the posterior telencephalon of singing male zebra finches. All birds were exposed to females for 30 min, and aromatase activity was measured in whole brain homogenate in birds that sang at least one song ('singers') versus birds that did not sing ('nonsingers'). Aromatase activity was significantly elevated (*$P = 0.032$) in singers within the posterior telencephalon only (A), and was not different in anterior telencephalon (A), nucleus taeniae (B) or anterior hypothalamus (B). Data are presented as the mean ± SEM, sample sizes are in parentheses. All biochemical data were analysed by nonparametric Mann–Whitney U-tests.

Singing experiment 2

In Experiment 2, for a separate group of birds, we sought to determine the subcellular compartment (S2 microsomes versus synaptosomes) that accounted for differences in aromatase activity between singers and nonsingers. Results (including log-transformed data) did not fit normal distributions (Shapiro–Wilk, $P < 0.0002$), so the results were analysed using nonparametric statistics. Once again, singers had higher aromatase activity than nonsingers in the posterior telencephalon. Mann–Whitney U-tests showed that synaptosomal aromatase activity (Fig. 3A) was significantly higher in singers versus nonsingers in the posterior telencephalon ($U = 32$; Bonferroni-corrected $P = 0.008$), but not in the anterior telencephalon ($P = 0.24$). Aromatase activity in the S2 fraction (microsomes; Fig. 3B) was not significantly different in singers versus nonsingers for either the posterior ($P = 0.93$) or anterior ($P = 0.26$) telencephalon. Similar to Experiment 1, there was no significant correlation between synaptosomal aromatase activity from posterior telencephalon and the number of songs each male sang ($R^2 = 0.104$; Spearman rank, $z = 1.68$; $P = 0.094$).

Unlike aromatase, the activity of synaptosomal androgen-conversion enzymes was not different between singers and nonsingers, for either brain region (data not shown). Results (including log-transformed data) did not fit normal distributions (Shapiro–Wilk $P < 0.0001$), so the results were analysed using nonparametric statistics. Mann–Whitney U-tests showed no significant differences between singers and nonsingers for synaptosomal 5α-reductase activity (posterior $P = 0.99$; anterior $P = 0.99$), 5β-reductase activity (posterior $P = 0.18$; anterior $P = 0.86$) and 17β-hydroxysteroid dehydrogenase activity (posterior $P = 0.10$; anterior $P = 0.24$). Other than singing behaviour itself, all other scored behaviours were again not significantly different between singers versus nonsingers (data not shown; chirps, $P = 0.70$; beak wipes, $P = 0.27$; flights, $P = 0.55$; preening, $P = 0.83$; drinking, $P = 0.07$; feeding, $P = 0.11$). There was again no significant difference ($P = 0.20$) between plasma oestradiol in singers (51.79 ± 20.83 pg/ml) versus nonsingers (21.61 ± 8.19). There was also no significant correlation between plasma oestradiol and the number of songs each male sang ($R^2 = 0.07$; $F = 0.754$; $P = 0.40$). Furthermore, there were no significant correlations between plasma oestradiol and PT aromatase activity or AT aromatase activity in either subcellular fraction (all $P > 0.23$).

Playback experiment

We reasoned that the differences in PT aromatase activity levels between singers versus nonsingers could be due to self-stimulated activation of the forebrain auditory circuit rather than singing behaviour *per se*. The results obtained (including log-transformed data) did not fit normal distributions (Shapiro–Wilk $P < 0.0000$), so the results were analysed using nonparametric statistics. There were no differential effects of playback stimuli on telencephalic aromatase activity, for either cellular compartment. Mann–Whitney U-tests tests showed that synaptosomal aromatase activity (Fig. 4A) was not significantly different between male song versus white noise treatments in the posterior telencephalon ($P = 0.33$) or anterior telencephalon ($P = 0.12$). Similarly, Mann–Whitney U-tests showed that S2 microsomal aromatase activity (Fig. 4B) was also not significantly different between male song versus white noise treatments in the posterior telencephalon ($P = 0.45$) or anterior telencephalon ($P = 0.74$). The qualitatively higher aromatase activity levels in the posterior versus anterior telencephalon are consistent with earlier reports for elevated aromatase expression (14), as well as aromatase activity (21), in the posterior versus anterior telencephalon of zebra finches.

Similar to aromatase, the activity of synaptosomal androgen-conversion enzymes was not different among playback groups,

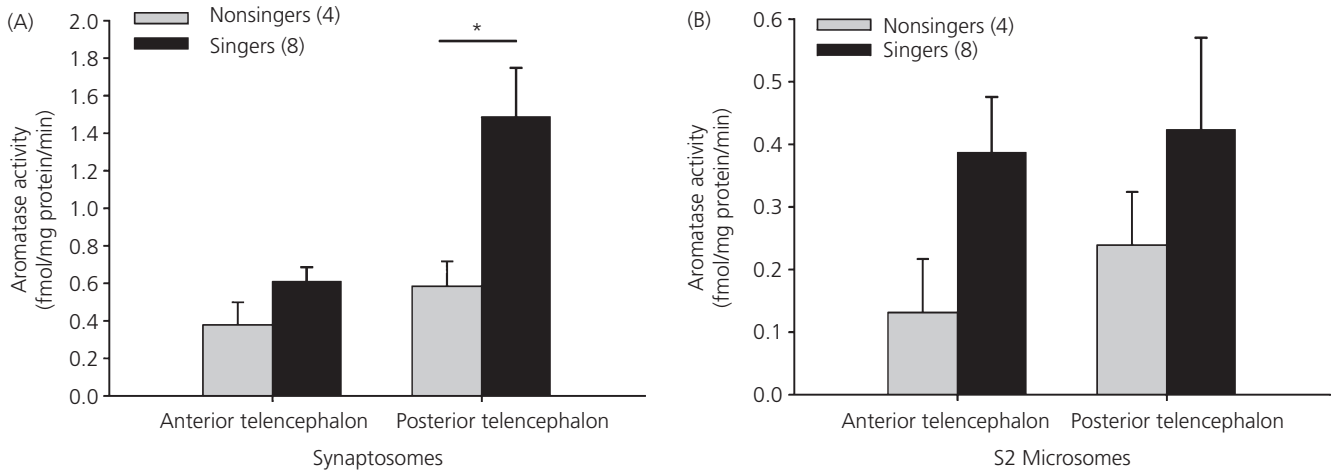

Fig. 3. Aromatase activity within synaptosomes is elevated in the posterior telencephalon of singing male zebra finches. A new set of birds was exposed to females for 30 min, and aromatase activity was measured in subcellular compartments that contain synaptic terminals (synaptosomes) or an S2 fraction containing microsomes in singers versus nonsingers. Aromatase activity was significantly elevated (*$P = 0.008$) in singers within the synaptosomal fraction of posterior telencephalon only (A), and not within anterior telencephalon (A) or within S2 microsomes from either telencephalic region (B). Data are presented as the mean $\pm$ SEM, sample sizes are presented in parentheses.

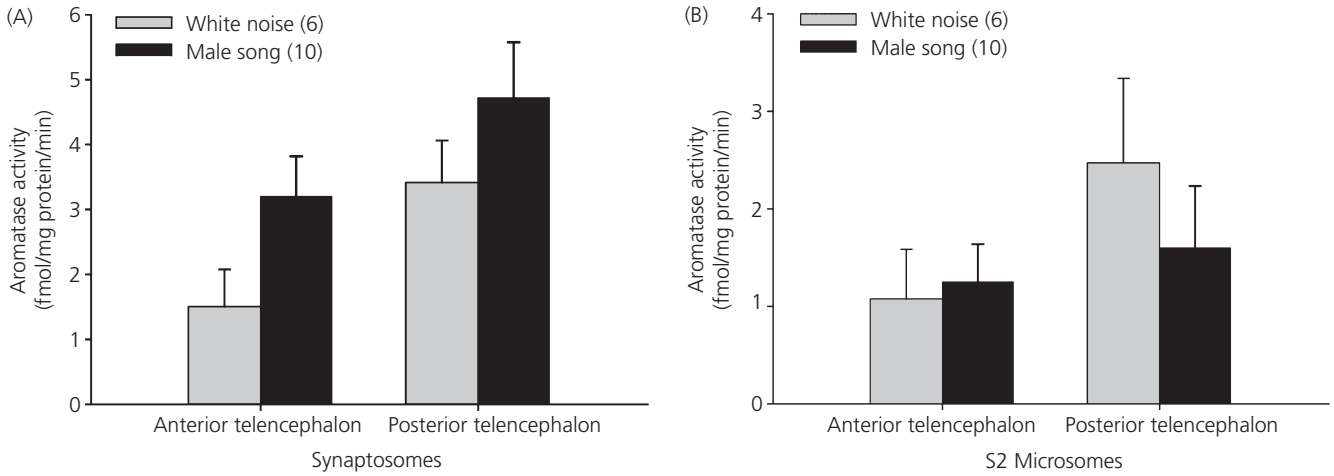

Fig. 4. Aromatase activity does not change in either subcellular compartment in response to acoustic playback. All birds were exposed playback of male song or white noise for 30 min, and aromatase activity was measured in synaptosomes (A) and S2 microsomes (B). Data are presented as the mean ± SEM, sample sizes are presented in parentheses.

for either brain region (data not shown). The results obtained (including log-transformed data) did not fit normal distributions (Shapiro–Wilk $P < 0.0001$), so the results were analysed using nonparametric statistics. Mann–Whitney U-tests showed no significant differences between male song and white noise treatment for synaptosomal 5α-reductase activity (posterior $P = 0.66$; anterior $P = 0.89$), 5β-reductase activity (posterior $P = 0.16$; anterior $P = 0.66$) and 17β-hydroxysteroid dehydrogenase activity (posterior $P = 0.16$; anterior $P = 0.93$). For birds in Experiment 3, observed behaviour scores were not significantly different between male song versus white noise treatments (data not shown; chirps, $P = 0.12$; beak wipes, $P = 0.71$; flights, $P = 0.55$; preening, $P = 0.41$; drinking, $P = 0.55$; feeding, $P = 0.99$). Plasma oestradiol levels for this experiment were as reported in a previous study (22); there were no differences between playback groups (male song: 41.96 ± 10.16; white noise: 51.24 ± 24.65; $P = 0.70$).

Quantitative PCR analysis showed no effect of playback treatment on aromatase mRNA expression in either anterior or posterior telencephalon (Fig. 5). Delta C_t values were log-transformed to achieve normality (Shapiro–Wilk $P > 0.05$) and analysed by two-way ANOVA. In the two-way factorial ANOVA, there was no significant effect of playback treatment on aromatase mRNA expression ($F = 0.307$; $P = 0.74$), but there was a significant effect of brain region on aromatase mRNA expression ($F = 4.59$; $P = 0.042$). Tukey's post-hoc tests showed that aromatase mRNA expression was elevated in the posterior telencephalon versus anterior telencephalon in all three experimental groups ($P < 0.03$ for 'male song', 'white noise' and 'male song singers'). Interestingly, there were no significant correlations between telencephalic aromatase activity and quantitative mRNA expression level, (synaptosomes: anterior telencephalon, $R^2 = 0.02$, $F = 0.22$, $P = 0.65$; posterior telecephalon, $R^2 = 0.05$, $F = 2.14$, $P = 0.16$; S2 microsomes: anterior telencephalon, $R^2 = 0.02$, $F = 0.001$, $P = 0.99$; posterior telecephalon, $R^2 = 0.029$, $F = 0.29$, $P = 0.59$).

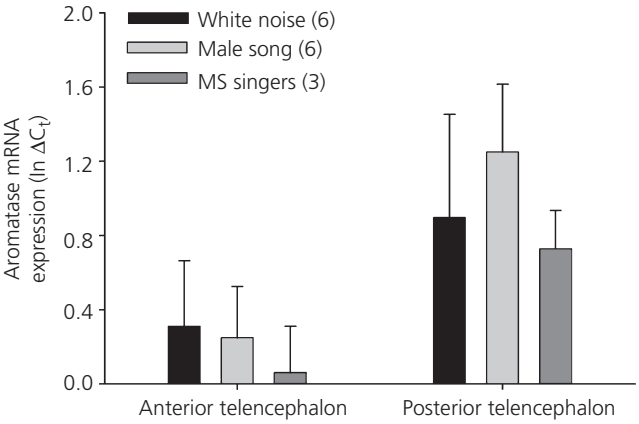

Fig. 5. Aromatase mRNA expression level, as measured by quantitative polymerase chain reaction, does not change in response to acoustic playbacks. All birds were exposed playback of male song or white noise for 30 min, and three males in the 'male song' group sang at least once ('MS singers'). Aromatase mRNA levels are significantly elevated in posterior versus anterior telencephalon within all three treatment groups ($P < 0.03$), but do not significantly differ among treatments (e.g. white noise versus male song). Data are presented as the mean ± SEM, sample sizes are presented in parentheses. Data were analysed by factorial ANOVA following log-transformation.

Discussion

We observed elevated aromatase activity in the posterior telencephalon of singing male zebra finches compared to nonsinging males. Importantly, singers have elevated aromatase activity in the posterior telencephalon that is localised to the subcellular fraction containing synaptosomes. This indicates that aromatase in synaptic terminals within the posterior telencephalon (19, 21) has a functional role in association with song production. A series of recent findings complimentary to the results of the present study show that social interactions with females increases local oestradiol levels

within the caudal forebrain in zebra finches, as measured by *in vivo* microdialysis (22). Local forebrain oestradiol and testosterone levels determined by this method also change rapidly in response to infusion of the neurotransmitters glutamate and GABA. Collectively, these results are consistent with the hypothesis that local oestrogen synthesis within the caudal forebrain is regulated at the presynaptic level by acute (e.g. neurotransmitter-dependent) mechanisms. The present study emphasises that the neural circuits that underlie song production and auditory processing in zebra finches have the capacity for acute modulation by local steroidal microenvironments.

The elevated synaptosomal aromatase activity levels in singing versus nonsinging males observed in the present study could be due to either fast upregulation of aromatase activity during the period of singing itself, or a 'prior condition' of constitutively elevated aromatase activity in the telencephalon of singers. There is experimental precedent for rapid (within 5 min) regulation of brain aromatase activity in birds. In quail hypothalamus, aromatase activity is rapidly regulated in males during copulation (9) and glutamate is a potent modulator of aromatase activity in *in vitro* hypothalamic explants (30). Future experiments could assess whether similarly rapid changes in aromatase activity occur within the zebra finch forebrain within time periods of < 30 min. Complementary tests of this hypothesis could include the short-term administration of aromatase inhibitors such as fadrozole, which has been shown to reduce song and other courtship behaviours following long-term (days to weeks) implantation in a wide range of vertebrates (11, 31–34).

A rapid increase in synaptic aromatase activity within the posterior telencephalon of singing males would be associated with similarly rapid oestrogen actions. Synaptic aromatase activity is most likely regulated by acute interactions between steroids and neurotransmitters because aromatase is colocalised with NMDA-receptors (35) and forebrain oestradiol levels are regulated by glutamate (22). There is now strong evidence for a multitude of rapid downstream oestrogen effects on neuronal activity and intracellular second messengers (36, 37), some mediated via membrane oestrogen receptors (38, 39). Although no evidence exists to date for rapid (seconds to minutes) oestrogen actions in the avian song system, oestradiol rapidly affects the motor circuit that patterns vocalisations in a teleost fish, the plainfin midshipman (40, 41). Therefore, rapid steroid actions on vocal pathways may be conserved among vertebrates. Alternatively, changing forebrain oestrogens could also influence the activity of other steroidogenic enzymes (42), leading to secondary modulatory effects. Finally, oestrogens could interact with neurotransmitter/neuropeptides systems in the song circuit, such as catecholamines like dopamine (43, 44), to acutely modulate song production.

Support for the interpretation of a 'prior condition' of brain aromatase activity comes from two previous observations. In rats, medial preoptic aromatase activity levels are higher in copulating versus noncopulating males (10) and these differences are also region-specific (i.e. no differences were reported for the bed nucleus of the stria terminalis, medial amygdala and ventral medial nucleus of the hypothalamus). Similarly, quail hypothalamic aromatase activity is elevated in highly-aggressive males and the stability of aggression propensity is linked to the 'prior condition' of high versus low hypothalamic aromatase activity (4). Aromatase activity is positively correlated with behavioural score in rats and quail (copulatory or aggressive events, respectively), whereas, in contrast, the findings of the present study demonstrate that song bouts and aromatase activity in posterior telencephalon are not directly linked. Therefore, it remains possible that a threshold relationship exists between telencephalic aromatase activity and the onset of singing behaviour in zebra finches, or that aromatase activity is related to auditory processing (i.e. self stimulation or song memory retreival) within the posterior telencephalon. Despite these differences, these studies are among a growing number indicating that aromatisation provides a localised mechanism within the brain to allow plasticity in reproductive behaviours.

Aromatase activity in the posterior telencephalon is higher in male versus female zebra finches and this sex difference is most pronounced in synaptosomes (21). The present study suggests that sex differences in synaptosomal aromatase activity are linked to the sex-specific production of song in male zebra finches. A comprehensive review showed that telencephalic aromatase activity is elevated in bird species that produce learned song versus nonlearners (45). The present study predicts that aromatase activity is particularly pronounced within forebrain synaptic terminals of song learning species, and that synaptic aromatase is acutely regulated during the production of learned song.

We observed that aromatase activity does not differ between males that heard conspecific song playback versus white noise in both anterior and posterior telencephalon, in either subcellular compartment. This is in contrast to a recent observation in which song playback (and not white noise) causes acute elevations in local oestradiol levels within auditory forebrain, as measured by *in vivo* microdialysis (22). The evidence for laterality of auditory processing in the zebra finch telencephalon emphasises the left NCM (46, 47) and the previous microdialysis experiments and current biochemical experiments analysed the left telencephalon. The discrepancy in previous microdialysis versus current biochemical results could be due to: (i) Changes in oestradiol levels within NCM as measured by microdialysis are localised and do not occur in adjacent telencephalic regions, and they therefore reflect localised increases in aromatase activity. Thus, homogenisation of the posterior telencephalon in the present study may not yield sufficient spatial resolution to detect such changes in aromatase activity during auditory activation. (ii) Local changes in oestradiol could also be the result of changing levels of androgen precursors, which are then aromatised to result in increased oestradiol levels, without active changes in aromatase activity. (iii) The methods employed in the present study may have missed the peak in aromatase activity by sampling after 30 min of playback, whereas microdialysis experiments collect oestradiol for the entire 30-min period and reveal a two-fold surge in oestradiol levels. Microdialysis methods may therefore be more sensitive to recent neuroendocrine events, whereas biochemical measurement of aromatase activity (which determines enzymatic capacity) could be predictive of future brain steroid levels. Similarly, the utilisation

(i.e. half-life) of local oestrogens *in vivo* may be considerably different as compared to *in vitro*, leading to differences in detection by these two methods.

Quantitative PCR analysis showed that aromatase mRNA expression was elevated in the posterior versus anterior telencephalon of males, consistent with immunohistochemical results for zebra finch aromatase protein (14) and aromatase biochemical activity (21). The present study also reported no effect of playback stimulus (male song versus white noise) on quantified aromatase mRNA expression levels in either anterior or posterior telencephalon. Furthermore, aromatase mRNA expression was not elevated in birds that sang during male song playback compared to birds that did not sing. Therefore, the present study reports that singing male zebra finches have elevated aromatase activity in the posterior telencephalon within synaptic terminals, and provides limited evidence that telencephalic aromatase mRNA levels are not similarly elevated during singing. Interestingly, there was no strict correlation between telencephalic aromatase mRNA levels and biochemical activity, indicating that post-transcriptional regulation of aromatase [e.g. protein phosphorylation (48)] could be a primary mechanism for controlling aromatase (and thereby local oestrogen levels) in zebra finch telencephalon. Together with a lack of evidence for the regulation of androgen conversion enzymes during singing and audition, the present study focuses attention for future work on the acute changes in the biochemical activity of the aromatase protein that are associated with singing behaviour in male songbirds.

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